BME Design-Spring 2025 - Althys Cao
Complete Notebook

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JULIA SALITA

on

May 07, 2025 @11:21 AM CDT

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Project description

Emily Rhine - Sep 10, 2024, 3:0

Course Number: BME 400

Project Name: CRISPRi screening in cancer spheroids to investigate factors in genome stability

Short Name: CRISPRi Screening in Cancer Spheroids

Project description/problem statement:

Previous CRISPR screening in 2D monolayers has provided extensive knowledge on cancer drivers and therapeutic susceptibilities, but it can fail to accurately identify factc to the 3D environment of in vivo tumors, including genes that regulate genome stability/DNA damage. Therefore, we want to develop a cell culture method that recapitulates environment and is compatible with CRISPR screening to identify sources of DNA damage affected by the tumor environment compared to 2D culture.

The main objectives of the BME team would involve: selecting the cell line for the screen, creating and optimizing a spheroid formation protocol, and developing a protocol γH2AX. Longer term goals could involve: investigating how biomaterial properties affect DNA damage, conducting the screen collaboratively with members of the Hess lat downstream analysis.

About the client:

Ms. Carley Schwartz
Biomolecular Chemistry and Center for Human Genomics and Precision Medicine
Biochemistry
cischwartz@wisc.edu

Alternate Contact:
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Relevant Journal Articles and Websites:
CRISPR screen on growth in spheroids:
https://www.nature.com/articles/s41586-020-2099-x#Sec11
H2AX:
https://www.sciencedirect.com/science/article/pii/S0887233312001567#:~:text=Histone%20H2AX%20is%20rapidly%20phosphorylated,in%20in%20vitro%20mechanistic
CRISPR Screening: https://www.idtdna.com/pages/education/decoded/article/overview-what-is-crispr-screening
https://www.nature.com/articles/s41580-022-00571-x
CRISPR interference (CRISPRi)
https://en.wikipedia.org/wiki/CRISPR_interference#:~:text=The%20technology%20uses%20a%20catalytically,sgRNA)%20to%20the%20genomic%20locus.
Genomic Instability: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/genome-instability
γH2AX screening: https://www.cell.com/molecular-cell/fulltext/S1097-2765(23)00472-0
γH2AX screening: https://www.cell.com/molecular-cell/fulltext/S1097-2765(23)00518-X
ECM and DNA Repair: https://www.science.org/doi/full/10.1126/sciadv.abb2630

Team contact Information

Emily Rhine - Feb 16, 2025, 2:29 PM CST

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Fall 2024 CRISPRi Cancer Spheroid Final Notebook

Althys Cao - Jan 24, 2025, 10:52 AM CST


BME Design-Fall 2024 - Althys Cao
Complete Notebook

JULIA SALITA

Table of Contents

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Timeline_1/31/25

Title: Client Meeting 1_Timeline

Date: 1/31/2025

Content By/Present: Entire team

Content:

Bureaucracy

Experiments

Week	Monday	Tuesday	Wednesday	Thursday	
1/27 - 1/31	-Passage 1		-Passage 2	-Progress report 1	-P -C
2/3 - 2/7	-Spheroid Formation			-Progress report 2	-P -S
2/10 - 2/14				-Progress report 3	
2/17 - 2/21				-Progress report 4	
2/24 - 2/28				-Progress report 5	
3/3 - 3/7				-Progress report 6	
3/10 - 3/14				-Progress report 7	
3/17 - 3/21				-Progress report 8	
3/24 - 3/28	Spring Break				
3/31 - 4/4				-Progress report 9	
4/7 - 4/11				-Progress report 10	- E
4/14 - 4/18				-Progress report 11	
4/21 - 4/25					
4/28 - 5/2					

NOTE: Red = clients' answers/notes

1. Thaw a new vial of A549s
    a. Freeze 3 new vials of cells too after 1-2 passages
2. Redo spheroid experiment with 50k and 75k seeding densities to confirm results
    a. Alter methylcellulose levels to confirm optimization
    b. Test spheroid dissociation with Accutase protocol
    c. Test percent cell viability
        i. Question for client: are we still doing live/dead staining (ideal)
            1. Celltiter glo- Hess lab uses – no dissociation (would get % viability but wouldn't know individual cells)
                a. Can do a control group with no cells (just media) for comparison
                b. Kyle has experience - might want to consult
            2. Almar blue – no dissoc (see above)
                a. Don't have in lab
            3. Sytox green - stains DNA of dead cells
                a. Have to dissociate to count
            4. Both are extracellular assay
            5. Hess Lab uses flow cytometry for percent viability - they use the dye to test permeability (if cells are dead → dyes can permeate)
        ii. Do flow cytometry (for dissociated spheroids) with staining (for spheroids to see necrotic core) - do staining first then flow cytometry later

1. If we are doing flow cytometry for dissociated spheroids, we should expect results to be similar to 2D cells → will also do 2D cells as a contı comparison
2. Make sure cells are not in clumps (70um filter?)

d. "By real histology, necrotic cores were defined as areas in which the extracellular matrix was lacking (total loss of collagen by picrosirius red staining) and ı (fragmented or no nuclei by hematoxylin and eosin staining) and lipid-rich cellular debris."

i. https://www.ahajournals.org/doi/10.1161/circimaging.109.919357#:~:text=By%20real%20histology%2C%20necrotic%20cores,and%20lipid%2Dric

e. Carley still thinks flow cytometry works, but using Celltiter glo would give cleaner data for presentation

f. Select ideal seeding density based on data gathered

3. Calculate whether, using the ideal seeding density, it is possible/realistic to scale up spheroid formation to the 50 million cells necessary for the genome wide screer

a. Calculate time, materials necessary, and complexity

4. Use qPCR to determine whether or not expression of SOX2 increases in our spheroids to confirm ideal cellular conditions and similarity to tumor environment

a. do we do this in a different set of spheroids to the spheroids used for live/dead staining (can we even do this?)

b. Look for primers relevant to our cell line → are GADPH/beta actin okay to use for organoids/spheroids?

Answer: GAPDH protein was used as internal control for organoid

1. https://www.nature.com/articles/s41419-019-1453-0

c. "A549 with CRISRPi to add gRNA and look for specific mutation --> See loss in staining if specific proteins are selected to be removed with gRNA" -Gaele

5. Lentiviral transduction of cells with γH2AX, a biomarker for DNA double strand breaks (dsbs), so the team can understand the effects of gene knockdown via lenti

a. See "Spheroid Formation Protocols_10/25/2024" entry excerpt below

b. See if methylcellulose levels alter γH2AX presentation - Carley S.

i. Question for client: So we are seeding cells at different methylcellulose concentrations (but same density to save time I assume), do CRISPRi, and γl if the methylcellulose concentrations affect γH2AX presentation?

1. alternative proposal: 5 concentration, 5 densities x 3 trials each = 75 wells (enough to fit in a 96-well plate), but should we do it though?
2. For now, pick 1 density and 1 methylcellulose concentration for gammaH2AX staining.

c. Question: how do we actually confirm our protocol for gamma-H2AX staining works - because this step happens <u>after</u> CRISPRi screening but we won't be Do the clients do it for us and then we stain or do we do a faux CRISPR (that we know how the results will look - positive control)

i. It would be valuable to do trial CRISPRi run to induce DNA damage (as a positive control) to confirm our gammaH2AX staining protocol works → (3-6) to get set up and ran

1. CRISPRi will give more relevant information
2. Alternatively (from Gaelen): use DNA break-inducing drugs → more speedy
    a. Still a good option if we are *just* testing if the gammaH2AX staining protocol works
    b. Use <u>etoposide</u> to compare DSB signal
        i. Can either look at IC-50 from online, or just go ahead and do a higher-end dosage to make sure there is enough damage

ii. Will do gammaH2AX staining alongside cells in 2D culture as a point of comparison/positive control: looking for similar results between the two

1. 3 trials of:
    a. Treated spheroids
    b. Untreated spheroids
    c. Treated 2D cells
    d. Untreated 2D cells

d. Question: can we do multiple protocols to check them?

i. Probably yes?

ii. Should start with the one currently in the lab, identify problems, and then tweak 1 variable as we go.

6. ~~CRISPRi genome-wide screen~~ (Technically not a goal for us to complete according to the client(s))

a. Will still need to do a faux CRISPRi to check if the spheroid formation protocol actually is compatible with it, and to check whether γH2AX also works

γ-H2AX staining process (basic procedure - as if don't have spheroids) ***Takes 4-4.5 hours

1. Infect cells with lentivirus

a. Will introduce plasmid with guideRNA, which will target some gene that is involved in gamma-H2AX/DNA damage

i. The plasmid also has fluorescence: mCherry (positive)

1. Therefore, associate mCherry+ cells with guideRNA infection into cell

b. Will not get 100% infection → ideal so we can have controls (with no plasmid aka no vector and no mCherry - negative)

c. Wait 3-5 days so that DNA damage caused by lentivirus infection itself will be "healed" → only downstream data and can be ignored

2. Gather population of cells (3-5 million)

3. Fix cells

a. Immobilize and kill cells
b. This allows us to stain, image, sort, and other downstream steps
c. Dissociate cells from spheroid here?

4. Permeabilize cells
    a. Makes holes in cells so “stuff” can get in them (because we are doing an intracellular staining)
    b. Dissociate cells from spheroid here?
5. Antibody 1
    a. In our case, this will be gamma-H2AX
    b. We actually have a primary-conjugated antibody (already has fluorescence so do not need a secondary antibody)
        i. Our fluorophore will be APC - Alexa fluorophore 647
    c. Dissociate cells from spheroid here?
6. Flow Cytometry
    a. Will have graph: mCherry vs. FSC
    b. In each population, will have some cells positive for mCherry and some negative for mCherry (control, not lentivirally infected)
    c. Will “gate” mCherry+ cells and average their gamma-H2AX expression
        i. Two more subplots:
            1. mCherry+ only with APC gamma-H2AX
                a. Expected to see increase in APC gamma-H2AX
                b. On plot, “peak” will be shifted higher/to the right on x-axis (APC gamma-H2AX)
            2. mCherry- only with APC gamma-H2AX
                a. Expect to see decrease in APC-gamma-H2AX (because no lentiviral infection = gene was not knocked down = no increase in DNA da
                b. On plot, “peak” will be shifted lower/to the left on x-axis (APC gamma-H2AX)

NOTE: will need to refer to current spheroid CRISPR screening gamma-H2AX staining protocols to see the process (do we dissociate spheroids before fixing, aft

- **Possible: if earlier dissociation, may be losing some of DNA markers)**
- **Our job: find a “starting point” for when to dissociate based on literature, then test staining at alternative disassociation points and compare whetl**

Conclusions & Action Items:

- Outline planned experiments and determine how many replicates are needed
    - Hold off on large scale expansion, might go up to T-150 Flask as needed
- Experiments:
    1. Repeat spheroid experiments with 50k and 75k cells
        - Alter methylcellulose levels
        - Only 3 replicates (3 wells) necessary if experimental results have a low range only replicate the experiment if absolutely necessary
    2. Select cell: drug treated or gRNA
    3. Follow gamma-H2AX and change one variable at a time until ideal protocol is found
- Continue 2D cell passaging as a control
    - High yH2AX seen in treated with drug for both 2D and 3D culture *If drug route is taken (Etoposide)
    - Low yH2AX seen in untreated 2D and 3D culture * If drug route is taken

Client Meeting 2_qPCR.v.s.Immunostain_2/7/25

Emily Rhine - Feb 07, 2025, 1:13 PM CST

Title: Client Meeting 2_qPCR.v.s.Immunostain

Date: 2/7/2025

Content By: Emily, Julia, & Jayson

Content:

- qPCR
  - Human source SOX2- fam small to xs side of things (MGD represents quencher, not super important)
  - TaqMan - spans exons
  - Housekeeping: Beta-Actin (recommend because it expresses well for many cell types)
  - Housekeeping: GAPDH (fine with it because of literature found but more looking into)
  - More quantitative for base level expression
- Immunostaining is overall cheaper and faster- staining for SOX2 would have to stain in spheroids and we don't know how
  - Downstream- check for changes in expression level
  - Cancer-associated fibroblasts suppress SOX2-induced dysplasia in a lung squamous cancer coculture
  - Sox2 is associated with cancer stem-like properties in colorectal cancer
- Cell-titer Glo
  - Measures live cells through ATP
  - Buffer1 - Luciferan
  - Buffer2- ultra glo luciferase
  - Oxyluciferin - glows!
  - Experiment
    - Wells with just cells for positive experiment control
    - just media (no cell) well for negative assay control
      - Average then normalize to this level of fluorescence
    - Discuss more 2/10
  - For 2D culture (Promega) modify to 3D instructions
    - Add lyse cell step
    - Add longer incubation times
    - End of life for cells :(
  - Need 100uL Cell-titer Glo:1uL media (2/10)
    - Spin down spheroids, aspirate media
    - Add 1:1 ratio
    - See Han protocol
  - Celltiter glo mechanism
    - Celltiter glo reagent added to cells
    - The reagent lyses cell membranes releasing ATP
    - The reagent provides luciferin and luciferase
    - Luciferase reacts with luciferin and ATP to produce oxyluciferin and release energy as luminescence
    - The amount of luminescence is proportional to the amount of ATP present
- SFM (needed for spheroid formation)
  - Add penicillin streptomycin 1% (Need to thaw a day before)

Conclusions/Action Items:

- Follow up on these research articles
    - Housekeeping gene: Choice of endogenous control for gene expression in nonsmall cell lung cancer
    - SOX2 Immunostain for Lung Cancer Spheroid: Cancer-associated fibroblasts suppress SOX2-induced dysplasia in a lung squamous cancer coculture
    - Resuspend spheroids:
- Seed spheroids
- Update timeline and notebook

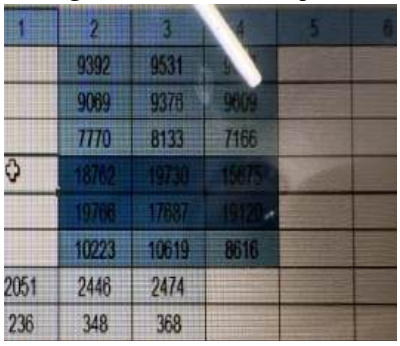
Client Meeting 3_2/14/25

Emily Rhine - Feb 14, 2025, 4:33 PM CST

Title: Client Meeting
Date: 2/14/25
Present: Emily, Ana, Julia, & Jayson
Content:

- Revisit timeline:

2/10 - 2/14	~~- Celltiter glo~~ ~~- Passage 7~~ ~~(Althys + Julia)~~	Example Primer link: https://www.novusbio.com/products/gapdh-primer_nbp1-71650?srsltid=AfmBOoonuNZhzD0tr-UtfhYgyXTL1Tj5l6EC309CpwJo51bbFjdWigMof	~~- Passage 8 (Ana)~~ ~~- Make plates (Althys)~~	~~- ALTHYS IS GONE~~ ~~- Progress report 3~~ ~~**- ImageJ Analysis done by the latest -> find best density and protocol (to make new spheroids for qPCR)**~~ ~~- Hopefully Carley takes plates to the fridge~~	- ALTHYS IS GONE - Passage 9 - Make spheroids (retry experiments from last Friday 8/2) **- Talk to Dr Hess and Carley about: qPCR vs immunostaining for SOX2, possibility of live/dead staining (highly likely not go with this), 3D kit for CellTiter Glo**
2/17 - 2/21	-Passage 10 -Image spheroids, then suck out media and add in new 100uL serum free media - Seed cells for positive and add media for negative control		-Passage 11 -ALTHYS IS GONE - 2D Cell TiterGlo for plates on 2/14	- ALTHYS IS GONE - Progress report 4	- ALTHYS IS GONE - Passage 12

- Cell-titer Glo stain (2/14)
  - Measures ATP in media
  - We measured 20 minutes after changing media
  - Needs 1 Cell-titer Glo:1 Old media
- Analyze Cell-titer Glo results
  - A higher number correlates to a higher level of metabolic cells and a higher level of ATP
  - Some variations between the groups
  - Average, find SD, and compare to control
  - 

- Order Cell-titer Glo 3D?
  - Gaelen said that inner cells will mostly be dead, so it way be useless to show ATP concentration
  - 2D Assay is proven to work for these comparisons
  - $152
- Order SOX2 Antibody or qPCR primers?
  - Antibody
    - Let incubate 1 hour
    - PFA (4%) for spheroids to fix
  - Same as Gamma-H2AX stain, so go with qPCR
- SOX2 Primer for qPCR
  - Send to Gaelen ASAP
- Repeat spheroid experiment (see timeline above)
  - Image spheroids Monday
  - Spin down and replace media with 100uL

    - Wait 2 days then use Cell-titer Glo

Conclusions & Action Items:

- Start next spheroid experiment by seeding cells (2/14)
- Analyze Cell-Titer Glo experiment 1
- Ask Carley to book BioTek for imaging and luminescence measurement
- Set meeting for qPCR workflow breakdown (2/21)
    - Read protocols Carley sends us
    - Send a follow up email to schedule meeting for (2/21)

Client Meeting 4_2/21/2025

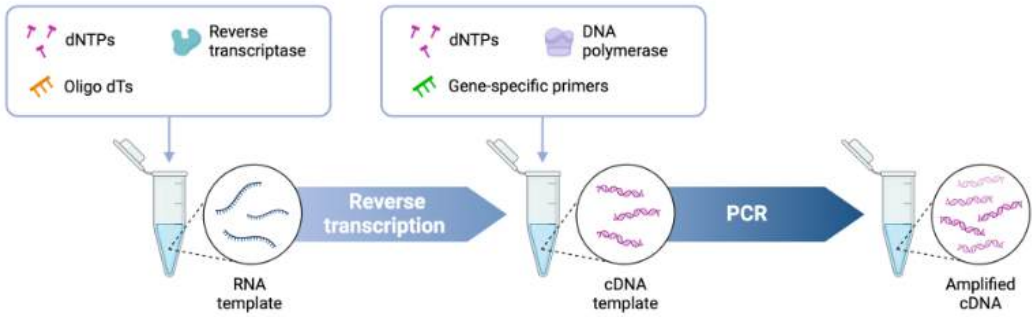
Emily Rhine - Feb 21, 2025, 8:06 PM CST

Title: Client Meeting_RT-qPCR
Date: 2/21/25
Present: Emily, Ana, Julia, & Jayson
Content:
***CARLEY GONE MARCH 13TH-15TH (interview)**

- **Busy at work Mon 2/24, Fri 2/28 end of afternoon**

Please fill out your availability to help plan out each section of RT-qPCR!
https://www.when2meet.com/?29177370-GDEJR
2-3 people per step, preferably different people each time!

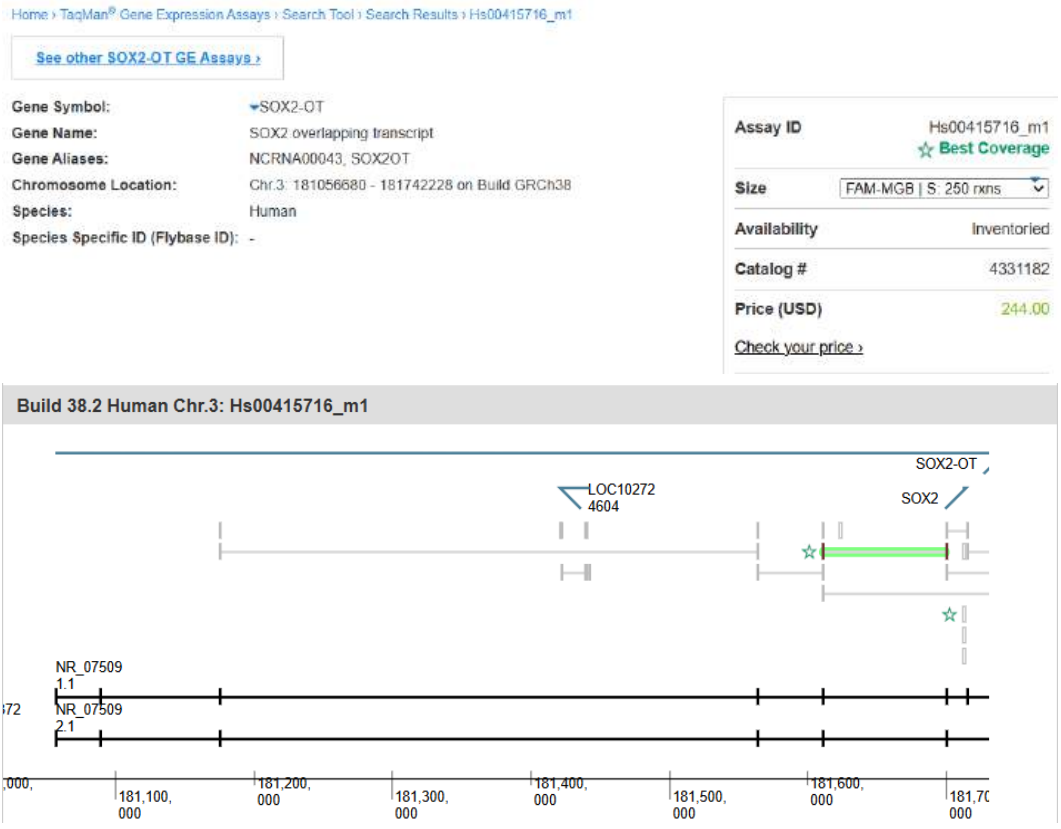
- Dissociate spheroids and count doubling time
  - Transfer from 96 well to 24 well to get 2 million cells per sample
- qPCR Workflow Lecture
  - See benchling for complete protocol
  - Main Steps
    - RT
      - Our protocol is “two-step” - RT step is not a stand-alone step



      - Can go from messenger RNA (mRNA) → complementary DNA (cDNA)
    - qPCR
  - Steps Overview
    - Day 1 (est. 1-2 hours → generous)
      - RNA extraction - reverse transcriptase
        - Use protocol that uses Qiashredder
        - *Need 2-3 million cells per sample → 3x spheroid, 3x 2D
    - Day 2 (or 3)
      - cDNA synthesis - made from RNA extracted (est. 2hrs incubate, 3hrs total)
        - Use an Oligo dT - single DNA strand with all “T’s” that binds to poly-A tail of mRNA (of ALL cells, not just SOX2)
          - Acts like a “primer” and reaction with DNTPs (single nucleotides) to synthesize cDNA
        - **NOT SOX2 specific at this point**
        - Add water, buffers, enzymes (to get bases for cDNA). heat and then ice for 5 min for

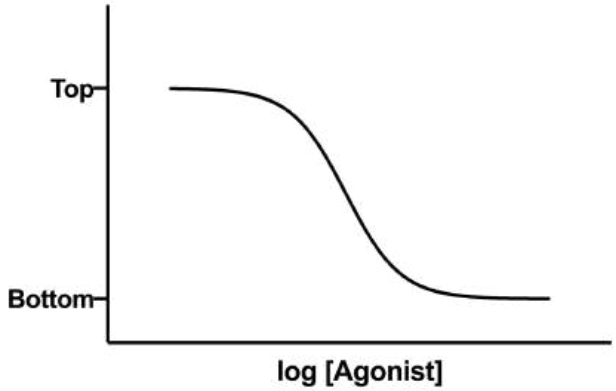
reaction
    - ** Optional stop: at -80 for 6 months, at -20 for 1 month
- qPCR (est. 1hr setup, 4-5hr total)
    - Spheroid cDNA x4 (x3 for RNA extraction tubes), 2D cDNA x4 (x3 for RNA extraction tubes) → 24 total tubes
    - Master mix (Taqman) for master mix for aliquot (round up to estimate 30 tubes/sets)
        - M1: 1uL B-actin , 4uL RNAse free water, 12uL Master mix
        - M2: (M1)*0.5, add spheroid/2D cDNA to each M2 tube (each will have 4x 20uL replicates)

- qPCR primer research
    - TaqMan (spans exons), human, SOX2, and extra small
    - https://www.thermofisher.com/taqman-gene-expression/product/Hs00415716_m1?CID=&ICID=&subtype=

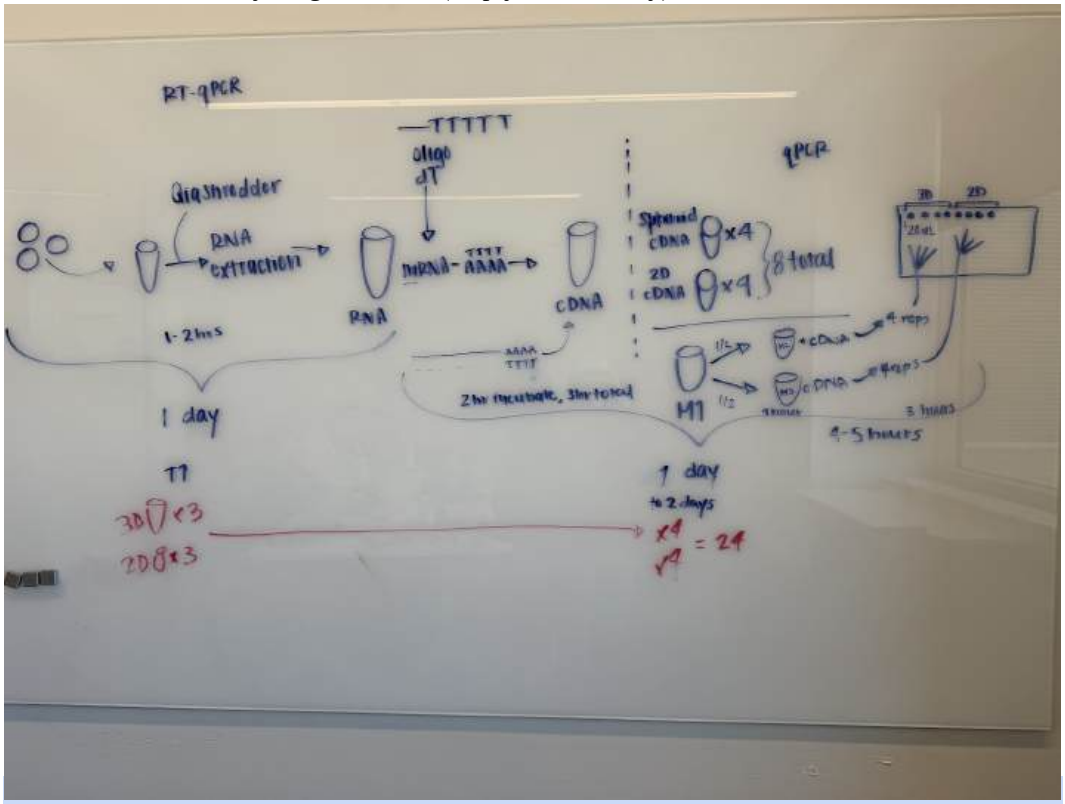


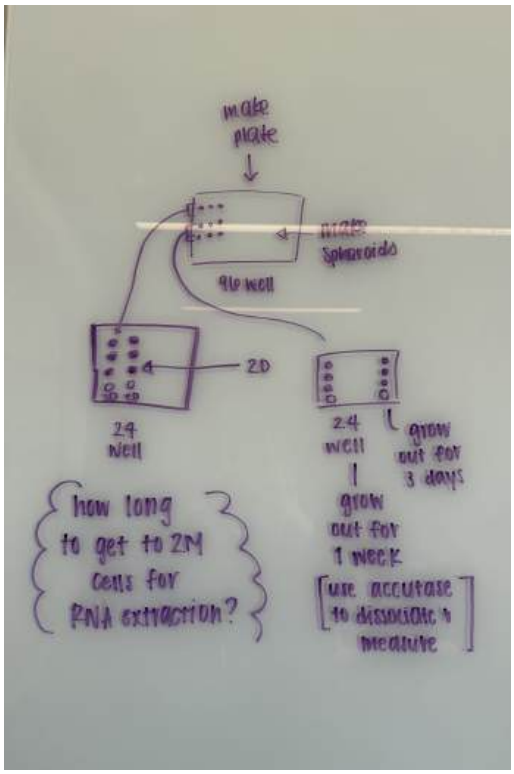
- Discussed Timeline

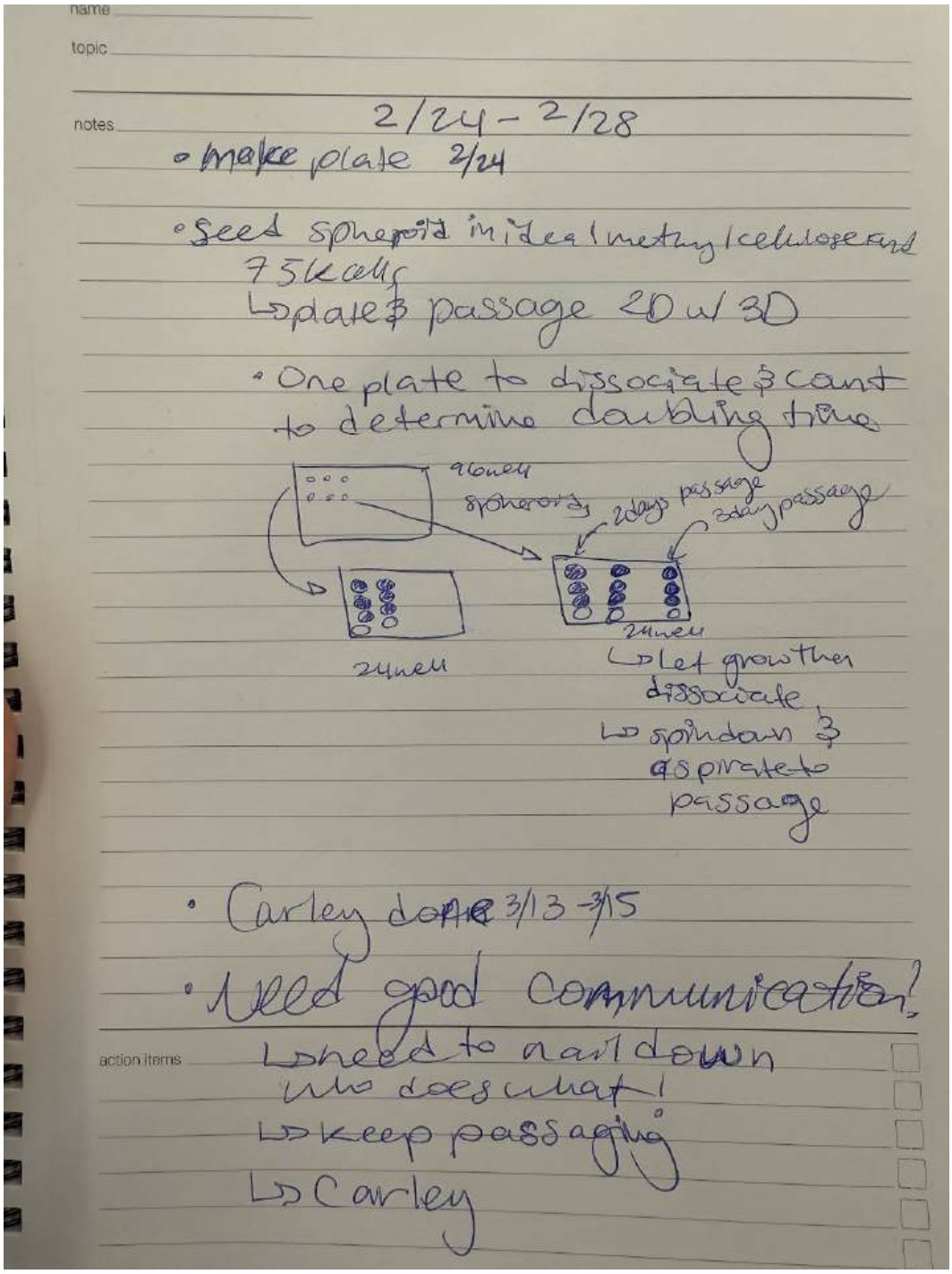
2/10 - 2/14	~~- Celltiter glo~~ ~~- Passage 7~~ ~~(Althys + Julia)~~	Example Primer link: https://www.novusbio.com/products/gapdh-primer_nbp1-71650?srsltid=AfmBOoomNZhzD0tr-UtfhYgyXTL1Ij5I6EC309CpwIo51bbFjdWigMof	~~- Passage 8 (Ana)~~ ~~- Make plates (Althys)~~	~~- ALTHYS IS GONE~~ ~~- Progress report 3~~ ~~**- ImageJ Analysis done by the latest -> find best density and protocol (to make new spheroids for qPCR)**~~ ~~- Hopefully Carley takes pintes to the fridge~~	- ALTHYS IS GONE - Passage 9 - Make spheroids (retry experiments from last Friday 8/2) **- Talk to Dr Hess and Carley about: qPCR vs immunostaining for SOX2, possibility of live/dead staining (highly likely not go with this), 3D kit for CellTiter Glo**
2/17 - 2/21	~~-Passage 10~~ ~~-Image spheroids, then suck out media and add in new 100uL serum free media~~ ~~- Seed cells for positive and add media for negative control (Althys, Julia, Ana)~~		-Passage 11 -ALTHYS IS GONE - 2D Cell TiterGlo for plates on 2/14	- ALTHYS IS GONE - Progress report 4	- ALTHYS IS GONE - Passage 12 - Analysis of spheroid size (Julia) - CellTiter Glo data analysis (Julia & Gang) **Choose density & methylcellulose concentration**

- Higher density is fine in terms of cell death for Cell-Titer Glo
  - Complete the rest of the analysis and select ideal methylcellulose concentration
  - Ask about graph type?
    - IC-50 type curve
      - Y-axis: luminescence
      - X-axis: condition (density, MC)
  - 

- Normalize everything to control (empty - media only)




make plate
96 well
2D
24 well
2.4 well
grow out for 3 days
grow out for 1 week
how long to get to 2M cells for RNA extraction?
use accutase to dissociate + measure



Conclusions & Action Items:

- SOX2 primer ordered
- Plan ahead timeline/get more definitive with all team members
- Start making plates monday 2/23/25
- Update notebook for those who haven't
- Please fill out your availability to help plan out each section of RT-qPCR!
    - https://www.when2meet.com/?29177370-GDEJR
- Plan out step by step breakdown

Client Meeting 5_Data Analysis_2/28/25

Emily Rhine - Feb 28, 2025, 1:30 PM CST

Title: Client Meeting
Date: 2/28/25
Present: Emily, Ana, Julia, & Jayson
Content:

- Discussed Timeline

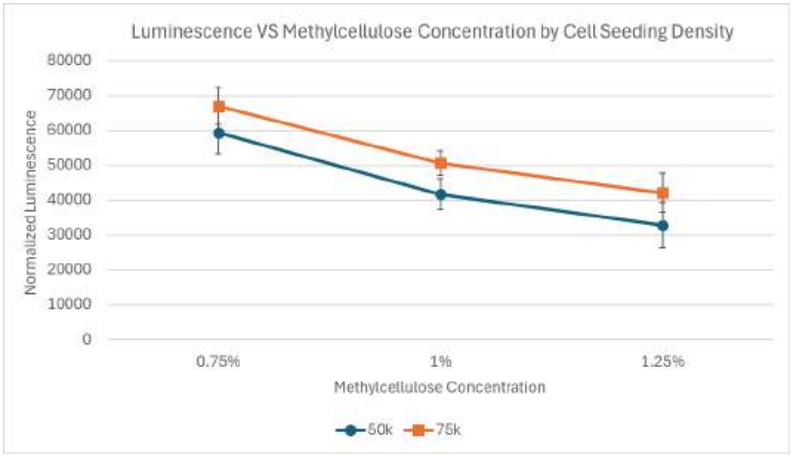
2/24 - 2/28	~~-Passage 13 -Make plates (Ana) -Carley Busy~~	-Team Meeting	**PRELIMINARY REPORT DUE** -Passage 14 (Althya) - Make 1x96-well & 2x24-well plates (Althya) -Seed spheroids with chosen density and methylcellulose concentration (set 1) (Emily)		-Progress report 5 -Hopefully Carley takes plate to the fridge	- Passage 15 - receive primers for SOX2/qPCR - seed spheroids (set 2) (Jayson)
3/3 - 3/7	-Passage 16 - Passage spheroids (set 1-5 days, set 2-3 days) - dissociate via Accutase - determine doubling time for both populations, if not 2 million, reseed and wait for however long based on td/if 2 million, seed new population of spheroids)	-Team Meeting	-Passage 17		-Progress report 6	-Carley busy later
3/10 - 3/14	-Passage 18 (Vial3) -Passage 1 (Vial 4) - qPCR for SOX2 (tentatively) Step 1 mRNA extraction (2hr)		-Passage 19 (Vial3) OR KILL -Passage 2(Vial 4) -qPCR (step2) cDNA isolation (2hr)		-Progress report 7 *CARLEY GONE MARCH 13TH-15TH (interview)	-JULIA IS GONE *CARLEY GONE MARCH 13TH-15TH (interview)
3/17 - 3/21	-qPCR for SOX2 step 3				-Progress report 8	JAYSON MIGHT BE GONE

Agenda:

- how best to interpret luminescence data
  - How to standardize? (Current method: Compare with no cell/only media - deduct the mean from obtained data)
- Show spheroid size and count graphs
  - Anything we can add/edit?
- Do we need to change optimal density and methylcellulose concentration?
  - Original choice: Condition D (75k cells/cm^2 cell density, 0.75% methylcellulose)
- (show graphs!)

Content:

- Result analysis - show graphs, luminescence data, spheroid size and count graphs (which one to choose), do we need to change optimal density and mc concentration?
  - Don’t need to normalize what we seeded at
    - We expect the ones with more cells to have more luminescence
    - Average wells and normalize to media only control wells by subtracting background luminescence → don’t divide by seeding density (anything)
    - “Average all conditions/controls, then subtract average control from average of replicates”
    - Error bars can be done, standard deviation within replicates & control
  - Should we do a T-test for p-values? With significant asterisks?
    - PRISM? Yes Hess lab prism account available
  - How can we do a volume to volume comparison?

        - Sphere comparison rather than surface area
            - Sphere - know area → can find radius → volume = 4/3 * pi * r^3
        - Cells in 2D do have a thickness (assume 3-5 micron thick - look up literature?)
        - Per Carley, unneeded! No excessive cell death at either seeding density so cell death is not a concern
            - No plateau of cell confluency - cells are still growing
            - In implications: “per Cell Titer Glo, have not hit the limit, don’t wanna hit it so choose the higher seeding density”
                - Point of cell titer glo: no conditions are truly killing the cells, so good!
    - 

        - Higher seeding density is better for CRISPi and qPCR in addition to cell luminescence and ideal spheroid size/shape
    - New PolyHEMA protocol - Gaelen suggested?
        - Don’t need to filter! → just make sure bottle is sterile
        - **Powder added to ethanol** rather than ethanol being added to powder
            - Only affects dissolution rate, not filtration ease
        - UV sterilize each plate after dried (can do in TC hood, ~30 min)

Conclusions & Action Items:

- Continue passaging spheroids to determine doubling time (2/28)
- Finish/edit preliminary report based on finalized graphs
- Figure out dates/times that will work for team + Carley for qPCR
- Future Experiments:
    - Match amount of cells/confluency using standard cell doubling time and spheroid doubling time
    - At time = 3 days and t=5 days (good information to know)

Client Meeting 6_3/14/25

Emily Rhine - Mar 14, 2025, 1:27 PM CDT

Title: Client Meeting - Progress Update
Date: 14.3.25
Progress update:

- still struggling w finding doubling time for spheroids
    - Seeded new spheroids this week
    - Will do the accutase dissociation trial again next week
- (Ask for advice for accutase dissociation troubleshooting tips?)
    - Because accutase dissociation is still unsuccessful, can't get to gammaH2AX staining yet
- Wont start with qPCR and gammaH2AX staining until after spring break
    - Team will prioritize gammaH2AX staining

Content:

- Only remove 80uL using P200 when aspirating old media to limit amount of cells lost (this is for feeding for 1 well in a 96-well plate)
- During accutase dissociation:
    - Sometimes cell pellet still moves after centrifugation (PolyHema)
        - Spin down in the plate?
        - Compare 24 v.s. 96
    - Prevent transfer loss
        - Wash plate with PBS
        - Hard to pellet cells on the bottom
    - Prevent cell death
        - Bubbles
        - Drying out
- Some cell death is expected with spheroids due to necrotic core
    - Can we find an average cell death and predict/prepare for it for qPCR
- Doing well so far with changing/improving a few element with each iteration
    - Is loss predictable? Just account for loss?
        - (maybe account for it in the doubling time? Pretend nothing is lost?)
- Seed new PolyHema plates today (3rd patch)
    - Stock was not sterilized
    - Plates were sterilized after polyHEMA dried up under UV for 30 min
- Continue with SFM & full DMEM spheroid seeding
    - Redo CellTiter-Glo comparison
- About seeding spheroids and stuff:
    - Everything was done on serum-free media
    - Serum has a bunch of growth factors - biggest difference compared to SFM
    - Don't know if SOX2 expression is serum-driven or not

Conclusions & Action Items:

- Continue focus on spheroid formation and dissociation optimization
- Continue with SFM & full DMEM spheroid seeding
    - Redo CellTiter-Glo comparison
- What drives SOX2 expression?
    - Is it GFs in Full DMEM?

    - Does it require a starvation state: SFM?
        - Takes a day or two to adapt, meaning if we add full DMEM just before qPCR reaction it won't affect as much
- Send preliminary report to the client

Client Meeting 7_3/21/25

Emily Rhine - Mar 21, 2025, 3:05 PM CDT

Title: Client Meeting - Progress Update
Date: 3/21/25
Content:

- Will plan to do qPCR
- GammaH2AX staining?
- Option 1:
  - Seed 24 well
  - Transfer to 6 well after 5 days
- Option 2:
  - Start with 6 well plate right away
  - Dissociate 1 well at day 5 → if not by 1.5 million let grow a couple more days
  - When at 1.5 mill per well, combine two wells into 1 to get >2 mill per sample
    - If slightly below 2 million per sample that should work as well
- Some cell death is expected with spheroids due to necrotic core
  - Can we find an average cell death and predict/prepare for it for qPCR
- Dates in April Carley is gone
  - April 11, 14, 15th
- If split off,
  - 2 ppl on gammaH2AX staining
  - 3 ppl on RT-qPCR
- Staining timeline
  - Need ~1 million per sample
    - Exp group: 3D, control group: 3D, both treated with etoposide (treated for 24 hours ONLY after spheroid formation, then dissociate right away)
  - 45 min to dissociate spheroids?
  - 1 hour incubation with Ab
  - Imaging, Analysis
  - Total: 2-3 hours
  - Exp 1: Spheroid Gamma-H2AX stain with (Etoposide) and without drug
    - 6 well
    - 3 3D
    - 3 3D drug
  - Exp 2: Spheroid and Gamma-H2AX

Color key	Past day	Team member not in town	Deliverable due	upcoming day	upcoming day
Week	Monday	Tuesday	Wednesday	Thursday	Friday
3/24 - 3/28	Spring Break				
3/31 - 4/4	Make PolyHEMA	Coat 6 well plate Team meeting 4pm - 5pm	Seed 6 well plate	-Progress report 9	
4/7 - 4/11	- qPCR for SOX2 (tentatively) Step 1 mRNA extraction (2hr) -1st trial of 3D gamma H2AX (based on Hess Lab's 2D protocol) + 2D gammaH2AX	qPCR (step2) cDNA isolation (2hr)	-qPCR for SOX2 step 3 qPCR (5hrs)	-Progress report 10	- Engineering Expo (ask about running booth (tracy Puccinelli) for outreach) CARLEY GONE!

Conclusions & Action Items:

- Make more PolyHema

- Message Carley with passaging instructions
- Schedule rt-qPCR and Gamma-H2AX follow up meeting
- Enjoy break

Client Meeting 8_4/18/25

Emily Rhine - Apr 18, 2025, 1:21 PM CDT

Title: Client Meeting - Experiment Repeats and Data Analysis
Date: 4/18/25
Content:

- Preliminary report feedback to add to draft of final report
- Gamma-H2AX
    - Added drug 4/18 and seeded 2D
    - finish trial 2: 4/19 (9 am)
    - Analyze Gamma-H2AX data 4/21
        - Flo Jo (make figures)
        - Excel (raw data from gating) → do t-test through excel
- qPCR:
    - no amplification for SOX2 (concerning!)
    - low 20s Ct values for GAPDH for 3D cells (good expression of housekeeping gene) → nothing wrong with cDNA
        - Another concern, not enough cDNA → do smaller dilution (1:4 or 1:5 instead of 1:10)
    - Redo step 3 with *smaller* dilution (1:4), some errors *may* happen during master mix formation
        - Not enough time to redo all the way from step 1 :(
        - FAM (for SOX2) and VIC (for GAPDH) probes
- PRISM
    - Redo statistics for spheroid size and quantity
    - TBD for Gamma-H2AX

Conclusions & Action Items:

- Don’t need to thaw another flask
- Redo step 3 qPCR (Monday 4/21) 8am
- Finish final poster
    - Poster session April 25th 12-2pm
- Continue work on final report

Meeting 1_1/31/25

Emily Rhine - Jan 31, 2025, 12:19 PM CST

Title: Advisor Meeting 1_Timeline
Date: 1/31/25
Present: Entire team
Content:

- Redo spheroid experiment with 50k and 75k seeding densities to confirm results:
  - Alter methylcellulose levels to confirm optimization
  - Test spheroid dissociation with Accutase protocol
  - Live dead staining
- Make a reliable plan/timeline for the project and stick to it!
  - Caution: deadline is closer than it appears
- He has no other advice for things to add/change for the project
- Project outreach
  - At engineering Expo is definitely an option
    - Date: April 11th 9 am-12 pm (schools day), April 12th 9 am-12 pm (community day)
    - Might need to change delivery method a bit
  - Ask professor Tracy Puccinelli for more logistics

Conclusions & action items:

- Propose timeline to client
- Continue work on preliminary presentation
- Update notebook
- Continue research as needed not just "one entry a week"

Meeting 2_2/14/25

Emily Rhine - Feb 14, 2025, 4:31 PM CST

Title: Advisor Meeting
Date: 2/14/25
Present: Emily, Ana, & Julia
Content:

- Revisit timeline:

2/10 - 2/14	~~- Celltiter glo~~ ~~- Passage 7~~ ~~(Althys + Julia)~~	Example Primer link: https://www.novusbio.com/products/gapdh-primer_nbp1-71650?srsltid=AfmBOoomNZhzD0tr-UtfhYgyXTL1Ij5I6EC309CpwJo51bbFjdWigMof	~~- Passage 8 (Ana)~~ ~~- Make plates (Althys)~~	~~- ALTHYS IS GONE~~ ~~- Progress report 3~~ ~~**- ImageJ Analysis done by the latest -> find best density and protocol (to make new spheroids for qPCR)**~~ ~~- Hopefully Carley takes plates to the fridge~~	- ALTHYS IS GONE - Passage 9 - Make spheroids (retry experiments from last Friday 8/2) **- Talk to Dr Hess and Carley about: qPCR vs immunostaining for SOX2, possibility of live/dead staining (highly likely not go with this), 3D kit for CellTiter Glo**
2/17 - 2/21	-Passage 10 -Image spheroids, then suck out media and add in new 100uL serum free media - Seed cells for positive and add media for negative control		-Passage 11 -ALTHYS IS GONE - 2D Cell TiterGlo for plates on 2/14	- ALTHYS IS GONE - Progress report 4	- ALTHYS IS GONE - Passage 12

- Client meeting 2/14 1:00-1:30 over zoom
    - Order Cell-titer Glo 3D
    - Order SOX2 Antibody or primers?
    - Decide with finality qPCR or antibody
- Cell-titer Glo stain
    - Measures ATP in media
    - We measured 20 minutes after changing media
    - Needs 1 Cell-titer Glo:1 Old media
- Repeat spheroid experiment (see timeline above)
    - Image spheroids Monday
    - Spin down and replace media with 100uL
    - Wait 2 days then use Cell-titer Glo
- Recommended fluorescence imaging (Calcium)
- Antibody
    - Let incubate 1 hour
    - PFA (4%) for spheroids to fix

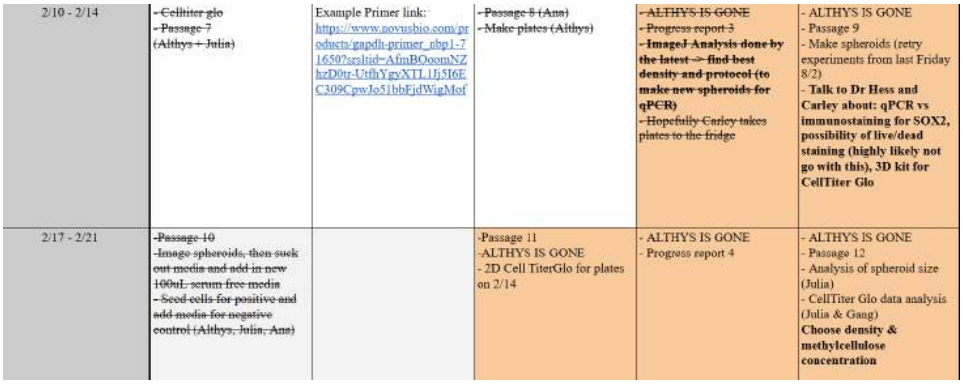
Conclusions & Action Items:

- Advisor approved plan
- Advisor recommended looking into fluorescence imaging (Calcium)
- Prepare for client meeting today

Meeting 3_2/21/25

Emily Rhine - Feb 21, 2025, 12:57 PM CST

Title: Advisor Meeting
Date: 2/21/25
Present: Emily, Ana, Julia, & Jayson
Content:

- Discussed Timeline
  - 

2/10 - 2/14	~~- Celltiter glo~~ ~~- Passage 7~~ ~~(Althys + Julia)~~	Example Primer link: https://www.novusbio.com/products/gapdh-primer_nbp1-71650?srsltid=AfmBOoomNZhzD0tr-UtfhYgvXTL1Ij5I6EC309CpwIo51bbFjdWigMof	~~- Passage 8 (Ana)~~ ~~- Make plates (Althys)~~	~~- ALTHYS IS GONE~~ ~~- Progress report 3~~ ~~**- ImageJ Analysis done by the latest -> find best density and protocol (to make new spheroids for qPCR)**~~ ~~- Hopefully Carley takes plates to the fridge~~	- ALTHYS IS GONE - Passage 9 - Make spheroids (retry experiments from last Friday 8/2) **- Talk to Dr Hess and Carley about: qPCR vs immunostaining for SOX2, possibility of live/dead staining (highly likely not go with this), 3D kit for CellTiter Glo**
2/17 - 2/21	~~-Passage 10~~ ~~-Image spheroids, then suck out media and add in new 100uL serum free media~~ ~~- Seed cells for positive and add media for negative control (Althys, Julia, Ana)~~		-Passage 11 -ALTHYS IS GONE - 2D Cell TiterGlo for plates on 2/14	- ALTHYS IS GONE - Progress report 4	- ALTHYS IS GONE - Passage 12 - Analysis of spheroid size (Julia) - CellTiter Glo data analysis (Julia & Gang) **Choose density & methylcellulose concentration**

- Discussed preliminary report due 2/26/25
  - We will discuss spheroid experiment results & luminescence there
- qPCR primer research
  - TaqMan (spans exons), human, SOX2, and extra small/small
  - https://www.thermofisher.com/taqman-gene-expression/product/Hs00415716_m1?CID=&ICID=&subtype=
- LabArchives Notebook
  - Keep up individual and team notebook to see contributions
  - Add individual research
  - Catchup before mid semester checkpoint
  - Can be used for a letter of recommendation for design work
- Feedback Fruits

Conclusions & Action Items:

- Update LabArchives
- Continue work on Preliminary Report
- Attend client meeting 1-2pm 2/21/2025

Meeting 4_2/28/25

Emily Rhine - Feb 28, 2025, 12:57 PM CST

Title: Advisor Meeting
Date: 2/28/25
Present: Emily, Ana, Althys, Julia, & Jayson
Content:

- Discussed Timeline

2/24 - 2/28	~~-Passage 13~~ ~~-Make plates (Ana)~~ ~~-Carley Busy~~	-Team Meeting	**PRELIMINARY REPORT DUE** -Passage 14 (Althys) - Make 1x96-well & 2x24-well plates (Althys) -Seed spheroids with chosen density and methylcellulose concentration (set 1) (Emily)		-Progress report 5 -Hopefully Carley takes plate to the fridge	- Passage 15 - receive primers for SOX2/qPCR - seed spheroids (set 2) (Jayson)
3/3 - 3/7	-Passage 16 - Passage spheroids (set 1-5 days, set 2-3 days) - dissociate via Accutase - determine doubling time for both populations, if not 2 million, reseed and wait for however long based on td if 2 million, seed new population of spheroids)	-Team Meeting	-Passage 17		-Progress report 6	-Carley busy later
3/10 - 3/14	-Passage 18 (Vial3) -Passage 1 (Vial 4) - qPCR for SOX2 (tentatively) Step 1 mRNA extraction (2hr)		-Passage 19 (Vial3) OR KILL -Passage 2(Vial 4) -qPCR (step2) cDNA isolation (2hr)		-Progress report 7 *CARLEY GONE MARCH 13TH-15TH (interview)	-JULIA IS GONE *CARLEY GONE MARCH 13TH-15TH (interview)
3/17 - 3/21	-qPCR for SOX2 step 3				-Progress report 8	JAYSON MIGHT BE GONE

- Discussed preliminary report due 2/26/25 (Monday 3/3 for us)
    - We will discuss spheroid experiment results & luminescence there
- qPCR primer research
    - TaqMan (spans exons), human, SOX2, and extra small/small
    - https://www.thermofisher.com/taqman-gene-expression/product/Hs00415716_m1?CID=&ICID=&subtype=
    - Order arrived at Hess lab
- LabArchives Notebook
    - Keep up individual and team notebook to see contributions
    - Add individual research
- Result analysis - show graphs, luminescence data, spheroid size and count graphs (which one to choose), do we need to change optimal density and mc concentration?
    - Spheroid area used - 2D imaging
    - How to interpret
    - How to account for scaling by initial cell count - seed in 2D control for 50k cells/cm^2 and 75k cells/cm^2
    - Aimed for 100% confluency in 2D by cell titer glo
    - Should we find the % of cells that form spheroids?
        - Probability of formation?
            - Spheroid count/total live cell count?
            - qPCR?
    - How can we do a volume to volume comparison?
        - Sphere comparison rather than surface area
            - Sphere - know area → can find radius → volume = 4/3 * pi * r^3
        - Cells in 2D do have a thickness (assume 3-5 micron thick - look up literature?)
- Future Experiments
    - Match amount of cells/confluency using standard cell doubling time and spheroid doubling time
    - Calculate starting seeding density for 2D - plan for 3 days and 100% confluency at the end

    - Account for different doubling times
    - Live count in 2D and 3D
- Ask the client if we can do some 3D imaging

Conclusions & Action Items:

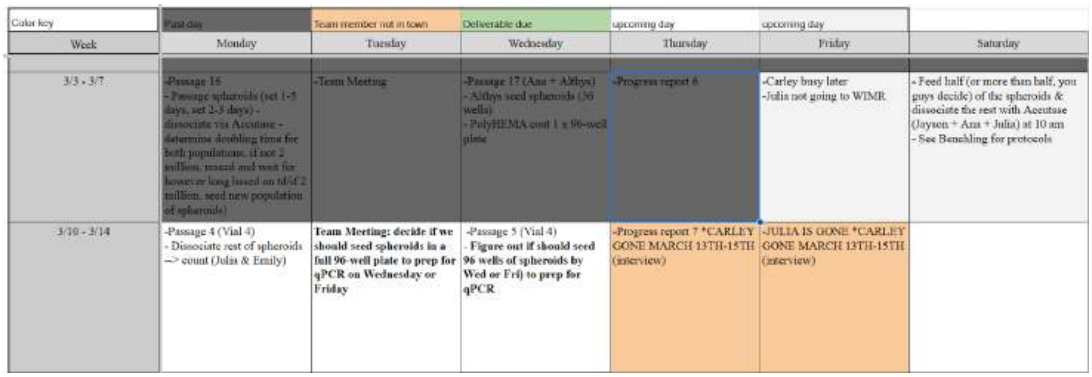
- Preliminary report due 2/26/25 (Monday 3/3 for us)
- Update LabArchives
- Attend client meeting 1-2pm 2/28/2025

Meeting 5_3/7/25

Emily Rhine - Mar 07, 2025, 12:32 PM CST

Title: Advisor meeting_Week 7
Date: 3/7/2025
Content:

- Discussed Timeline



Color key	Past day	Team member not in town	Deliverable due	upcoming day	upcoming day	
Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
3/3 - 3/7	-Passage 16 - Passage spheroids (set 1-5 days, set 2-3 days) - dissociate via Accutase - determine doubling time for both populations, if not 2 million, reseed and wait for however long based on tdif 2 million, seed new population of spheroids)	-Team Meeting	-Passage 17 (Ana + Althys) - Althys seed spheroids (36 wells) - PolyHEMA coat 1 x 96-well plate	-Progress report 6	-Carley busy later -Julia not going to WIMR	- Feed half (or more than half, you guys decide) of the spheroids & dissociate the rest with Accutase (Jaysen + Ana + Julia) at 10 am - See Benchling for protocols
3/10 - 3/14	-Passage 4 (Vial 4) - Dissociate rest of spheroids --> count (Julia & Emily)	**Team Meeting: decide if we should seed spheroids in a full 96-well plate to prep for qPCR on Wednesday or Friday**	-Passage 5 (Vial 4) - **Figure out if should seed 96 wells of spheroids by Wed or Fri) to prep for qPCR**	-Progress report 7 *CARLEY GONE MARCH 13TH-15TH (interview)	-JULIA IS GONE *CARLEY GONE MARCH 13TH-15TH (interview)	

- First time completing accutase protocol
  - Establish doubling time
- Scaled up to 24 well plate
  - 3 million cells/plate
- 1 T150 or 2 T75 flasks
- Pause steps over spring break
  - 2 steps before 1 step after break
- No 3D stain would still have to optimize for spheroids in Gamma-H2AX
  - Not doing SOX2 antibody
  - Paul: “Can stain with anything → not super hard to optimize” …
    - Emily’s brain cancer paper: used Trypan Blue for 3D staining
    - https://currentprotocols.onlinelibrary.wiley.com/doi/full/10.1002/cpz1.357
- Recap preliminary report
  - Write it as a protocol? (ex: Nature Protocols, Nature Methods – a bit less detail)
  - Write as cancer paper? (Cancer Research)
  - See comments left by advisor on emailed PDF

Conclusions & Action Items:

- Send prelim report to client!
- Read over Dr. C’s comments for prelim report

Meeting 6_3/14/25

Emily Rhine - Mar 14, 2025, 12:28 PM CDT

Title: Advisor Meeting_Week 8
Date: 3/14/2025
Content:

- Discussed Timeline
    - Seed today
    - Accutase wednesday (3/19)
- Progress update
    - Still struggle with finding doubling time
        - Biggest hypothesis: problem with accutase dissociation protocol
        - Have found ways to troubleshoot, will do it again next week (this week is for making plates and seeding more spheroids)
        - **How to avoid aspirating spheroids**
            - Focus on optimizing this!
    - gammaH2AX and qPCR together after spring break
        - will focus on gammaH2AX staining
- Advisor gone 9 days in April (will miss 2 meetings!)

Conclusions & Action Items:

- Send prelim report to client!
- Show and Tell next friday (3/21)
- Schedule a Thursday meeting (2/20) 5:15- 5:30 pm (Zoom)
- Main problem to address: How to avoid aspirating spheroids
    - One way: when feeding cells, only remove half of the media and replace with new media

Meeting 7_3/20/25

Emily Rhine - Mar 23, 2025, 4:42 PM CDT

Title: Advisor meeting_Week 9
Date: 3/20/2025
Content:

- Discussed Timeline
  - Accutase Friday (3/21)
- Show and tell tomorrow
- Progress update
  - Full DMEM
  - Optimize for 24 well plate rather than 96 well
    - Learned that spinning down on 96 well isn't efficient
    - Alternate: use PBS washes after transferring spheroids+media
  - gammaH2AX and qPCR together after spring break
    - will focus on gammaH2AX staining
- Advisor gone 9 days in April (will miss 2 meetings!)
- Gamma-H2AX
  - Antibody penetration – get through at most 30 micron (diameter) spheroids
    - Our spheroids were approx. 6000 um^2 (~87 micron)
    - May suggest we may have to dissociate, then stain
- Carley planning to passage our flasks for us over Spring Break!
  - Will talk to her with more details tomorrow at client meeting
- Make changes to preliminary report suggested by Paul
  - Work on a more formal prose
- Show and Tell tomorrow
  - Look at google doc/email before?
- Executive summary draft

Conclusions & Action Items:

- Attend show and tell
- Attend client meeting
- See timeline

Meeting 8_4/4/25

Emily Rhine - Apr 04, 2025, 12:28 PM CDT

Title: Advisor Meeting_Week 11
Date: 4/4/2025
Content:

- Advisor is gone
  - Meet advisor 4/8 at 4:00pm in-person (innovation meeting room)
  - Advisor gone 4/10- 4/19
- Discussed Timeline
  - Start qPCR monday
  - Seed second plate for gamma-H2AX
- 6 well plates to have ~6 million cells per sample
  - RT-qPCR team
    - Ana
    - Althys
    - Julia
  - gammaH2AX staining
    - Jayson
    - Emily
- Gamma-H2AX Recap
  - Antibody penetration – get through at most 30 micron (diameter) spheroids
    - Our spheroids were approx. 6000 um^2 (~87 micron)
    - May suggest we may have to dissociate, then stain?
- Executive summary draft
  - Advice/Feedback?

Conclusions & Action Items:

- Attend client meeting at 1pm in WIMR
- Begin final report – assign sections
- Poster session 4/25

Meeting 9_4/8/25

Emily Rhine - Apr 08, 2025, 4:14 PM CDT

Title: Advisor Meeting_Week 11
Date: 4/8/2025
Content:

- Advisor gone 4/10- 4/19
- Discussed Timeline
    - Start qPCR monday
    - Seed second plate for gamma-H2AX
- RT-qPCR team
    - Ana
    - Althys
    - Julia
- Gamma-H2AX Trial 1 4/8
    - Jayson
    - Emily
- Gamma-H2AX Redo: next week M-F
    - 6 well plates to have ~6 million cells per sample
    - Jayson
    - Emily
- Executive summary final draft due 4/18
    - Advice/Feedback?

Conclusions & Action Items:

- Expo this friday April, 11
    - Outreach
- Begin final report
- Poster session 4/25

Meeting 10_4/22/25

Emily Rhine - Apr 22, 2025, 4:19 PM CDT

Title: Advisor Meeting 10
Date: 4/22/2025
Content:

- qPCR
  - GAPDH good amplification
  - SOX2 bad primer or bad assay for A549
  - CT values on poster
- Gamma-H2AX
  - Not enough Drug
  - 2D and 3D
- Finish poster
- Submit final deliverables

Conclusions/ Action items:

- All deliverables due May 6th
- May 7th 5pm Dinner with Paul at the Terrace Mem U

Team Meeting 1_1/31

Emily Rhine - Feb 17, 2025, 11:16 AM CST

Title: Team meeting 1
Date: 1/31
Content:
Goals: discuss the following:

- Presentation
- Trial CRISPR or drug treatment for gammaH2AX staining
- Do we still want to do qPCR
- Experiment timeline
- PolyHEMA stock protocol + coating (should we do 2 coats??)

Bureaucracy

Experiments

Week	Monday	Tuesday	Wednesday	Thursday	Friday
1/27 - 1/31	-Passage 1		-Passage 2	-Progress report 1	-Passage 3 -Client/Advisor Meeting
2/3 - 2/7	-Passage 4 -PolyHema Plate	Filter PolyHema stock	-Passage 5 (Ana) -Make plates (Althys)	-Progress report 2 -Hopefully Carley takes the plates to the fridge	-Presentation -Make spheroids (Althys + Jayson)
2/10 - 2/14	Celltiter glo - Image spheroids: do 2 sets of photos per - Accutase dissociation + flow cytometry			-Progress report 3	
2/17 - 2/21				-Progress report 4	
2/24 - 2/28				-Progress report 5	
3/3 - 3/7				-Progress report 6	
3/10 - 3/14				-Progress report 7	
3/17 - 3/21				-Progress report 8	
3/24 - 3/28	Spring Break				
3/31 - 4/4				-Progress report 9	
4/7 - 4/11				-Progress report 10	- Engineering Expo
4/14 - 4/18				-Progress report 11	
4/21 - 4/25					Final poster presentation
4/28 - 5/2					

- Selecting a suitable journal
    - Nature: Cell death and disease
    - Cancer: an international interdisciplinary journal of the American Cancer Society
        - AACR
    - BMC Cancer

Team Meeting 2_2/11/25

Emily Rhine - Feb 11, 2025, 5:00 PM CST

Title: Spheroid Recap_Timeline Alterations
Date: 2/11/25
Present: Entire Team
Content:

2/10 - 2/14	~~- Celltiter glo~~ ~~- Passage 7~~ ~~(Althys + Julia)~~	Example Primer link: https://www.novusbio.com/products/gapdh-primer_nbp1-71650?srsltid=AfmBOoomNZhzD0tr-UtfhYgyXTL1Ij5I6EC309CpwIo51bbFjdWigMof	- Passage 8 (Ana) - Make plates (Althys)	- ALTHYS IS GONE - Progress report 3 ~~- ImageJ Analysis done by the latest -> find best density and protocol (to make new spheroids for qPCR)~~ - Hopefully Carley takes plates to the fridge	- ALTHYS IS GONE - Passage 9 - Make spheroids (retry experiments from last Friday 8/2) **- Talk to Dr Hess and Carley about: qPCR vs immunostaining for SOX2, possibility of live/dead staining (highly likely not go with this), 3D kit for CellTiter Glo**
2/17 - 2/21	- Image spheroids, then suck out media and add in new 100uL serum free media - Seed cells for positive and add media for negative control		- ALTHYS IS GONE - 2D Cell TiterGlo for plates on 2/14	- ALTHYS IS GONE - Progress report 4	- ALTHYS IS GONE - Passage 12

- Experiment 2:
  - 24 PolyHema
  - 8 non-PolyHema coated wells
    - + 25k cells (seed cells Monday 2/17)
    - - no cells, just media
- Spatial imaging for necrotic core - Althys
  - Good spatial information
  - Does not matter for the purposes of our project/goal
  - Not needed to infer cell viability
- Live dead
  - w/ accutase dissociation
- Ask Gaelen to purchase 3D Cell-Titer Glo
- Register for engineering EXPO
  - Confirmation email sent 4:50pm
  - Students will be able to make a model of the 3D double helix structure of DNA. The team will already completed part of the double helix structure and have the nucleic acids (building blocks of the model) cut out so the students can design and color them

Questions:

Talk to Dr Hess and Carley about: qPCR vs immunostaining for SOX2, possibility of live/dead staining (highly likely not go with this), 3D kit for CellTiter Glo

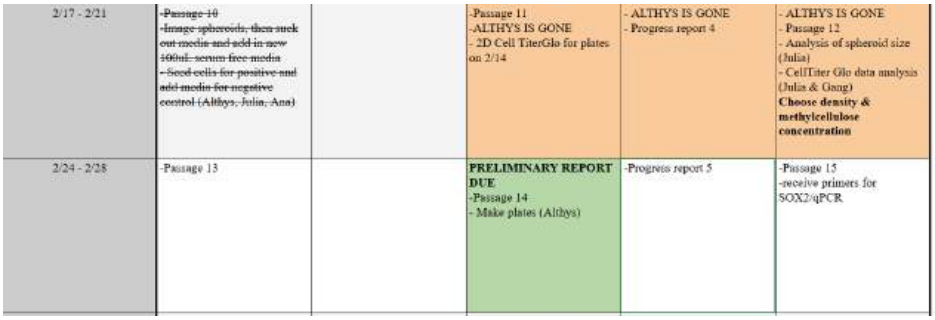
Conclusions & Action Items:

- Confirm with Tracy that our outreach project for the engineering EXPO is okay
- Meet with Gaelen over zoom at 1:00 pm
- Meet with Paul in-person at 12:00 pm

Team Meeting 3_2/18/2025

Emily Rhine - Feb 18, 2025, 4:19 PM CST

Title: Prelim Report
Date: 2/11/25
Present: Entire Team
Content:

- Discussed Timeline
  - 

2/17 - 2/21	~~-Passage 10 -Image spheroids, then suck out media and add in new 100uL serum free media - Seed cells for positive and add media for negative control (Althys, Julia, Ana)~~		-Passage 11 -ALTHYS IS GONE - 2D Cell TiterGlo for plates on 2/14	- ALTHYS IS GONE - Progress report 4	- ALTHYS IS GONE - Passage 12 - Analysis of spheroid size (Julia) - CellTiter Glo data analysis (Julia & Gang) **Choose density & methylcellulose concentration**
2/24 - 2/28	-Passage 13		**PRELIMINARY REPORT DUE** -Passage 14 - Make plates (Althys)	-Progress report 5	-Passage 15 -receive primers for SOX2/qPCR

- Discussed Preliminary Report and assigned sections
  - Introduction: Jayson
  - Methods:
    - Althys & Emily
  - Discussion: Ana
  - Results: Julia
- Follow up with Tracy about Engineering EXPO Outreach
- Meet Friday to
- Finish individual parts by monday night (2/24)
- Meet (2/25) at 4pm to discuss and make changes on preliminary report to prepare for submission
- Mention selected journal (AACR) to client
- qPCR primer research
  - TaqMan (spans exons), human, SOX2, and extra small

Conclusions & Action Items:

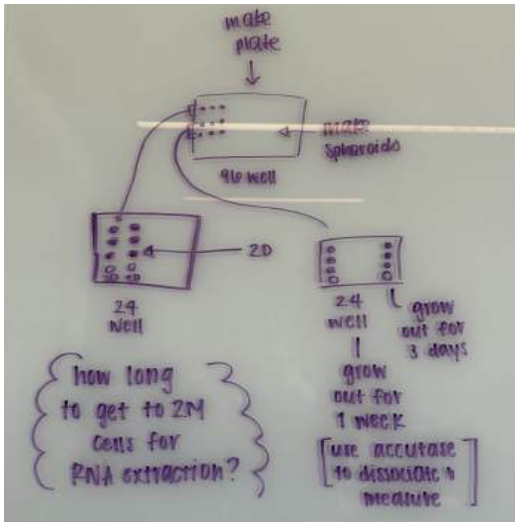
- Pitch SOX2 kit on Friday to Carley
  - https://www.thermofisher.com/taqman-gene-expression/product/Hs00415716_m1?CID=&ICID=&subtype=
- Further research qPCR to prepare for client meeting
- Continue work on prelim report

Team Meeting 4_2/25/25

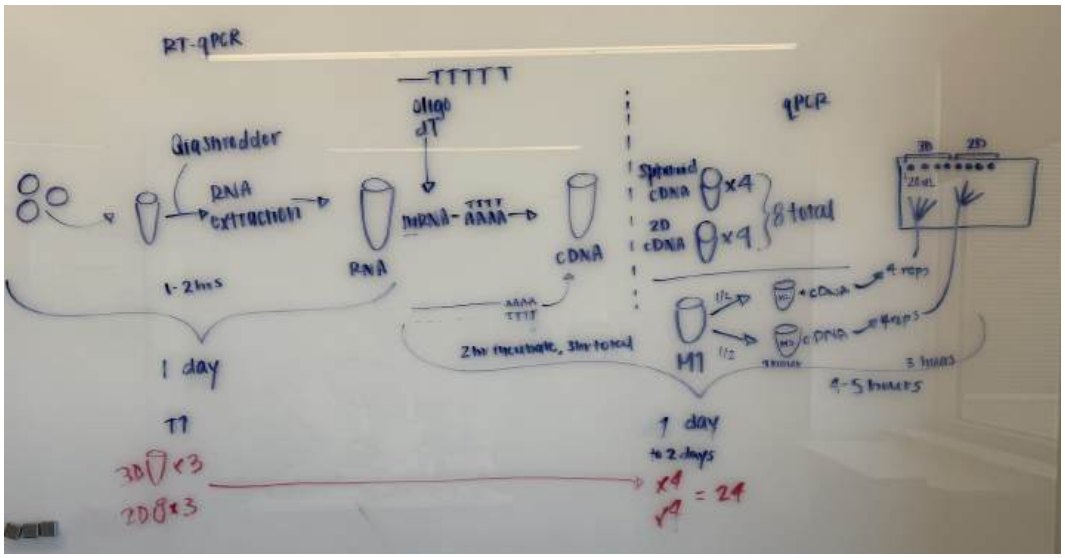
Emily Rhine - Feb 25, 2025, 5:03 PM CST

Title: Team Meeting_RT-qPCR
Date: 2/25/25
Present: Entire Team
Content:

- 2/24 - Ana
  - 96 well plate coated
  - Passage 13
- 2/26 - Emily & Althys
  - Seed cells & coat 24 well plate with PolyHEMA
    - 3D: 75k cells and 0.75% Methylcellulose
  - Passage 14
  - 96 well + 24 well
- 2/28 - Jayson & Emily
  - Dissociate some of cells from spheroid and determine doubling time
  - Transfer cells from 96 well to 24 well to get 2 million cells per sample
    - 2D: Confirm calculation
    - 3D:
  - Passage 15
- Continue work on preliminary report
- See benchling for complete RT-qPCR protocol



3/3 - 3/7	-Passage 16 - Passage spheroids (set 1-5 days, set 2-3 days) - dissociate via Accutase - determine doubling time for both populations, if not 2 million, reseed and wait for however long based on td/if 2 million, seed new population of spheroids)	-Team Meeting	-Passage 17	-Progress report 6	-Carley busy later
3/10 - 3/14	-Passage 18 (Vial3) -Passage 1 (Vial 4) - qPCR for SOX2 (tentatively) Step 1 mRNA extraction (2hr)		-Passage 19 (Vial3) OR KILL -Passage 2(Vial 4) -qPCR (step2) cDNA isolation (2hr)	-Progress report 7 *CARLEY GONE MARCH 13TH-15TH (interview)	-JULIA IS GONE *CARLEY GONE MARCH 13TH-15TH (interview)
3/17 - 3/21	-qPCR for SOX2 step 3 qPCR (5hrs)			-Progress report 8	JAYSON MIGHT BE GONE



Conclusions & Action Items:

- Update client and advisor at upcoming team meeting
- Upload preliminary report by Friday 2/28 if possible

Paul Campagnola
To: ANA L MARTINEZ CAVAZOS
Tue 2/25/2025 10:07 AM
Cc: Althys Cao; Julia Rose Salita; Emily Rhine; Jayson Lee O'Halloran

Hi all, next week is fine.

Best
paul

ANA L MARTINEZ CAVAZOS
To: Paul Campagnola
Tue 2/25/2025 10:00 AM
Cc: Althys Cao; Julia Rose Salita; Emily Rhine; Jayson Lee O'Halloran

Hi Paul,

I hope you are doing well.

Our team was wondering if we would be able to get an extension for Friday for the preliminary report. Several of our teammates have exams earlier this week, and since Althys was gone through the end of last week for grad school interviews she also had limited time to work on the report. We would really appreciate a bit longer time to turn in the best version of the report as possible.

Team Meeting 5_3/11/25

Emily Rhine - Mar 11, 2025, 8:43 PM CDT

Title: Planning
Date: 3/11/25
Present: Entire Team
Content:

- 3/12
    - Make PolyHema stock
    - Passaging (x2)
    - Seeding
        - 6x DMEM wells
        - 6x SFM wells
        - 6x only PolyHema wells (to see whether PolyHema)
-

Color key	Past day	Team member not in town	Deliverable due	upcoming day	upcoming day	
Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	[illegible] of spheroids)					
3/10 - 3/14	-Passage 4 (Vial 4) - Dissociate rest of spheroids --> count (Julia & Emily)	**Team Meeting: decide if we should seed spheroids in a full 96-well plate to prep for qPCR on Wednesday or Friday**	-Passage 5 (Vial 4) (Ana) **- Figure out if should seed 96 wells of spheroids by Wed or Fri) to prep for qPCR** **(NO PREP FOR qPCR :( )** - PolyHEMA stock preparation (Julia) - Seed 6 x full DMEM wells & 6 x SFM wells in 24 well plate (Althys)	-Progress report 7 *CARLEY GONE MARCH 13TH-15TH (interview) - Make polyHEMA plates (4 x 96-well plates) w/ new stock	-JULIA IS GONE *CARLEY GONE MARCH 13TH-15TH (interview) - Seed spheroids in 96 well plate to determine doubling time AGAIN - feed spheroids in 24 well plate (from Wednesday)	
3/17 - 3/21	- qPCR for SOX2 (tentatively) Step 1 mRNA extraction (2hr)		520 EXAM	-Progress report 8	JAYSON MIGHT BE GONE qPCR (step2) cDNA isolation (2hr)	
3/24 - 3/28	Spring Break					

Conclusions & Action Items:

- Meet with Gaelen this friday to discuss current difficulties 1:05pm-2:05pm
- Update LabArchives
- Begin planning qPCR steps based on spheroid doubling time

Team Meeting 6_4/1/25

Emily Rhine - Apr 01, 2025, 4:47 PM CDT

Title: Team Meeting
Date: 4/1/25
Content:

- Engineering Expo
  - https://www.yourgenome.org/theme/origami-dna/
  - Contact Tracy about materials
  - Brief Project Description: Students will be able to make a model of the 3D double helix structure of DNA. Our team will display a completed section of double helix structure and have nucleic acids (building blocks of the model) cut out so the students can color, label, and assemble them.
  - Our outreach is based on this activity Modeling the Structure of DNA.
  - "Typically for a table-top activity, you should have a flip book of concepts you can quickly go through as kids come and go. Also, a take home card if a fun addition and I can help you come up with something and help with printing materials." - Tracy
- Gamma-H2AX team
  - Emily
  - Jayson
- RT-qPCR team
  - Ana
  - Althys
  - Julia
- How much PolyHEMA (solid) is left
  - Ethanol+PolyHEMA stock "old" <15mL
  - Ethanol+PolyHEMA stock 3/12 <5mL
  - Ethanol+PolyHEMA stock 3/31 <23mL

3/31 - 4/4	Make PolyHEMA	Coat 6 well plate Team meeting 4pm - 5pm	Passage (Ana) & UV light Seed 1 x 6 well plate (Althys)	-Progress report 9 -Excutive summary due	2D Passaging (Emily & Jayson) Check on spheroids, brace ourselves -Seed 6 well plate for gamma-H2AX (Emily & Jayson)
4/7 - 4/11	- qPCR for SOX2 (tentatively) Step 1 mRNA extraction (2hr) (Althys & Ana & Julia) -1st trial of 3D gamma H2AX (based on Hess Lab's 2D protocol) + 2D gammaH2AX with drugs (Jayson & Emily)		qPCR (step2) cDNA isolation (2hr) (Althys & Ana & Julia)	-Progress report 10 -qPCR for SOX2 step 3 qPCR (5hrs) (team effort)	- Engineering Expo (ask about running booth (tracy Puccinelli) for outreach) CARLEY GONE!

Conclusions & Action Items:

- How much PolyHEMA (solid) is left?
  - We shouldn't order more unless we absolutely have to
- Draft executive summary
- Draft Expo booklet
- Contact Tracy

Cell Seeding/Spheroid Formation Protocol_11/18/2024

Althys Cao - Dec 17, 2024, 9:18 PM CST

Most recent protocol

Althys Cao - Dec 17, 2024, 9:17 PM CST

Title: Cell Seeding/Spheroid Formation Protocol

Date: 12/15/2024

Goals: Establish cell seeding protocol for spheroid formation.

Content By: Althys

Content:

NOTE: THIS PROTOCOL IS USED TO SEED CELLS IN A 96-WELL FLAT-BOTTOM PLATE. This protocol is currently the most full-fledged and can be used to seed at 4 different densities: 25k, 50k, 75k, 150k cells/cm^2.

1. Collect cells from that day's passage and obtain cell concentration using the CytoFLEX to obtain cell concentration (cells/mL).
2. Obtain a 1.5 mL cell solution of 500,000 cells/mL (750,000 cells in total)
    a. Volume needed to obtain 750,000 cells: 750,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)
    b. Obtain the volume calculated from the T75 flask and transfer into a 15 mL conical tube.
    c. Spin down the the tube at 200g for 5 minutes, then remove supernatant to collect cells using vacuum filter
    d. Resuspend cells in 1.5 mL of serum-free DMEM.
3. Prepare master mix of cell solutions for spheroid formation for 4 different densities. The master mix can be used for 6 wells in a 96-well plate and can be scaled to adjust for different numbers of wells if needed.
    a. Density 1: 25,000 cells/cm^2
        i. Into a new 1.5 mL eppendorf tube, add:
            1. 100 µL of 500,000 cells/mL cell solution (Step 2)
            2. 525 µL of serum-free DMEM
            3. 375 µL of 2% methylcellulose stock
        ii. Mix gently by pipetting up and down, make sure the solution is well-mixed
    b. Density 2: 50,000 cells/cm^2
        i. Into a new 1.5 mL eppendorf tube, add:
            1. 200 µL of 500,000 cells/mL cell solution (Step 2)
            2. 425 µL of serum-free DMEM
            3. 375 µL of 2% methylcellulose stock
        ii. Mix gently by pipetting up and down, make sure the solution is well-mixed
    c. Density 3: 75,000 cells/cm^2
        i. Into a new 1.5 mL eppendorf tube, add:
            1. 300 µL of 500,000 cells/mL cell solution (Step 2)
            2. 325 µL of serum-free DMEM
            3. 375 µL of 2% methylcellulose stock
        ii. Mix gently by pipetting up and down, make sure the solution is well-mixed
    d. Density 4: 150,000 cells/cm^2
        i. Into a new 1.5 mL eppendorf tube, add:
            1. 600 µL of 500,000 cells/mL cell solution (Step 2)

1. [illegible] cell solution (Step 2)
2. 25 µL of serum-free DMEM
3. 375 µL of 2% methylcellulose stock

ii. Mix gently by pipetting up and down, make sure the solution is well-mixed

4. Aliquot the prepared master mix into each well: add 160 µL of cell mixture into each well.
5. Grow spheroids in 37C, 5% CO2 incubator. Spheroids are split every 3-4 days.

References

[1] K. Han *et al.*, "CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities," *Nature*, vol. 580, no. 7801, pp. 136–141, Apr. 2020, doi: 10.1038/s41586-020-2099-x.

Action items:

- This protocol will be used next semester to seed cells at different densities. The biggest updated in this protocol from the previous version (used for trial 2, 11/22/2024, see below) is that all full DMEM will be removed so serum-free DMEM is used instead. This makes sure that there is no trace of FBS in the cellular media that can interfere with the spheroid formation process.
- This protocol will be rewritten for varying methylcellulose concentration.
- This protocol will be fully finalized once the optimal cell seeding density and methylcellulose concentration are found.

Althys Cao - Dec 17, 2024, 9:13 PM CST

Older versions of the protocol used for this semester + Notes

Emily Rhine - Nov 25, 2024, 11:22 AM CST

Title: Cell Seeding/Spheroid Formation Protocol

Date: 11/28/2024

Goals: Establish cell seeding protocol for spheroid formation.

Content By: Althys

Content:

NOTE: THIS PROTOCOL IS USED TO SEED CELLS IN A 96-WELL FLAT-BOTTOM PLATE. SEE FULL PROTOCOL AT THE END

- Diameter of one well of a flat-bottom 96-well plate: 6.35 mm → Surface area: 0.32 cm2
- Each well in a **flat-bottom 96-well plate** is 0.32 cm^2
- In Han's protocol: 500uL/cm^2 → will need 160 µL/well (max volume per well: 300 uL)
- Han's protocol: 20,000 cells/cm^2 → 150,000 cells/cm^2 to find peak 30% cell death
- 160 µL of total media (0.75% methylcellulose + cells) PER WELL:
- x µL of 2% methylcellulose stock:
- x µL * 2% = 160 µL * 0.75% → x = 60 µL of 2% methylcellulose stock
- Add additional 100 µL of cell media to reach 160 µL of total media and desired cellular concentration.

Emily Rhine - Nov 25, 2024, 11:22 AM CST

Table 1 - Number of Cells per Well

	A	B	C	D	E	F
1		Density 1	Density 2	Density 3	Density 4	X
2	Final cell density (cells/cm2)	25000	50000	75000	150000	X
3	# of cells in one well in a 96-well plate	8,000 cells	16,000 cells	24,000 cells	48,000 cells	X
4	Cell concentration at each well (cells/µL) (160 µL per well)	50 cells/µL	100 cells/µL	150 cells/µL	300 cells/µL	X
5	Volume of 500,000 cells/mL needed (µL)	16 µL	32 µL	48 µL	96 µL	X
6	6 trials - Total number of cells	48,000 cells	96,000 cells	144,000 cells	288,000 cells	Total # of cells needed = 576,000 cells

Table 2 - Relevant Protocol Values

	A	B	C	D	E	F
1		Cellular Concentration (cells/mL)	Volume needed from passage to get a total of 750,000 cells (mL)	Do we need to add extra serum-free DMEM to reach final volume of 1.5 mL? If yes, how much mL should be added?	Do we need to spin down cells and resuspend in 1.5 mL of serum-free DMEM?	
2		**ADD CELLULAR CONCENTRATION FROM CYTOFLEX (CELLS/ML), NOT TOTAL NUMBER OF CELLS**	#VALUE!	#VALUE!	#VALUE!	
3	11/19/2024	~~3350000~~	~~0.223880597~~	~~1.276119403~~	NO	**NOTE: Put in incorrect value (put in total number of cells instead of cell concentration), scrap this experiment**
4	11/22/2024	298500	2.5125628141	NO	YES	

Emily Rhine - Nov 25, 2024, 11:27 AM CST

6 wells for each concentration → 960 µL of total media is needed for each concentration, round up to **1000 µL of total media (methylcellulose + cells) for each concentration** to account for pipetting error

- Amount of 2% methylcellulose stock: x µL * 2% = 1000 µL * 0.75% → x = **375 µL of 2% methylcellulose stock**
- Amount of 500,000 cells/mL (or 500 cells/µL) solution needed: cell concentration of a well (Table 1, row 4) * 1000 µL / (500 cells/µL). Amount of extra serum-free DMEM needed = 1000 µL - amount of 500,000 cells/mL solution - amount of 2% methylcellulose solution
- **Density 1: 25,000 cells/cm^2:**
  - 50 cells/µL * 1000 µL / (500 cells/µL) = **100 µL of 500,000 cells/mL**

    - 1000 µL - 100 µL - 375 µL = **525 µL of serum-free DMEM**
- **Density 2: 50,000 cells/cm^2**
    - 100 cells/µL * 1000 µL / (500 cells/µL) = **200 µL of 500,000 cells/mL**
    - 1000 µL - 200 µL - 375 µL = **425 µL of serum-free DMEM**
- **Density 3: 75,000 cells/cm^2**
    - 150 cells/µL * 1000 µL / (500 cells/µL) = **300 µL of 500,000 cells/mL**
    - 1000 µL - 300 µL - 375 µL = **325 µL of serum-free DMEM**
- **Density 4: 150,000 cells/cm^2**
    - 300 cells/µL * 1000 µL / (500 cells/µL) = **600 µL of 500,000 cells/mL**
    - 1000 µL - 600 µL - 375 µL = **25 µL of serum-free DMEM**

FULL PROTOCOL:

1) Collect cells from that day's passage, run Cytoflex to obtain cell concentration, **write in CELL CONCENTRATION (# OF CELLS/ML), NOT TOTAL NUMBER OF CELLS in *Table 2 - Relevant Protocol Values, Column B***
2) Obtain 1.5 mL cell solution of 500,000 cells/mL (750,000 cells in total)
3)Volume needed to get from the passage to obtain a total of 750,000 cells in total is in Column C.
4)Column C = 750,000 / Cellular Concentration (A2)
4)If Column C value is less than 1.5 mL: "Extract" Column C volume of cells (mL) from the T75 flask into a 15 mL conical tube (1.5 mL eppendorf should be sufficient too).
5)Add C2 value of serum-free DMEM (mL) to tube.
6)If Column C value is more than 1.5 mL:
"Extract" Column C volume of cells (mL) from the T75 flask into a 15 mL conical tube
7)Spin down tube at 200 x g for 5 min, remove supernatant using vacuum filter
8)Resuspend pellet in 1.5 mL of serum-free DMEM, pipette up and down to mix the cell mixture
9)If Column C value is equal to 1.5 mL:
Lucky you, just need to "extract" Column C volume of cells (mL) from the T75 flask into a 15 mL conical tube
10)Prepare full spheroid formation cell solutions for concentrations 1-4. Aliquot into each well.
- Density 1: 25,000 cells/cm^2: Into a new 1.5 mL eppendorf tube, add 100 µL of 500,000 cells/mL cell solution + 525 µL of serum free DMEM + 375 µL of 2% methylcellulose stock. Mix gently by pipetting up and down, make sure the solution is well-mixed. Add 160 µL of cell mixture into each well
- Density 2: 50,000 cells/cm^2: Into a new 1.5 mL eppendorf tube, add 200 µL of 500,000 cells/mL cell solution + 425 µL of serum free DMEM + 375 µL of 2% methylcellulose stock. Mix gently by pipetting up and down, make sure the solution is well-mixed. Add 160 µL of cell mixture into each well
- Density 3: 75,000 cells/cm^2: Into a new 1.5 mL eppendorf tube, add 300 µL of 500,000 cells/mL cell solution + 325 µL of serum free DMEM + 375 µL of 2% methylcellulose stock. Mix gently by pipetting up and down, make sure the solution is well-mixed. Add 160 µL of cell mixture into each well
- Density 4: 150,000 cells/cm^2: Into a new 1.5 mL eppendorf tube, add 600 µL of 500,000 cells/mL cell solution + 25 µL of serum free DMEM + 375 µL of 2% methylcellulose stock. Mix gently by pipetting up and down, make sure the solution is well-mixed. Add 160 µL of cell mixture into each well
11) Grow spheroids in 37C, 5% CO2 incubator. Spheroids are split every 3-4 days

Althys Cao - Dec 17, 2024, 9:12 PM CST

Conclusions/Action Items:

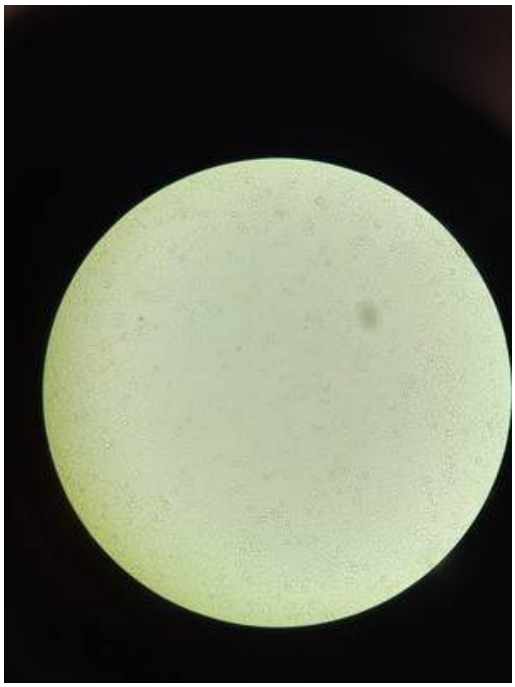
- Trial 1: 11/18/2024
    - In this trial, there were a few things that were miscalculated along the way, including: polyHEMA plates were not left to dry correctly (before seeding, there was still polyHEMA solution left in the wells), full

DMEM was used instead of serum-free DMEM which can prevent spheroid formation, and not enough cells were added.

- Trial 2: 11/22/2024
  - Mistakes from trial 1 were taken into account and fixed --> spheroids formed!
- Updated protocol to make it clearer: 2 times
  - First time: for trial 2 11/22/2024
  - Second time: 12/15/2024
- Added protocol to appendix of final report

Passage4_Vial 3

Emily Rhine - Feb 03, 2025, 10:57 AM CST



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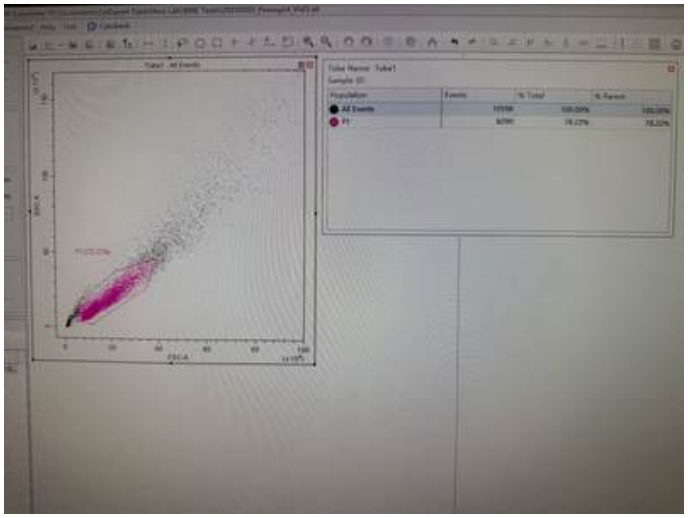
Emily Rhine - Feb 03, 2025, 11:28 AM CST



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A549 Passaging Table_2/9/25

Emily Rhine - Feb 09, 2025, 11:06 PM CST

A549 Confluency Tracker2

	Date	Passage Number	Cytoflex Confluency	Cells per mL	Total cells in solution	Doubling time	Cut Back To	mL to Keep
1	1/24/2025	0 (thaw)	N/A	N/A	1000000	Thaw	1000000	
2	1/27/2025	1	6687	668700	6687000	26.3	1750000	2.6
3	1/29/2025	2	6587	658700	6587000	25.1	1700000	2.6
4	1/31/2025	3	6419	641900	6419000	28.2	1700000	2.6
5	2/3/2025	4	8290	829000	8290000	29.7	1700000	2.1
6	2/5/2025	5	8194	819400	8194000	21.2	1650000	2.0
7	2/7/2025	6	7450	745000	7450000	22.1	1550000	2.1

https://benchling.com/uw_hesslab/f/lib_tD5E8G7L-cell-line-maintenance/etr_5Skd74TL-a549-cell-line-maintenance/edit

PolyHEMA Protocol_2/9/25

Emily Rhine - Apr 13, 2025, 4:14 PM CDT

Title: Stock PolyHEMA Protocol
Original Date:10/23/2024

Content by: Carley, Julia, Emily

Content:

Poly-HEMA hydrogel coating: [2]
Link: SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine
A total 1.3 g of poly-HEMA (Sigma-Aldrich Co.) was dissolved in 33 mL of 99% ethanol, and the solution was mixed overnight at 37°C. Fifty microliters or 3.2 mL of the poly-HEMA stock solution was added to 96-well plates and 10 cm dishes, respectively, in the tissue culture hood, and plates and dishes were swirled using a plate rotator for 10 minutes. Plates were left to dry overnight and then washed with PBS immediately before use. [1]

Current Date: 2/9/25
Alternate Protocol: https://pmc.ncbi.nlm.nih.gov/articles/PMC9006308/#S6 [2]

- Materials:
  - Poly(2-hydroxyethyl methacrylate) (PolyHEMA) (2g) (Sigma, P3932)
  - 95% (v/v) ethanol
  - Vacuum desiccator (VWR, cat. no. 24987–004)
  - 0.22 μm cellulose acetate membrane with bottle top vacuum filter system (Corning, cat. no. 430756)
  - Hot plate and magnetic stirrer
  - Magnetic stir bar
  - 200 mL beaker
  - Parafilm (Millipore Sigma, cat. no. P7793)
  - Glass thermometer (Thomas Scientific, cat. no. 9313A27)
  - Aluminum foil
  - Biosafety Class II tissue culture hood
- Steps (4 hours) [2]

Weigh and add 2g PolyHEMA to 100 mL 95% ethanol in a glass beaker.
Add a magnetic stir bar to the beaker and place the beaker on a hot plate with a magnetic stirrer and stir at 65°C until fully dissolved (typically ≥3 hours). Cover first with aluminum foil, followed by generous amounts of Parafilm to prevent evaporation.
Poke a glass thermometer through the top of the aluminum foil and Parafilm to ensure that the temperature of the PolyHEMA solution is as close to 65°C as possible.
Allow the PolyHEMA solution to cool to room temperature.
In a tissue culture hood, filter the PolyHEMA solution through a 0.22 μm filter using the vacuum filter system.
In a tissue culture hood, place the bottle of filtered PolyHEMA solution (with cap off) in a vacuum desiccator for 30 minutes to degas the solution.
In a tissue culture hood, after the solution is degassed, replace the screw cap and use Parafilm to seal the cap-bottle junction on the outside of the bottle, as an extra measure to prevent unwanted gas exchange.
This protocol describes how to make the PolyHEMA solution used to coat glass-bottom dishes in Basic Protocol 1. PolyHEMA solution should be kept under tight seal at room temperature when not in use, and after it is sterile filtered it

should only be opened in a Biosafety Class II tissue culture hood using aseptic technique. We suggest using a hot plate with a feedback system or probe to monitor the actual temperature of this solution as it is being mixed. Otherwise, take great care to monitor and adjust the hot plate temperature so that the PolyHEMA solution reaches 65°C for 3 hours while stirring, or until all PolyHEMA crystals are dissolved. Undissolved PolyHEMA interferes with organoid imaging. Once this reagent is made, it can be stored for up to 3 months at room temperature. We caution against leaving coated plates with 1x PBS in the incubator for more than 3 weeks. The coatings may degrade by this time. Always visually check coating integrity before using it for culture. [2]

References

[1] C. Choe, H. Kim, S. Min, S. Park, J. Seo, and S. Roh, “SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine,” OncoTargets and therapy, vol. 11, p. 6197, Sep. 2018, doi: 10.2147/OTT.S175810.

[2] H. M. Cambra, N. P. Tallapragada, P. Mannam, D. T. Breault, and A. M. Klein, “Triple-decker sandwich cultures of intestinal organoids for long-term live imaging, uniform perturbation and statistical sampling,” Curr Protoc, vol. 2, no. 1, p. e330, Jan. 2022, doi: 10.1002/cpz1.330.

Conclusions & Action Items:

- PolyHEMA plates can be sterilized under 30 min of UV in BSC rather than sterile filtered
- Sterile filtering is difficult, time consuming, and causes some of the PolyHEMA to solidify in the filter "gumming" it up and making the PolyHEMA stock have less PolyHEMA % and making it more difficult

Spheroid Formation Experiment_2/7/25

Emily Rhine - Feb 09, 2025, 11:13 PM CST

Title: Spheroid Formation Experiment

Date: 2/7/2

Content by: Althys & Emily

Content:

Link: https://benchling.com/uw_hesslab/f/lib_wSFKaFAF-spheroid-formation/etr_F867pmcX-cell-seeding-protocol-272025/edit

PROTOCOL

NOTE: USE SERUM-FREE DMEM IN **ALL** STEPS OF CELL SEEDING!

1. Collect cells from that day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL)**.
2. Make a **1.5 mL cell solution of 500,000 cells/mL (750,000 cells in total)**
    a. **Volume needed to obtain 750,000 cells from the day's cell passage: 750,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)**
        I. Can also put in **cell contration (cells/mL) from Step 1** into **Cell J2** from the table below, the volume needed for step 2a will be in **Cell K2.**
    b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**.
    c. Spin down the the tube at **200g for 5 minutes**, then **remove supernatant** to collect cells using vacuum filter
    d. Resuspend cells in **1.5 mL of serum-free DMEM**.
3. Prepare **master mix of cell solutions** for spheroid formation for **6 conditions**. The master mix can be used for **4 wells in a 96-well plate** and can be scaled to adjust for different numbers of wells if needed.
    a. Note: in the calculations you will see that I scale up to 5 instead of 4, this is to ensure we have enough materials for 4 wells.
    b. **Condition 1: 50,000 cells/cm^2, 0.75% methylcellulose**
        I. Into a new 1.5 mL eppendorf tube, add:
            1. 340 µL of serum-free DMEM
            2. 300 µL of 2% methylcellulose stock
            3. 160 µL of 500,000 cells/mL cell solution (Step 2)
        II. Mix gently by pipetting up and down, make sure the solution is well-mixed
    c. **Condition 2: 50,000 cells/cm^2, 1% methylcellulose**
        I. Into a new 1.5 mL eppendorf tube, add:
            1. 240 µL of serum-free DMEM
            2. 400 µL of 2% methylcellulose stock
            3. 160 µL of 500,000 cells/mL cell solution (Step 2)
        II. Mix gently by pipetting up and down, make sure the solution is well-mixed

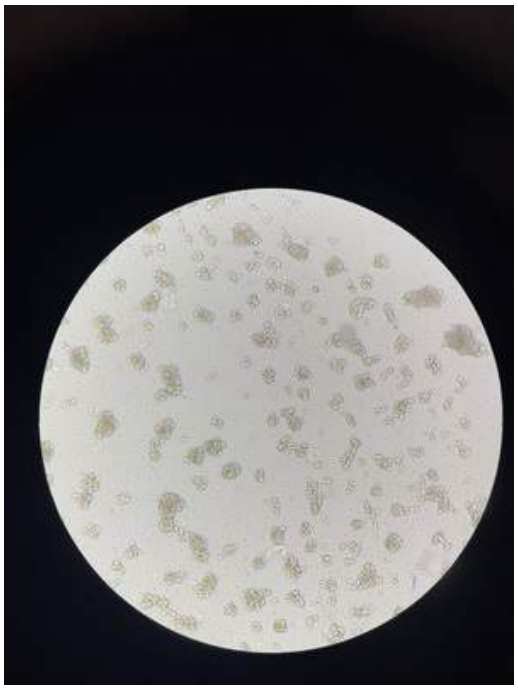
d. **Condition 3: 50,000 cells/cm^2, 1.25% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 140 µL of serum-free DMEM
      2. 500 µL of 2% methylcellulose stock
      3. 160 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
e. **Condition 4: 75,000 cells/cm^2, 0.75% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 260 µL of serum-free DMEM
      2. 300 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
f. **Condition 5: 75,000 cells/cm^2, 1% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 160 µL of serum-free DMEM
      2. 400 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
g. **Condition 6: 75,000 cells/cm^2, 1.25% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 60 µL of serum-free DMEM
      2. 500 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed

4. **Mix gently by pipetting up and down then Aliquot** the prepared master mix into each well: add **160 µL of cell master mix** into each well **(4 wells total)**.
5. Grow spheroids in **37C, 5% CO2 incubator**. Spheroids are split every 3-4 days.

Cell Density (cells/cm^2)	Methylcellulose concentration (%)	Amount of methylcellulose 2% needed (microL) for 1 well	Cell number (cells)	Volume of (500,000 cells/mL) needed (microL) for 1 well	Volume of (500,000 cells/mL) needed (microL) for master mix	Amount of methylcellulose 2% needed (microL) for master mix	Amount of serum-free DMEM (microL) for master mix	Total number of cells needed	Put in cell concentration from CytoFlex (cells/mL)	Volume needed to obtain 750,000 cells (step 2a) (mL)
50000	0.75	60	16000	32	160	300	340	600000	745000	1.0067114094
50000	1	80	16000	32	160	400	240			
50000	1.25	100	16000	32	160	500	140			
75000	0.75	60	24000	48	240	300	260			
75000	1	80	24000	48	240	400	160			
75000	1.25	100	24000	48	240	500	60			

Conclusions & Action Items:

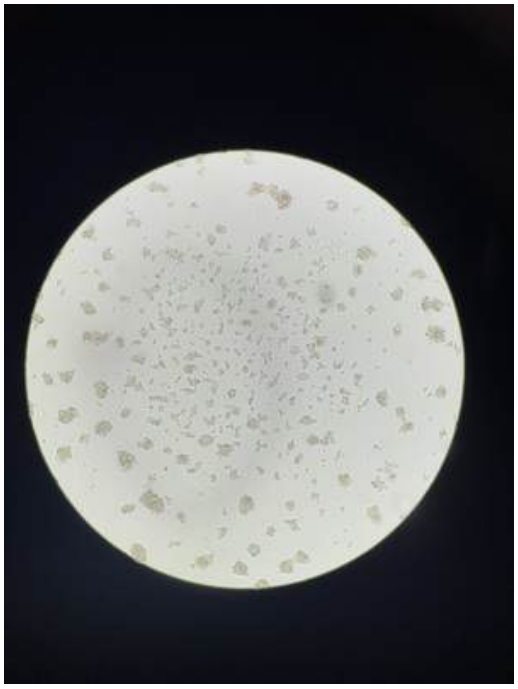
- Protocol followed on 2/7/25 by entire team (Jayson and Julia mainly)
- Condition 1 wells 1-3 may have an incorrect volume, so they may be excluded from the data collection & analysis.
- Follow up with Cell-Titer Glow protocol 2/10/25 with Carley (Julia, Althys, & Emily).
- Update final protocol after data analysis and conclusions on ideal seeding density and methylcellulose concentration.



Download

IMG_6522.jpeg (3.93 MB) 75k cells/cm^2

Emily Rhine - Feb 27, 2025, 4:12 PM CST



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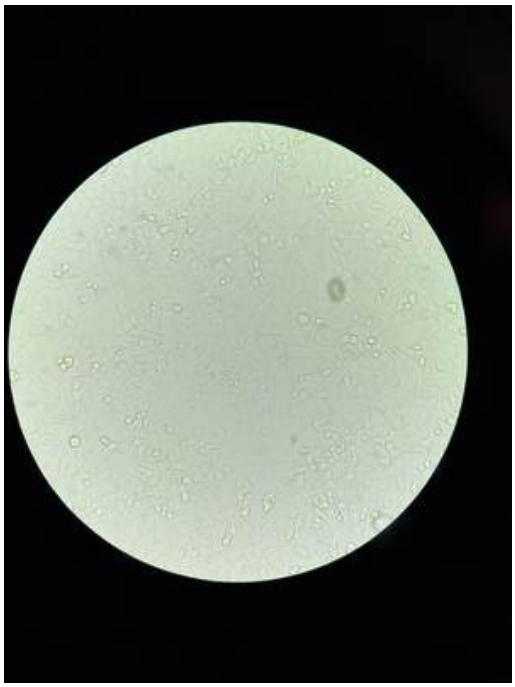
IMG_6523.jpeg (2.44 MB) 50k cells/cm^2

Passage 7_Vial 3

Emily Rhine - Feb 10, 2025, 10:26 AM CST



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Passage 10_Vial 3_2/17/25

Emily Rhine - Feb 17, 2025, 10:09 AM CST

Cell Number at the beginning of the growth phase (N_0) [cells]

1550000

Cell Number at the end of the growth phase (N_t) [cells]

5083000

Time the cells were growing (Δt) [hours]

68

The growth rate (μ) of your cells is [1/day] :

0.42

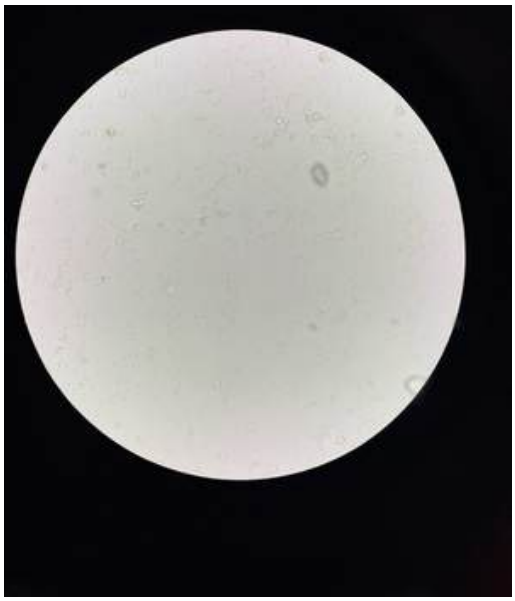
The population doubling time of your cells is [hours] :

39.7

	Date	Passage Number	Cytoflex Confluency	Cells per mL	Total cells in solution	Doubling time	Cut Back To	mL to Keep
5	2/3/2025	4	8290	829000	8290000	29.7	1700000	2.1
6	2/5/2025	5	8194	819400	8194000	21.2	1650000	2.0
7	2/7/2025	6	7450	745000	7450000	22.1	1550000	2.1
8	2/10/2025	7	6695	669500	6695000	33.2	1600000	2.4
9	2/11/2025	8	7415	741500	7415000	21.7	1550000	2.1
10	2/14/2025	9	7147	714700	7147000	21.8	1550000	2.2
11	2/17/2025	10	5083	508300	5083000	39.7	1600000	3.1

Cells sat out for 25 min (flask was placed vertically so that fewer cells adhered to the surface) --Carley's advice

Emily Rhine - Feb 17, 2025, 9:32 AM CST



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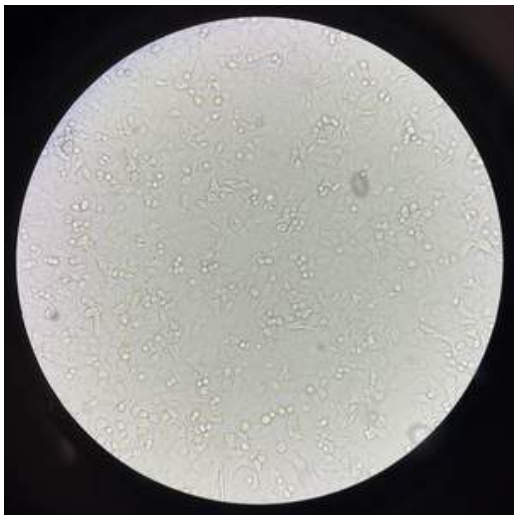
Passage 14_20x mag_2/26/25

Emily Rhine - Feb 27, 2025, 12:53 PM CST



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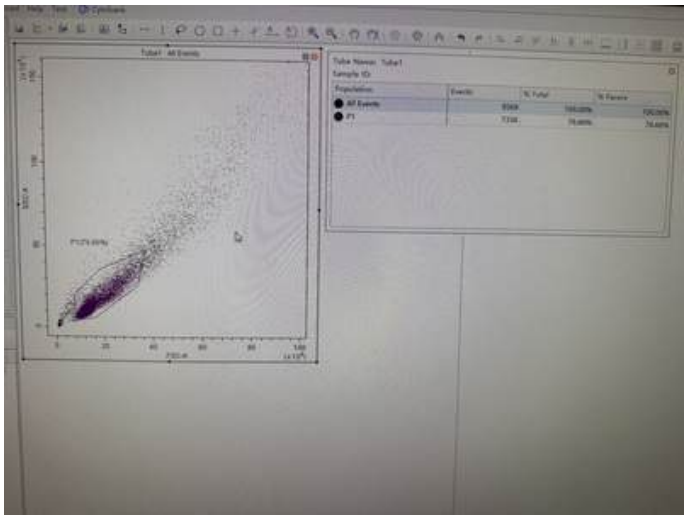
Emily Rhine - Feb 27, 2025, 12:53 PM CST



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IMG_6616.jpg (5.1 MB)

Emily Rhine - Feb 27, 2025, 12:57 PM CST

(7,336,000)/ 5,000,000 = 147% confluency

Passage 6_Vial 4_Flasks 1&2

Emily Rhine - Mar 14, 2025, 8:21 PM CDT

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910185B6-0259-4946-97D1-7A14AD4DA872.jpg (329 kB)

Emily Rhine - Mar 14, 2025, 8:21 PM CDT

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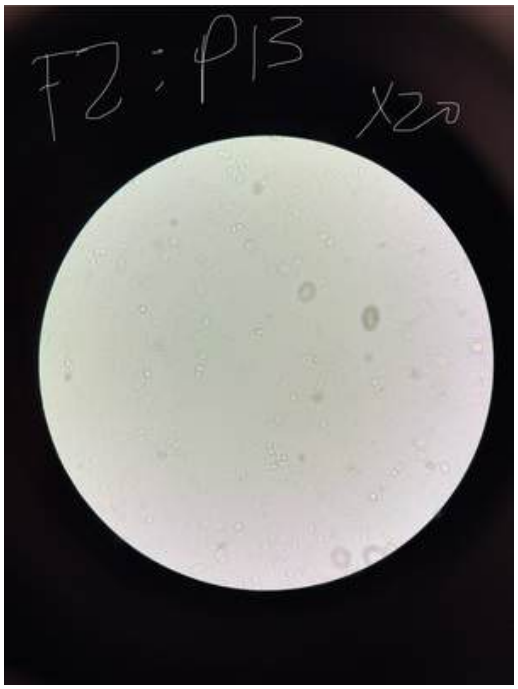
Passage13_Vial4_20x_3/31/25

Emily Rhine - Mar 31, 2025, 11:08 AM CDT



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Emily Rhine - Mar 31, 2025, 11:08 AM CDT

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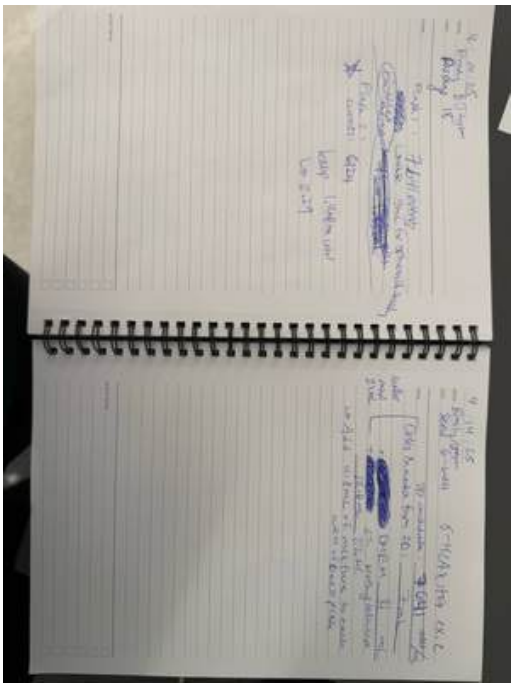
Passage19_Vial4_4/14/25

Emily Rhine - Apr 14, 2025, 11:56 AM CDT



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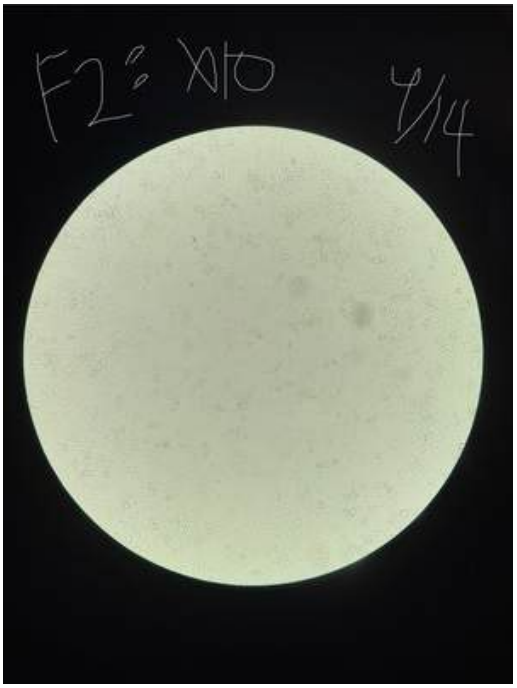
Emily Rhine - Apr 14, 2025, 11:56 AM CDT



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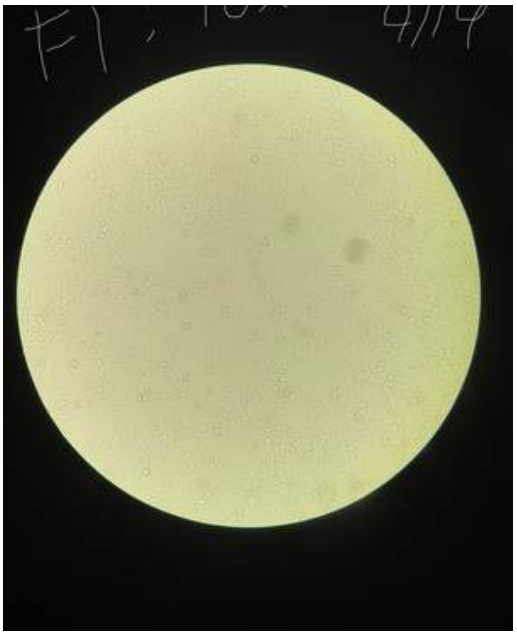
Emily Rhine - Apr 14, 2025, 11:56 AM CDT



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Exp1_Protocols_2/7-2/10

Emily Rhine - Feb 16, 2025, 2:17 PM CST

Title: Spheroid Formation Experiment

Date: 2/7/2

Content by: Althys & Emily

Content:

Link: https://benchling.com/uw_hesslab/f/lib_wSFKaFAF-spheroid-formation/etr_F867pmcX-cell-seeding-protocol-272025/edit

PROTOCOL

NOTE: USE SERUM-FREE DMEM IN **ALL** STEPS OF CELL SEEDING!

1. Collect cells from that day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL)**.
2. Make a **1.5 mL cell solution of 500,000 cells/mL (750,000 cells in total)**
    a. **Volume needed to obtain 750,000 cells from the day's cell passage: 750,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)**
        I. Can also put in **cell contration (cells/mL) from Step 1** into **Cell J2** from the table below, the volume needed for step 2a will be in **Cell K2.**
    b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**.
    c. Spin down the the tube at **200g for 5 minutes**, then **remove supernatant** to collect cells using vacuum filter
    d. Resuspend cells in **1.5 mL of serum-free DMEM**.
3. Prepare **master mix of cell solutions** for spheroid formation for **6 conditions**. The master mix can be used for **4 wells in a 96-well plate** and can be scaled to adjust for different numbers of wells if needed.
    a. Note: in the calculations you will see that I scale up to 5 instead of 4, this is to ensure we have enough materials for 4 wells.
    b. **Condition 1: 50,000 cells/cm^2, 0.75% methylcellulose**
        I. Into a new 1.5 mL eppendorf tube, add:
            1. 340 µL of serum-free DMEM
            2. 300 µL of 2% methylcellulose stock
            3. 160 µL of 500,000 cells/mL cell solution (Step 2)
        II. Mix gently by pipetting up and down, make sure the solution is well-mixed
    c. **Condition 2: 50,000 cells/cm^2, 1% methylcellulose**
        I. Into a new 1.5 mL eppendorf tube, add:
            1. 240 µL of serum-free DMEM
            2. 400 µL of 2% methylcellulose stock
            3. 160 µL of 500,000 cells/mL cell solution (Step 2)
        II. Mix gently by pipetting up and down, make sure the solution is well-mixed

d. **Condition 3: 50,000 cells/cm^2, 1.25% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 140 µL of serum-free DMEM
      2. 500 µL of 2% methylcellulose stock
      3. 160 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
e. **Condition 4: 75,000 cells/cm^2, 0.75% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 260 µL of serum-free DMEM
      2. 300 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
f. **Condition 5: 75,000 cells/cm^2, 1% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 160 µL of serum-free DMEM
      2. 400 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
g. **Condition 6: 75,000 cells/cm^2, 1.25% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 60 µL of serum-free DMEM
      2. 500 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed

4. **Mix gently by pipetting up and down then Aliquot** the prepared master mix into each well: add **160 µL of cell master mix** into each well **(4 wells total)**.
5. Grow spheroids in **37C, 5% CO2 incubator**. Spheroids are split every 3-4 days.

Cell Density (cells/cm^2)	Methylcellulose concentration (%)	Amount of methylcellulose 2% needed (microL) for 1 well	Cell number (cells)	Volume of (500,000 cells/mL) needed (microL) for 1 well	Volume of (500,000 cells/mL) needed (microL) for master mix	Amount of methylcellulose 2% needed (microL) for master mix	Amount of serum-free DMEM (microL) for master mix	Total number of cells needed	Put in cell concentration from CytoFlex (cells/mL)	Volume needed to obtain 750,000 cells (step 2a) (mL)
50000	0.75	60	16000	32	160	300	340	600000	745000	1.0067114094
50000	1	80	16000	32	160	400	240			
50000	1.25	100	16000	32	160	500	140			
75000	0.75	60	24000	48	240	300	260			
75000	1	80	24000	48	240	400	160			
75000	1.25	100	24000	48	240	500	60			

Conclusions & Action Items:

- Protocol followed on 2/7/25 by entire team (Jayson and Julia mainly)
- Condition 1 wells 1-3 may have an incorrect volume, so they may be excluded from the data collection & analysis.
- Follow up with Cell-Titer Glow protocol 2/10/25 with Carley (Julia, Althys, & Emily).
- Update final protocol after data analysis and conclusions on ideal seeding density and methylcellulose concentration.

Emily Rhine - Feb 16, 2025, 2:17 PM CST

Title: Cell-Titer Glo 2D Protocol (2/10/25)

Content By: Emily & Carley

Present: Emily, Althys, & Julia

Content:

1. Spheroids must be centrifuged at 800g for 15 min (sterile)
2. Remove media carefully from spheroid pellets (in hood)

3. Add 100uL SFM to each well (in hood)
4. Add 1X 10uL of Cell Titer Glo mix to each well (at bench, not in hood)
    - We are thawing the Cell titer glo mix the day of at room temp but since this might take some time in downstream experiments it may be beneficial to thaw the night before at 4c.
    - Cover wells with cell-Titer Glo with aluminum foil as you go to reduce light exposure.
5. Mix plates for 2 minutes at RT on an orbital shaker. Cover plate to reduce light exposure.
6. Incubate plates for 10 minutes at room temperature. Cover plate to reduce light exposure.
7. This plate can now be analyzed on a chemiluminescence machine (peak emission wavelength of cell titer glo is 560nm).
8. Analyze data: Normalized triplicate cell viability numbers for each drug treatment to vehicle only numbers (column 2). Plot on a log-linear plot and determine approximate IC50 values.

Conclusions & Action Items:

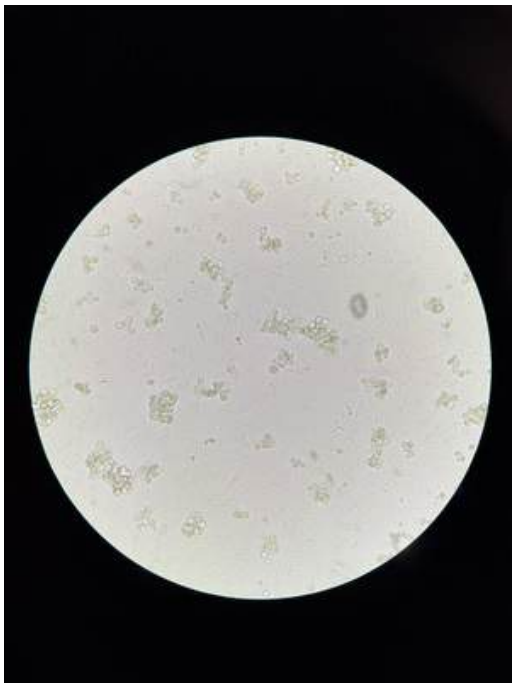
- Julia will complete this step and add it to the drive & notebook
- Update protocol as needed to optimize and add relevant details

Emily Rhine - Feb 16, 2025, 3:31 PM CST

******Since Cell-Titer Glo measures the amount of ATP in media and a high number indicates a large amount of live cells Carley noted that our results may be impacted by the fact that we measured the ATP luminescence only 15-20 minutes after changing media*******

Spheroid Formation Check_2/10/25

Emily Rhine - Feb 10, 2025, 10:29 AM CST



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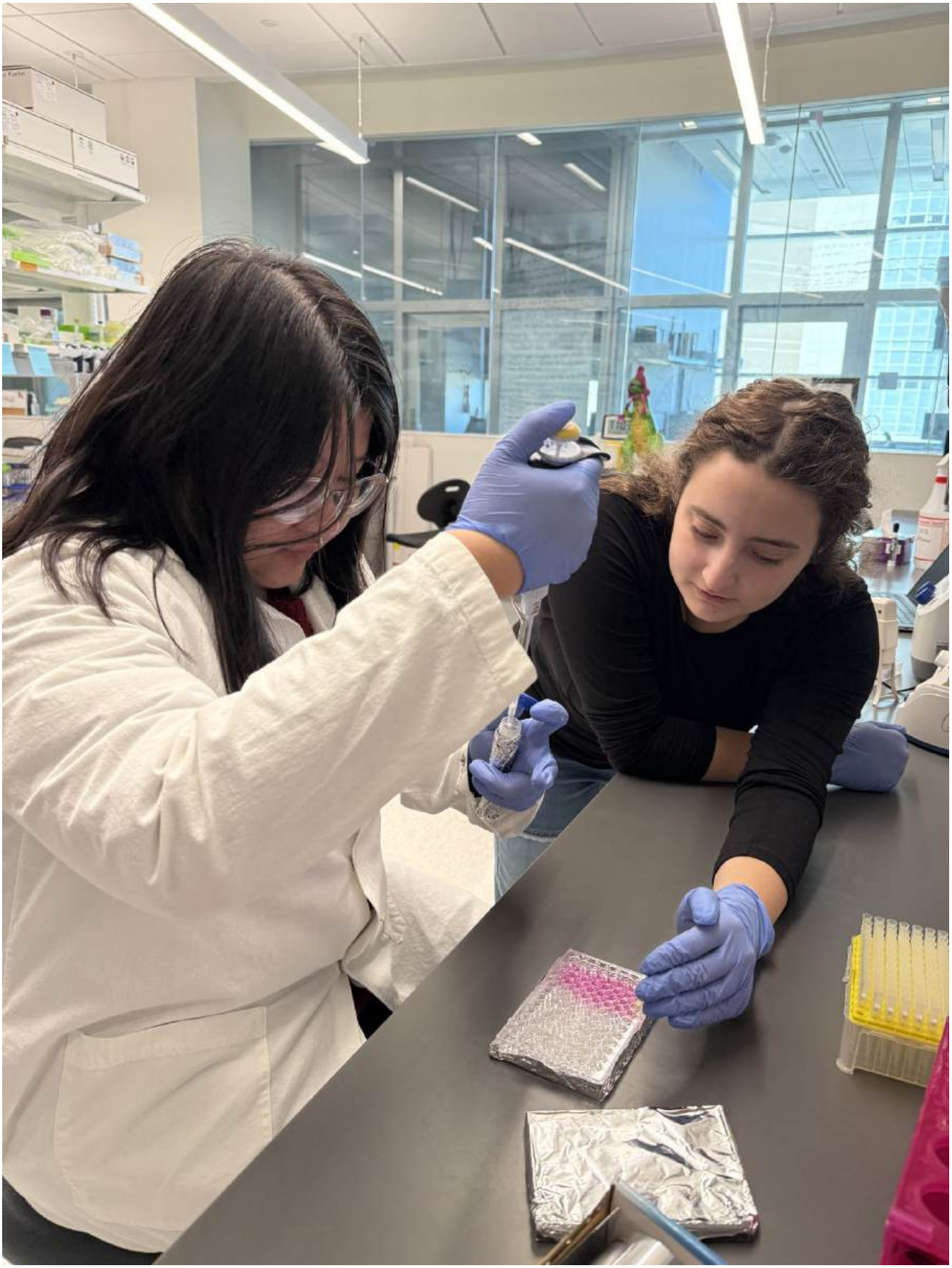
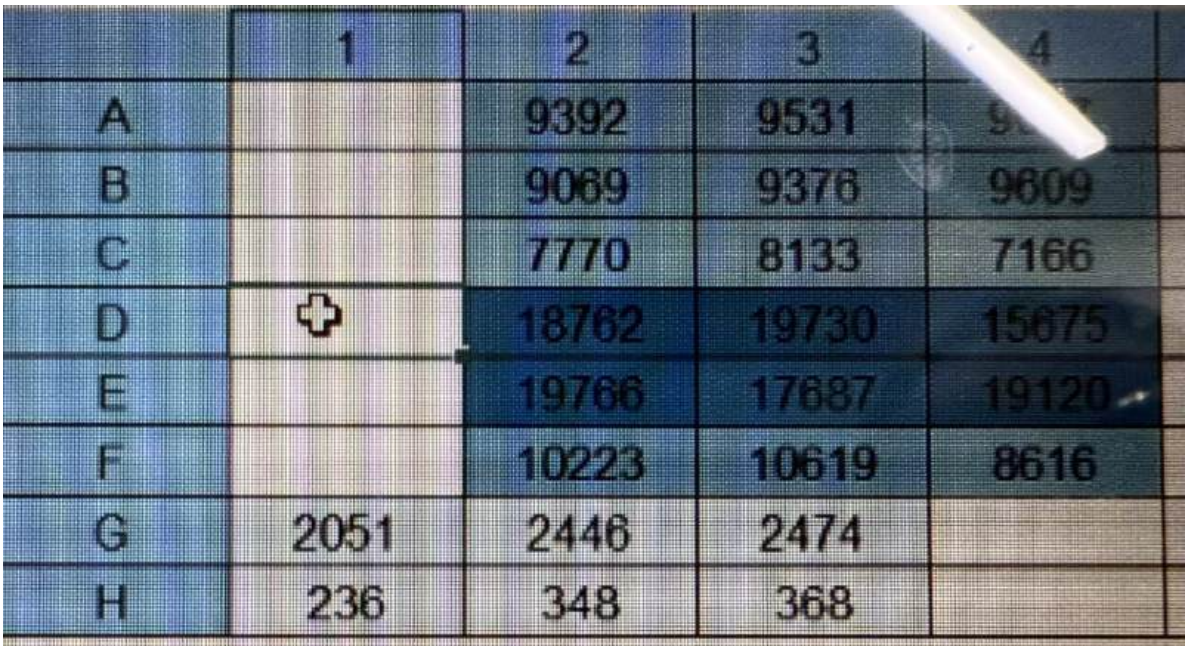
0698FA67-EC66-4952-9D1A-05AB87027647.jpg (346 kB)

Cell-Titer Glo Images_2/10/25

Emily Rhine - Feb 16, 2025, 2:13 PM CST



Emily Rhine - Feb 16, 2025, 2:15 PM CST



	1	2	3	4
A		9392	9531	
B		9069	9376	9609
C		7770	8133	7166
D		18762	19730	15675
E		19766	17687	19120
F		10223	10619	8616
G	2051	2446	2474	
H	236	348	368	

*Actual data will be uploaded by Julia at a later date

Exp2_Protocols_2/14-2/19

Emily Rhine - Feb 16, 2025, 2:21 PM CST

Title: Spheroid Formation Experiment

Date: 2/10/25

Content by: Emily

Present: Ana, Jayson, and Emily

Content:

Link: https://benchling.com/uw_hesslab/f/lib_wSFKaFAF-spheroid-formation/etr_F867pmcX-cell-seeding-protocol-272025/edit

PROTOCOL

NOTE: USE SERUM-FREE DMEM IN **ALL** STEPS OF CELL SEEDING!

1. Collect cells from that day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL)**.
2. Make a **1.5 mL cell solution of 500,000 cells/mL (750,000 cells in total)**
    a. **Volume needed to obtain 750,000 cells from the day's cell passage: 750,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)**
        I. Can also put in **cell contration (cells/mL) from Step 1** into **Cell J2** from the table below, the volume needed for step 2a will be in **Cell K2.**
    b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**.
    c. Spin down the the tube at **200g for 5 minutes**, then **remove supernatant** to collect cells using vacuum filter
    d. Resuspend cells in **1.5 mL of serum-free DMEM**.
3. Prepare **master mix of cell solutions** for spheroid formation for **6 conditions**. The master mix can be used for **4 wells in a 96-well plate** and can be scaled to adjust for different numbers of wells if needed.
    a. Note: in the calculations you will see that I scale up to 5 instead of 4, this is to ensure we have enough materials for 4 wells.
    b. **Condition 1: 50,000 cells/cm^2, 0.75% methylcellulose**
        I. Into a new 1.5 mL eppendorf tube, add:
            1. 340 µL of serum-free DMEM
            2. 300 µL of 2% methylcellulose stock
            3. 160 µL of 500,000 cells/mL cell solution (Step 2)
        II. Mix gently by pipetting up and down, make sure the solution is well-mixed
    c. **Condition 2: 50,000 cells/cm^2, 1% methylcellulose**
        I. Into a new 1.5 mL eppendorf tube, add:
            1. 240 µL of serum-free DMEM
            2. 400 µL of 2% methylcellulose stock
            3. 160 µL of 500,000 cells/mL cell solution (Step 2)
        II. Mix gently by pipetting up and down, make sure the solution is well-mixed

d. **Condition 3: 50,000 cells/cm^2, 1.25% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 140 µL of serum-free DMEM
      2. 500 µL of 2% methylcellulose stock
      3. 160 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
e. **Condition 4: 75,000 cells/cm^2, 0.75% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 260 µL of serum-free DMEM
      2. 300 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
f. **Condition 5: 75,000 cells/cm^2, 1% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 160 µL of serum-free DMEM
      2. 400 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed
g. **Condition 6: 75,000 cells/cm^2, 1.25% methylcellulose**
   I. Into a new 1.5 mL eppendorf tube, add:
      1. 60 µL of serum-free DMEM
      2. 500 µL of 2% methylcellulose stock
      3. 240 µL of 500,000 cells/mL cell solution (Step 2)
   II. Mix gently by pipetting up and down, make sure the solution is well-mixed

4. **Mix gently by pipetting up and down then Aliquot** the prepared master mix into each well: add **160 µL of cell master mix** into each well **(4 wells total)**.
5. Grow spheroids in **37C, 5% CO2 incubator**. Spheroids are split every 3-4 days.

Cell Density (cells/cm^2)	Methylcellulose concentration (%)	Amount of methylcellulose 2% needed (microL) for 1 well	Cell number (cells)	Volume of (500,000 cells/mL) needed (microL) for 1 well	Volume of (500,000 cells/mL) needed (microL) for master mix	Amount of methylcellulose 2% needed (microL) for master mix	Amount of serum-free DMEM (microL) for master mix	Total number of cells needed	Put in cell concentration from CytoFlex (cells/mL)	Volume needed to obtain 750,000 cells (step 2a) (mL)
50000	0.75	60	16000	32	160	300	340	600000	745000	1.0067114094
50000	1	80	16000	32	160	400	240			
50000	1.25	100	16000	32	160	500	140			
75000	0.75	60	24000	48	240	300	260			
75000	1	80	24000	48	240	400	160			
75000	1.25	100	24000	48	240	500	60			

Conclusions/Action Items:

- No inconsistencies, changes, or mistakes to note
- Continue following timeline
  - https://docs.google.com/spreadsheets/d/1qazQI04my1iPjsw7AaejbS4eu8blGuWJf2GprbxMNG0/edit?gid=0#gid=0
- Follow up with imaging and Cell-Titer Glo

Emily Rhine - Feb 16, 2025, 2:24 PM CST

Title: Cell-Titer Glo 2D Protocol (2/10/25)

Content By: Emily & Carley

Present: Emily, Althys, & Julia

Content:

1. Add 1X 10uL of Cell Titer Glo mix to each well (at bench, not in hood)

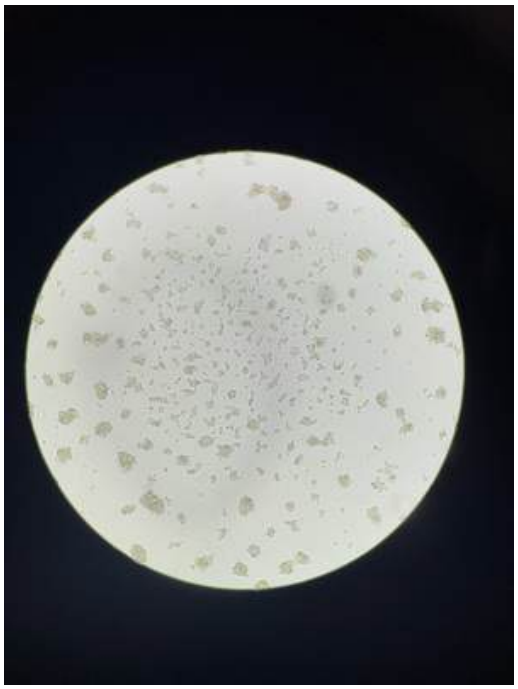
    - We are thawing the Cell titer glo mix the day of at room temp but since this might take some time in downstream experiments it may be beneficial to thaw the night before at 4c.
    - Cover wells with cell-Titer Glo with aluminum foil as you go to reduce light exposure.
2. Mix plates for 2 minutes at RT on an orbital shaker. Cover plate to reduce light exposure.
3. Incubate plates for 10 minutes at room temperature. Cover plate to reduce light exposure.
4. This plate can now be analyzed on a chemiluminescence machine (peak emission wavelength of cell titer glo is 560nm).
5. Analyze data: Normalized triplicate cell viability numbers for each drug treatment to vehicle only numbers (column 2). Plot on a log-linear plot and determine approximate IC50 values.

Conclusions & Action Items:

- Update protocol as needed to optimize and add relevant details
- Made sure previous steps 1- 3 (2/7-2/10 entry) were completed after spheroid imaging
    1. Spheroids must be centrifuged at 800g for 15 min (sterile)
    2. Remove media carefully from spheroid pellets (in hood)
    - 3. Add 100uL SFM to each well (in hood)
- Upload data to notebook and drive and compare to 2/10 data

Spheroid Confocal Images_2/17/25

Emily Rhine - Feb 17, 2025, 9:30 AM CST



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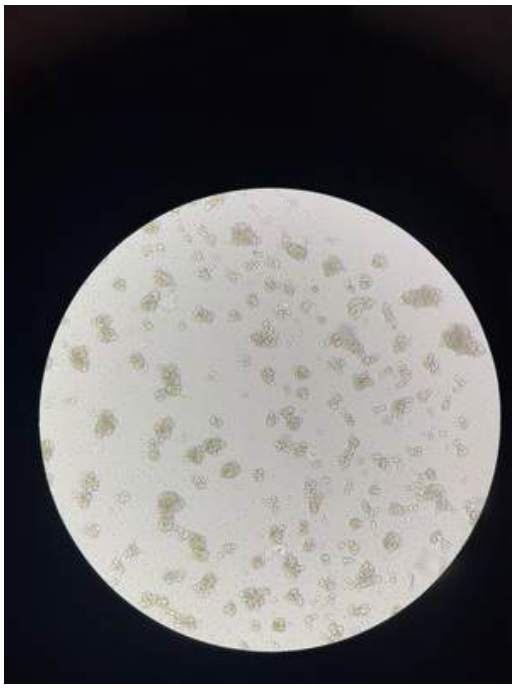

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Exp2_Images_2/17/25

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Spheroid Dissociation 1_2/28/25

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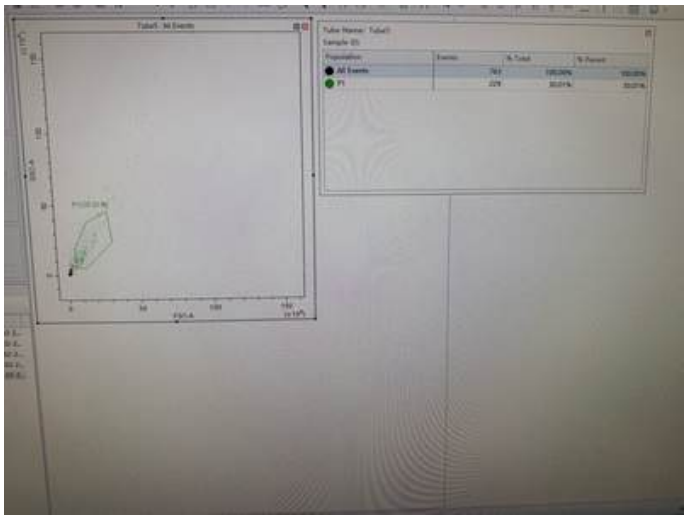

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Images_Accutase Spheroid Dissociation_Day 5_3/3/25

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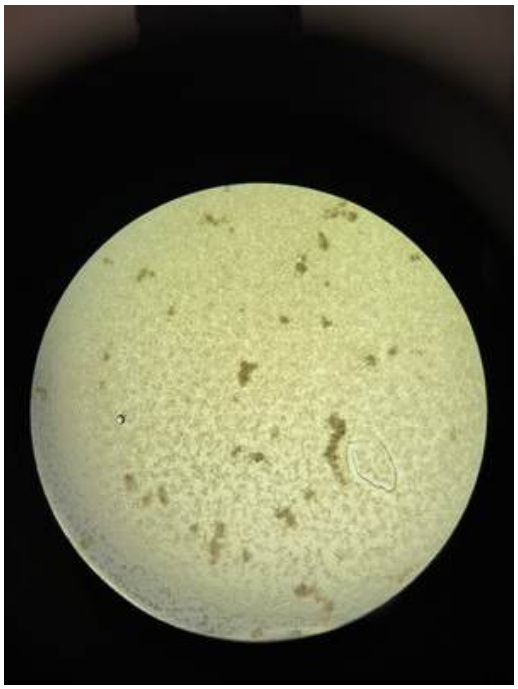

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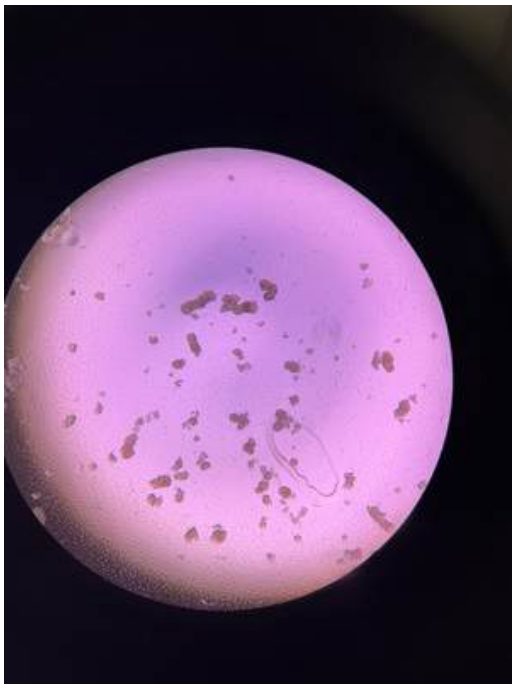

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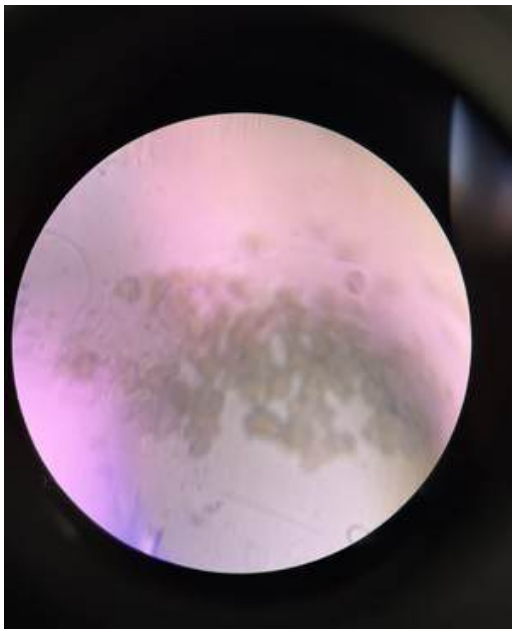

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1.5mL_before_centrifuge.jpg (212 kB)

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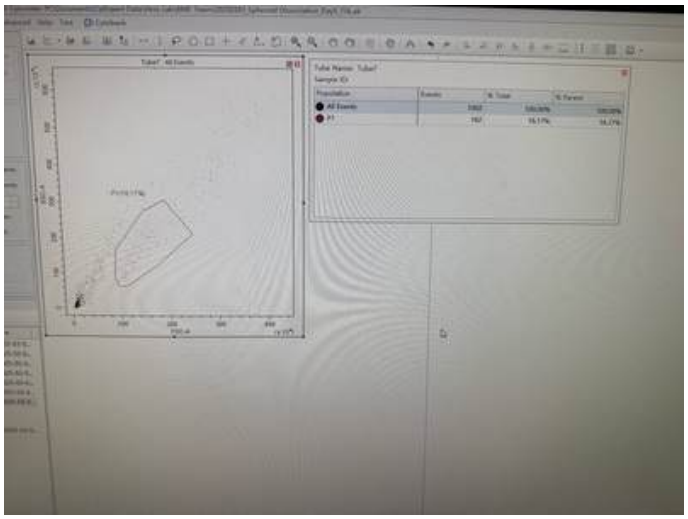

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Tube_7.jpg (301 kB)

Protocol_Accutase Spheroid Dissociation_Day 5_3/3/25

Emily Rhine - Mar 03, 2025, 1:29 PM CST

Accutase Spheroid Dissociation_3/3/25

FRIDAY, 2/28/2025

Materials

- Eppendorf tubes
- P200, P1000 pipettes
- Accutase
- Full DMEM (DMEM + 10% FBS + 1% Pen/Strep)
- Formed spheroids (5 days after seeding W-M)

Protocols:

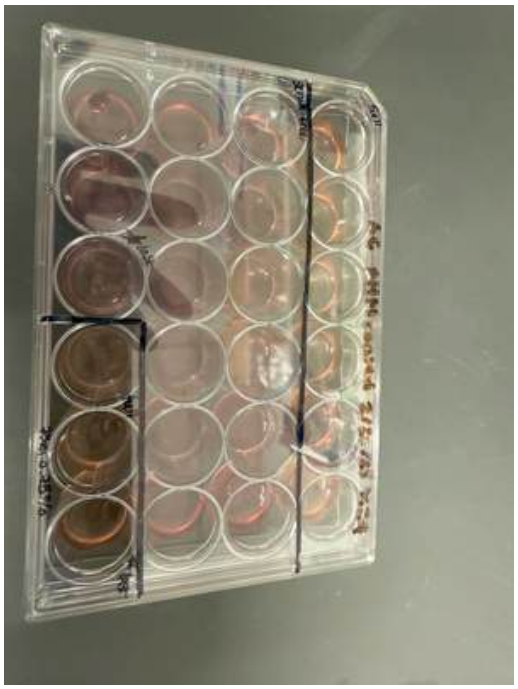
1. Prepare 9 eppendorf 1.5 mL tubes (for dissociation of 24 wells). Label them accordingly.
2. **Pipette contents of wells up and down using P1000 to break up spheroids and move them away from well walls**
3. Transfer the media (with cancer spheroids inside) from the wells into the according **eppendorf 1.5 mL tubes using a P1000** pipet tip set to **200µL.**
4. To each eppendorf 1.5 mL tube, **add 480 µL of PBS.**
5. Pellet the spheroids via centrifugation at **800g, 15 min,** then **remove the supernatant using a pasteur pipet.**
    a. Look at eppendorf tube under brightfield 4x microscope to confirm that the pellet is stuck to the side wall
    b. When removing supernatant make sure not to touch the side walls with the pasture pipet if the cell pellet is stuck there
    c. Aspirate with eppendorf tube tipped at a 45 degree angle and slowly aspirate until the pasture pipet barely brushes the bottom of the tube
6. **Resuspend the pellets in 150 µL Accutase** using a P200 pipette. Thoroughly mix the solution by pipetting up and down 10 times using a P200 pipette set to 100 µL.
7. Incubate the tubes in **37°C for 10 minutes.**
8. Pipette the solution in each tube **up and down 10 times** using a P200 pipette set to 100 µL.
9. Add **850 µL of DMEM so that the final volume reaches 1 mL.**

Spheroid Confluency & Doubling Time

	Events	Cells/mL	75,000*0.32 (Cells in Solution at t=0)	% Confluency
1	0	0	24000	0
2	45	4500	24000	18.75
3	39	3900	24000	16.25
4	99	9900	24000	41.25
5	89	8900	24000	37.0833333333
6	60	6000	24000	25
7	162	16200	24000	67.5
8	127	12700	24000	52.9166666667
9	137	13700	24000	57.0833333333

48hrs post seeding_3/7/25

Emily Rhine - Mar 08, 2025, 7:14 PM CST



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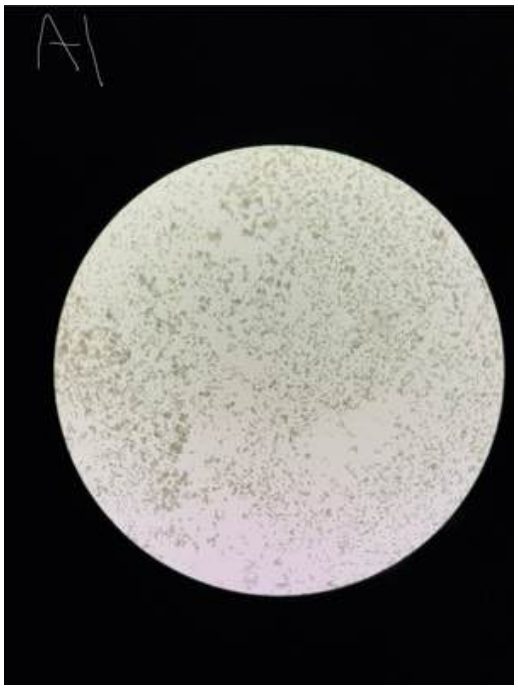

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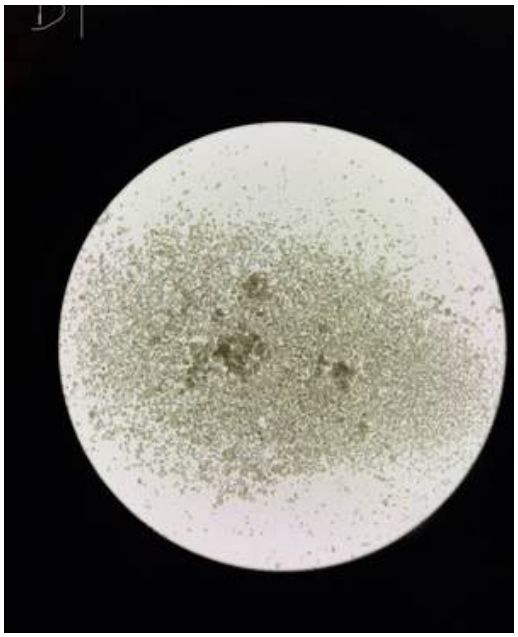

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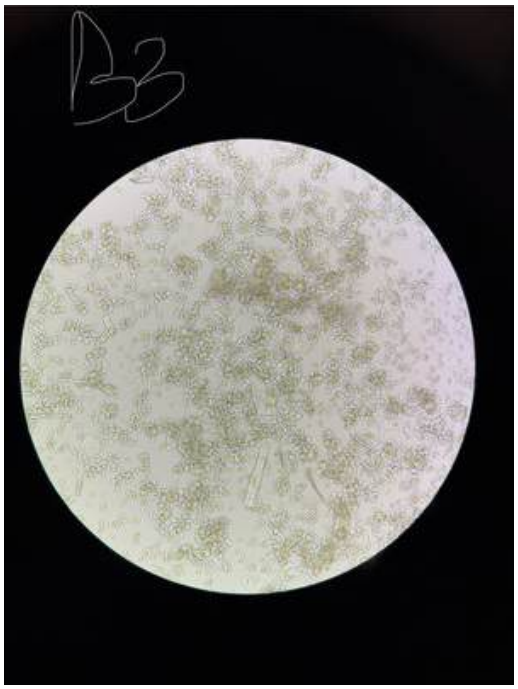

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72hrs post seeding_3/8/25

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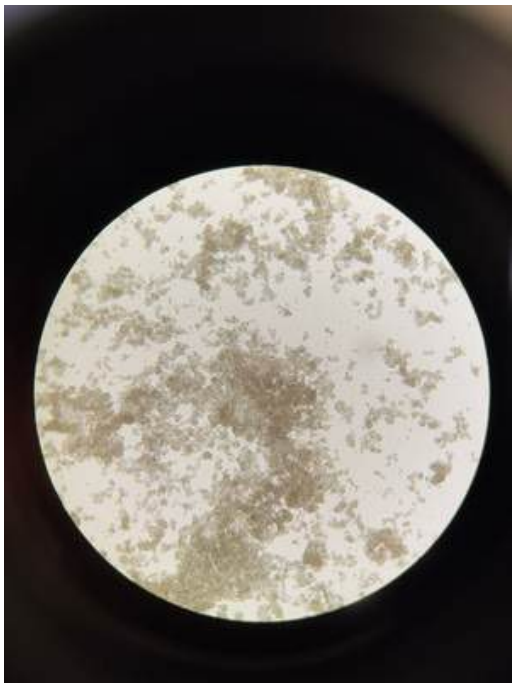

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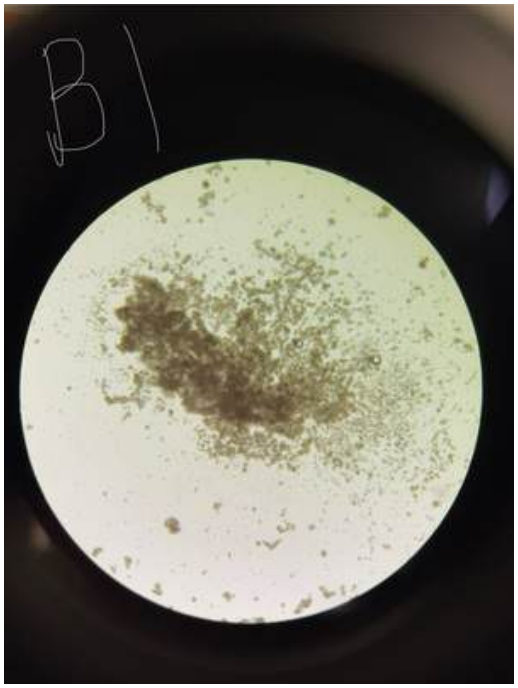

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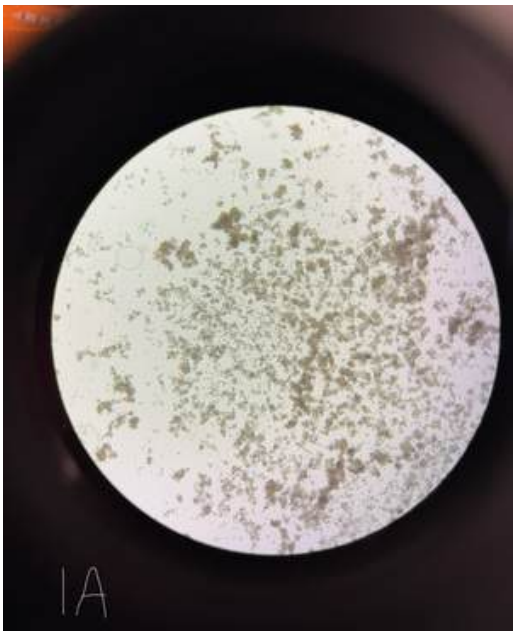

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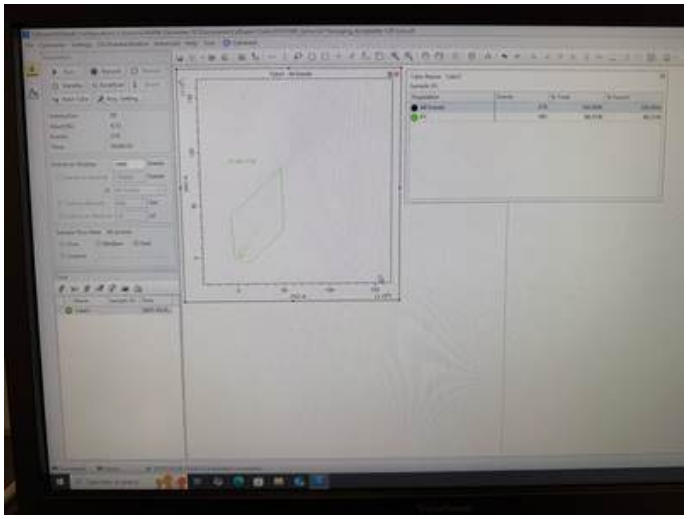

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Spheroid Passaging_3/9/25

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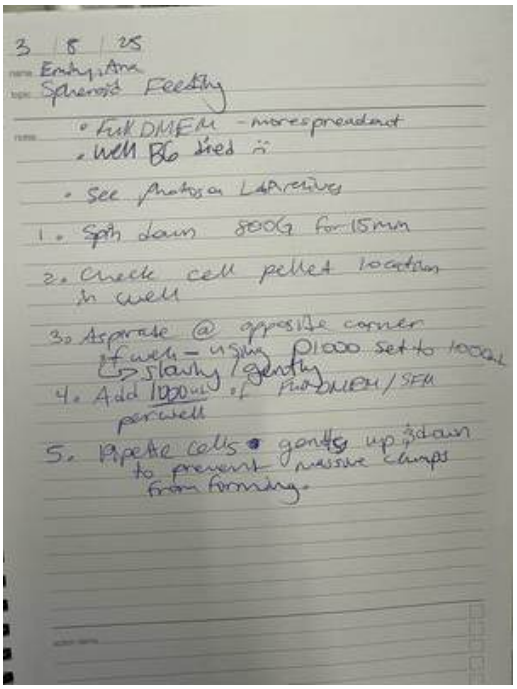

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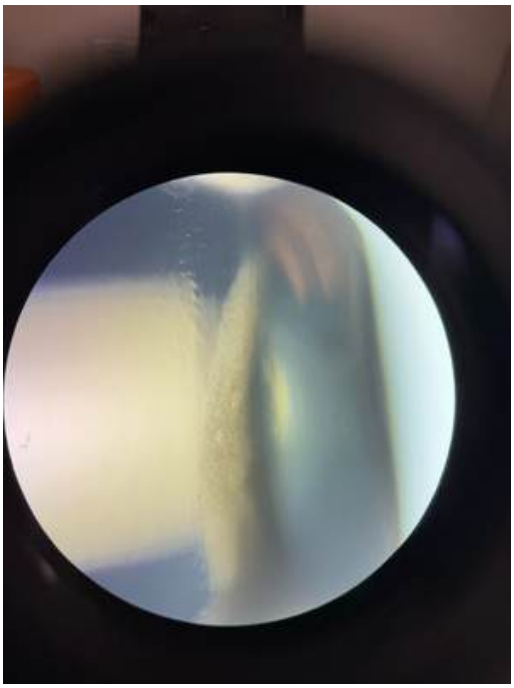

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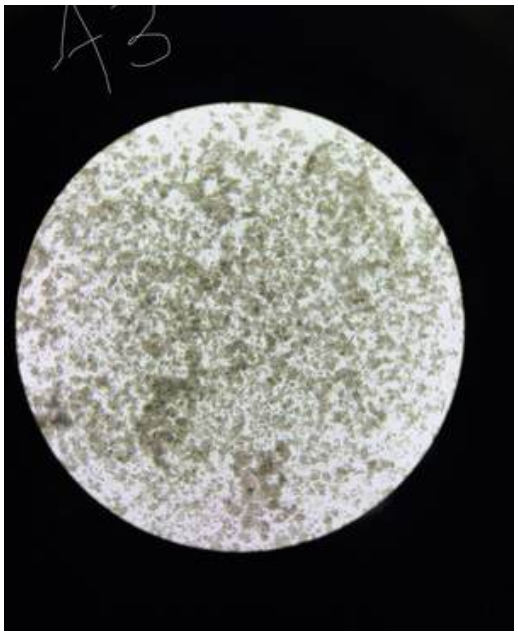

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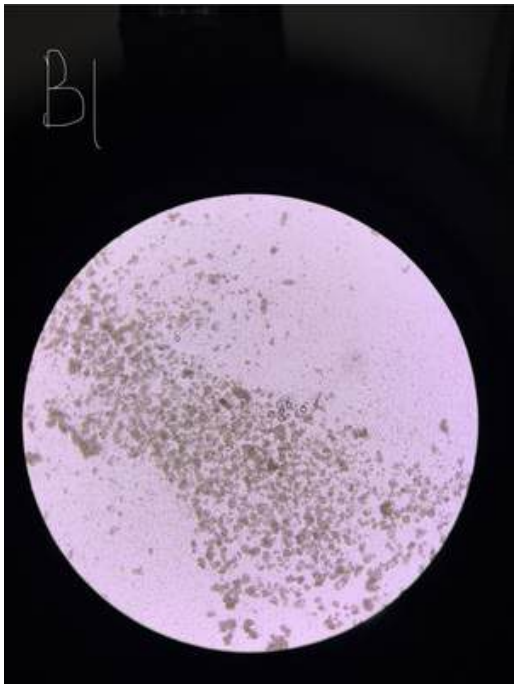

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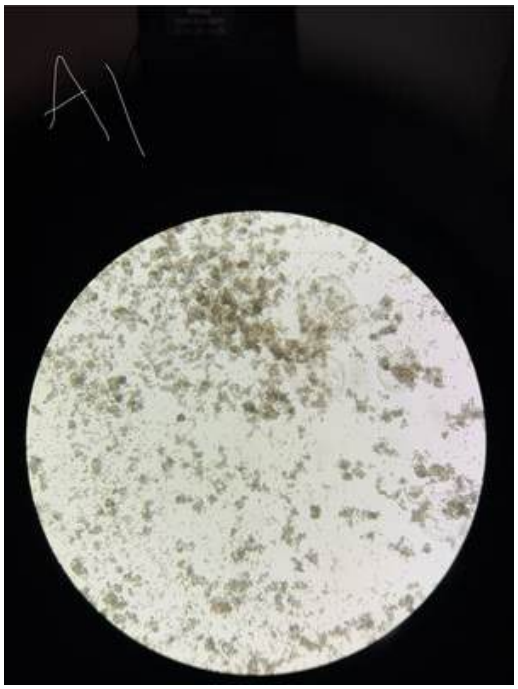

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48hrs

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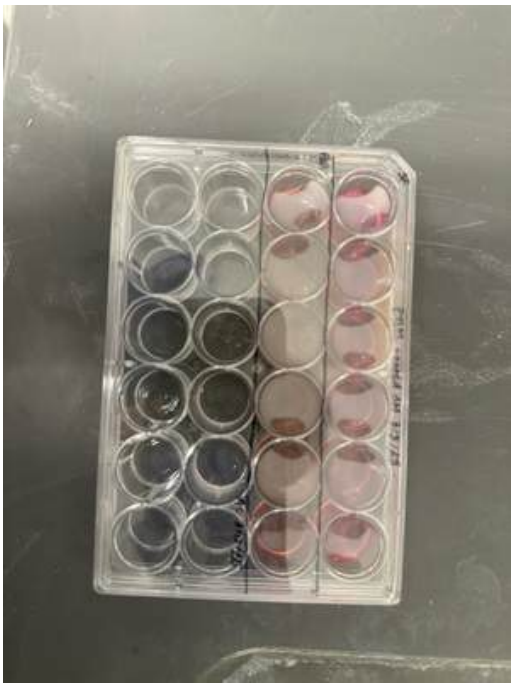

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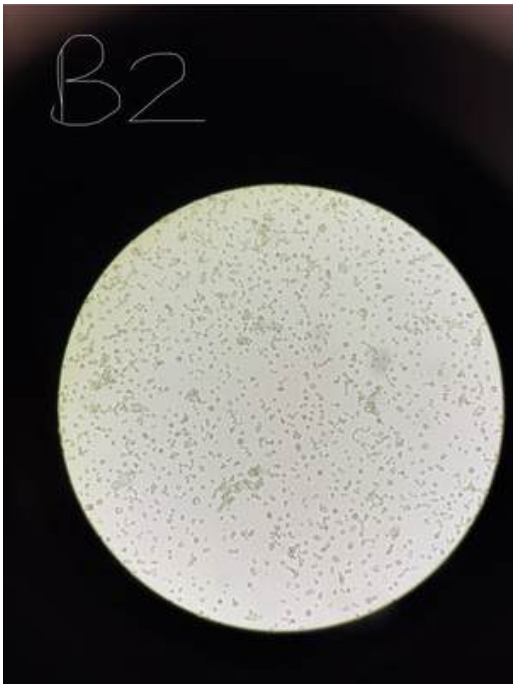

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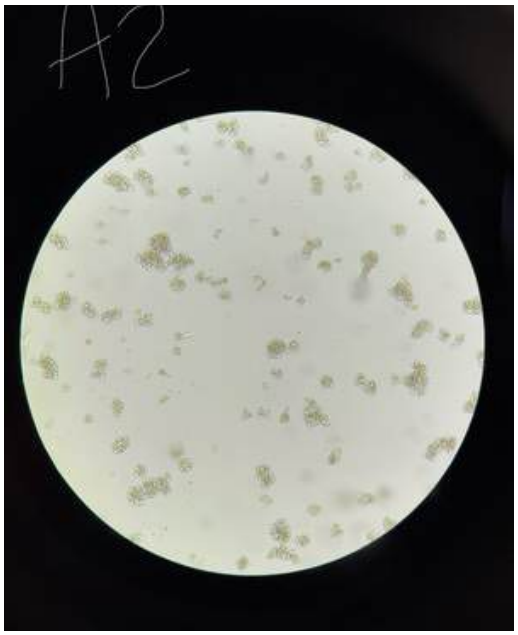

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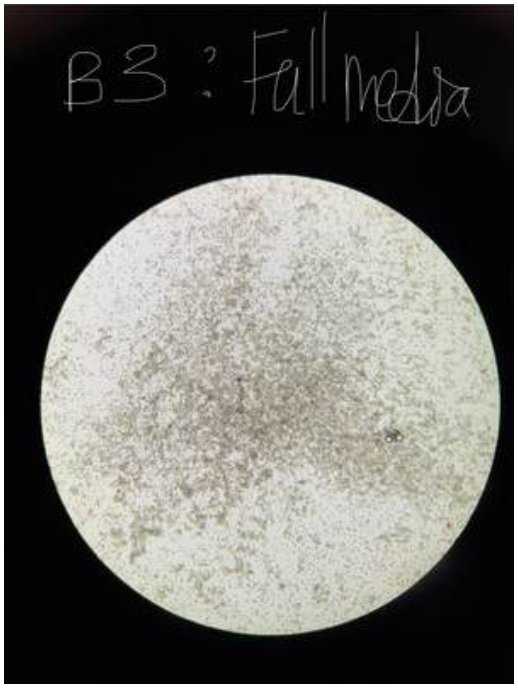

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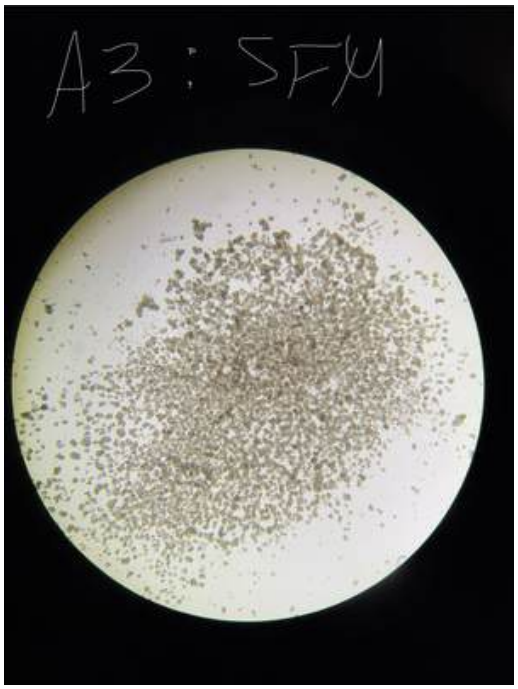

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132hrs

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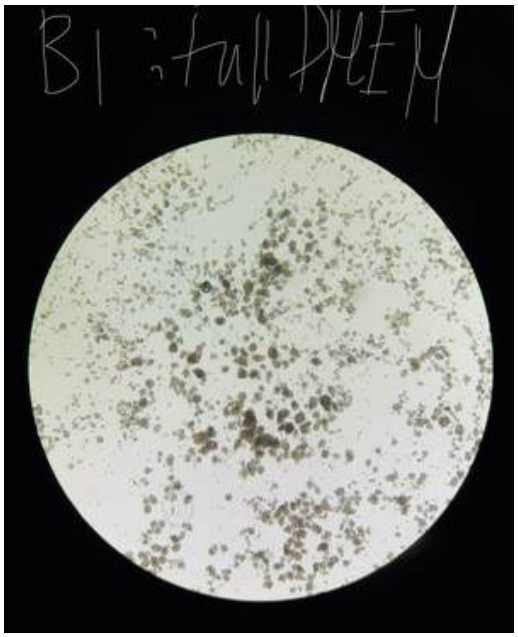

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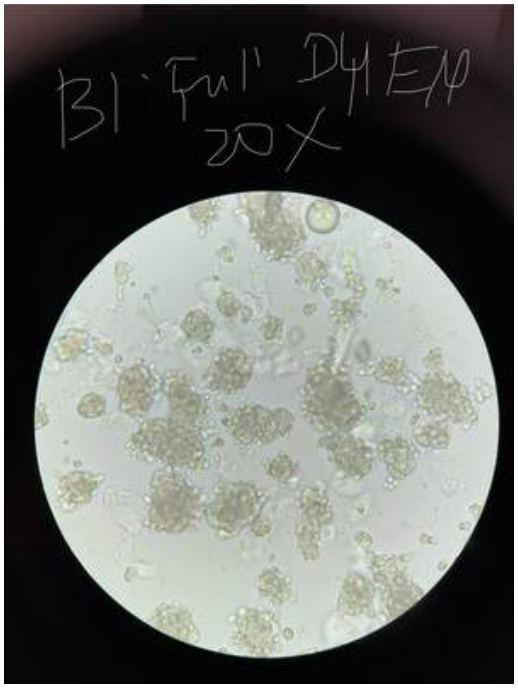

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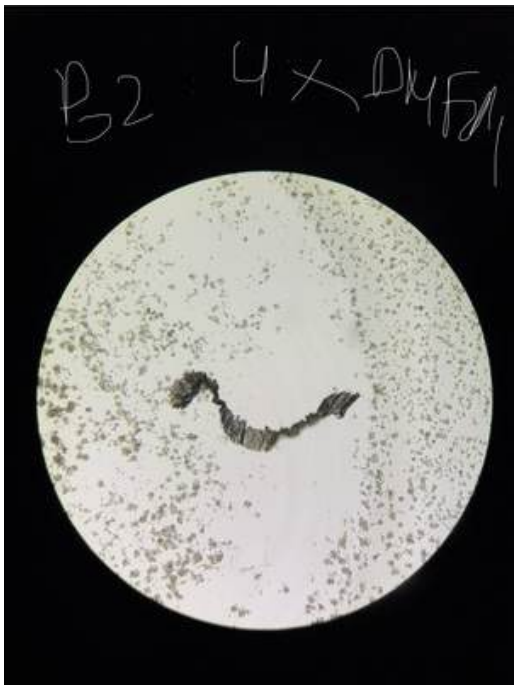

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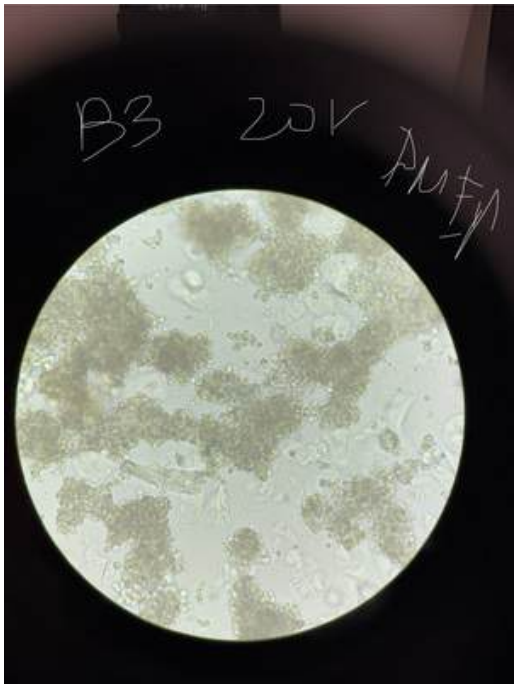

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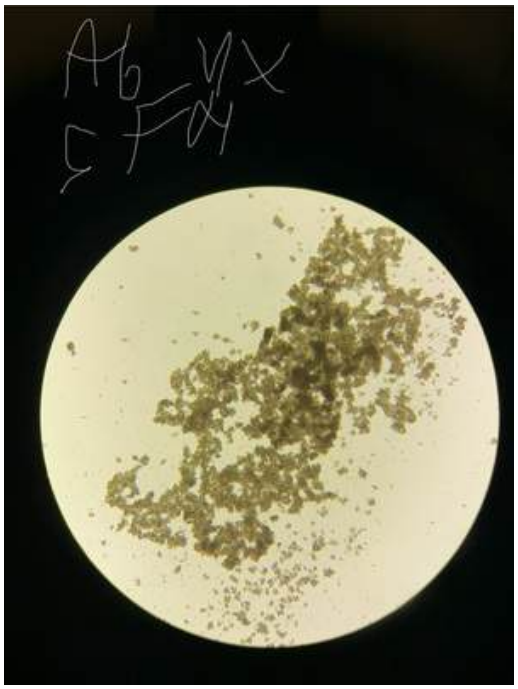

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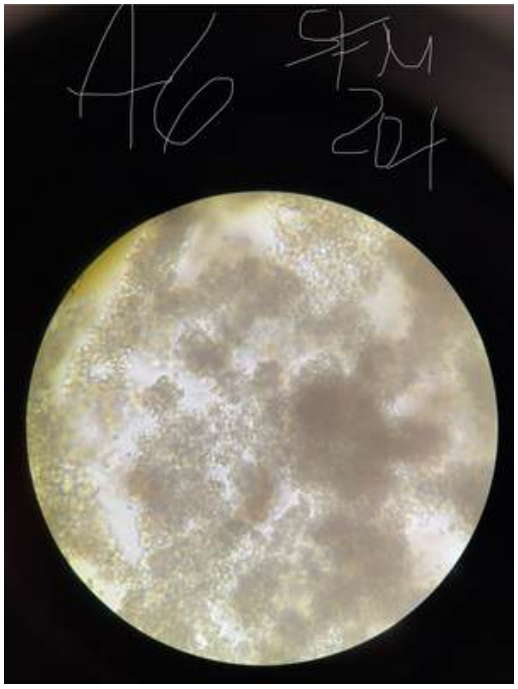

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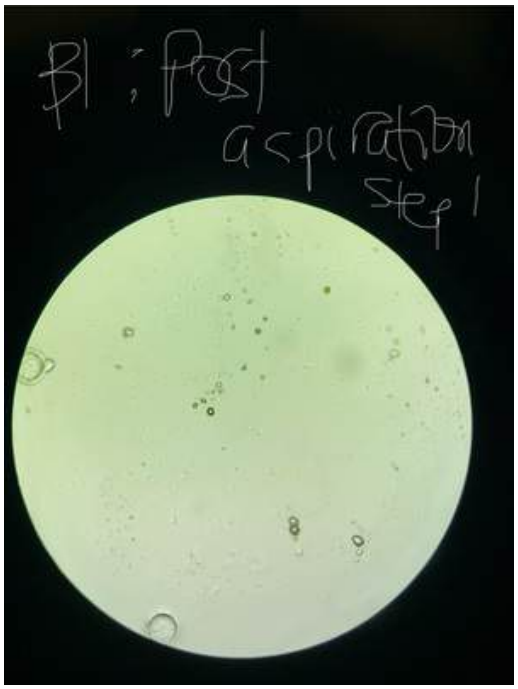

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Formation Protocol_3/17/25

Dissociation Protocol_3/17/25

Emily Rhine - Mar 17, 2025, 2:35 PM CDT

Accutase Spheroid Dissociation_3/17/25

FRIDAY, 2/28/2025

Materials

- Eppendorf tubes
- P1000 pipettes
- Accutase
- Full DMEM (DMEM + 10% FBS + 1% Pen/Strep)
- Formed spheroids (5 days after seeding W-M)

Protocols:

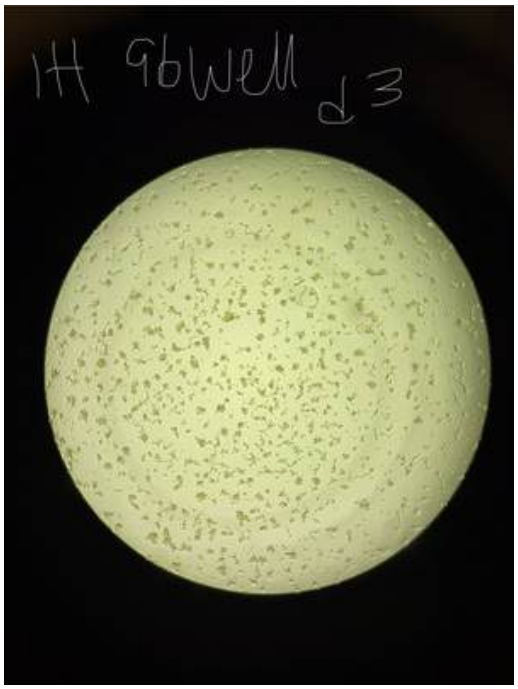
1. Prepare 12 eppendorf 15 mL tubes (for dissociation of 12 wells). Label them accordingly as either SFM or Full DMEM.
2. **Pipette contents of wells up and down using P1000 to break up spheroids and move them away from well walls**
    a. Note: Try to make as few bubbles as possible in this step
3. Transfer the media (with cancer spheroids inside) from the wells into the according connical **15 mL tubes using a P1000** pipet tip set to **400µL**.
    a. Wash well with 1mL PBS to collect any remain cells and add to the corresponding conical tube
    b. Note: Try to make as few bubbles as possible in this step
4. To each eppendorf 15 mL tube, **add 2 mL of PBS** (3mL total with the wash step)
5. Pellet the spheroids via centrifugation at **800g, 15 min**, then **remove the supernatant using a P1000 pipet**
    a. Remove all liquid from conical tube 1mL at a time using P1000 pipette making sure not to remove the cell pellet from the conical tube
    b. Note: Try to make as few bubbles as possible in this step
    c. Note: You dont have to change pipette tips as you remove the media unless you think the tip may be contaminated
6. **Resuspend the pellets in 600 µL Accutase** using a P1000 pipette.
    a. Thoroughly mix the solution by pipetting up and down 5 times (or until you can visably see the cell pellet has been broken up a little bit) using a P1000 pipette set to 600 µL.
    b. **Note: Complete this step concurently with the last step so that the cells do not dry out between the steps.** Once one tube is aspirated add accutase right away.
7. Incubate the tubes in **37°C for 10 minutes**.
8. Pipette the solution in each tube **up and down 5 times** using a P1000 pipette set to 1000 µL, add **1800 µL of DMEM so that the final volume reaches 2.4 mL**, and transfer 900uL of mixed cell solution to a 1.5mL eppendorf tube.
    a. Repeat untill all samples have been prepared for the cytoflex
9. Run cytoflex cell counting protocol
10. Calculate doubling time
    a. **https://www.cellseeker.org/cellcalc/growth-rate-and-doubling-time/**
    b. ~132 hours between seeding and dissociation

Spheroid Confluency & Doubling Time_Day 5_3/10/25

	Identity	Events	Cells/mL	Cells/ well	Starting amount of cells	Confluency (Cell#final/Cell #initial)
1	Full	253	28111.1111111111	67466.6666666666	142500	0.4734502924
2	Full-1 -tube 8	671	74555.5555555555	178933.3333333333	142500	1.2556725146
3	Full-2 -tube 9	1189	132111.1111111111	317066.6666666666	142500	2.2250292398
4	Full-3 -tube 10	826	91777.7777777778	220266.6666666667	142500	1.5457309942
5	Full-4 -tube 11	822	91333.3333333333	219200	142500	1.538245614
6	Full-5 -tube 12	854	94888.8888888889	227733.3333333333	142500	1.598128655
7	Full-6 -tube 13	899	99888.8888888889	239733.3333333333	142500	1.6823391813
8						
9						
10	Serum free	253	28111.1111111111	67466.6666666666	142500	0.4734502924
11	Serum free 1	127	14111.1111111111	33866.6666666666	142500	0.2376608187
12	Serum free 2	163	18111.1111111111	43466.6666666666	142500	0.3050292398
13	Serum free 3-tube 5	204	22666.6666666667	54400.0000000001	142500	0.381754386
14	Serum free 4-tube 6	280	31111.1111111111	74666.6666666666	142500	0.5239766082
15	Serum free 5-tube 7	315	35000	84000	142500	0.5894736842

72hrs

Emily Rhine - Mar 17, 2025, 2:10 PM CDT



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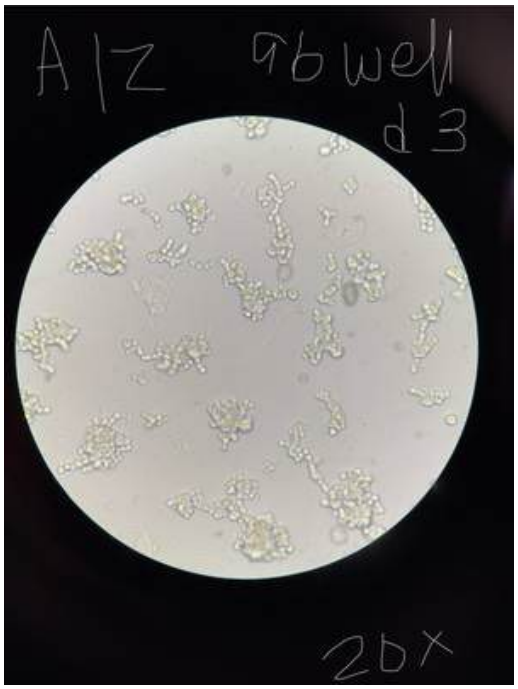

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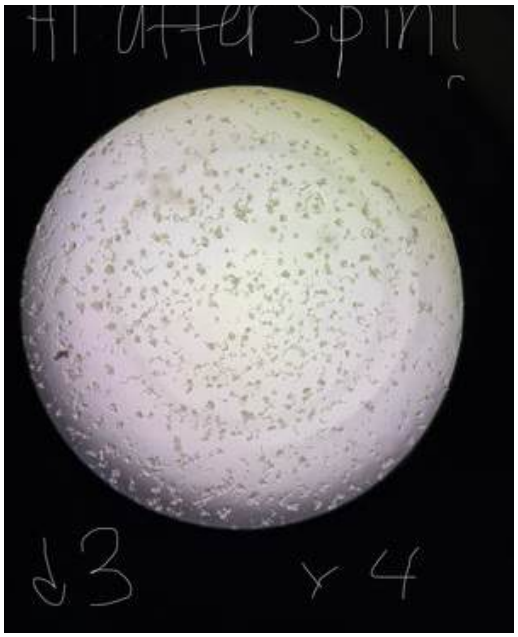

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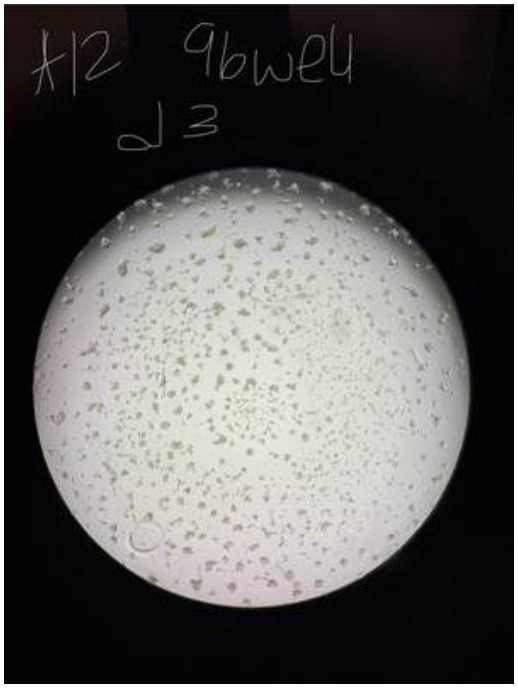

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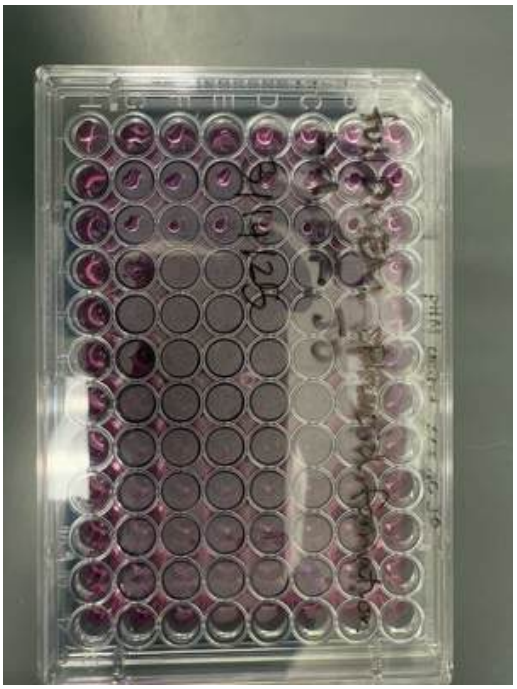

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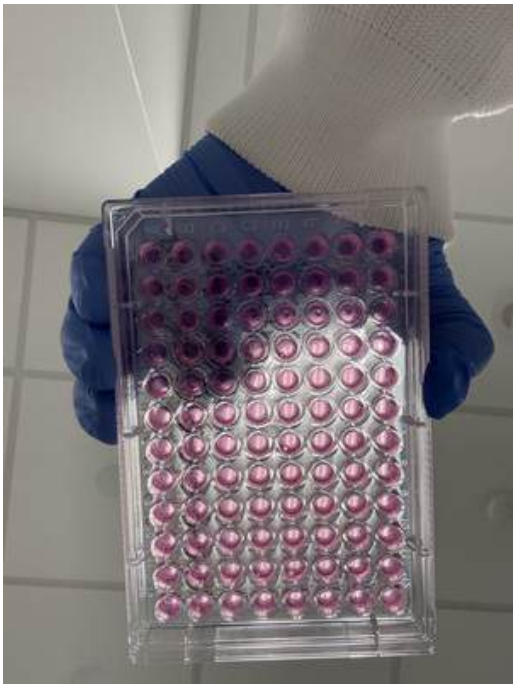

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Spheroid Formation Protocol_96 well_3/14/25

Emily Rhine - Mar 17, 2025, 2:32 P

Cell Seeding Protocol (3/14/2025) -- 96 well plate (full)

WEDNESDAY, 2/26/2025

NOTE: THIS PROTOCOL IS USED FOR 96 WELLS, EACH WELL HAS CELL DENSITY OF 75,000 CELLS/CM^2 AND METHYLCELLULOSE DENSITY OF 0.75%

1. Collect cells from that day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL)**.
2. Make a 8 **mL cell solution of 500,000 cells/mL (4000000 cells in total)**
    a. **Volume needed to obtain 4000000 cells from the day's cell passage: 4,000,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)**
        I. Can also put in **cell contration (cells/mL) from Step 1** into **Cell J2** from the table below, the volume needed for step 2a will be in **Cell K2.**
    b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**.
    c. Spin down the the tube at **200g for 5 minutes**, then **remove supernatant** to collect cells using vacuum filter
    d. Resuspend cells in **8 mL of full-serum DMEM**.
3. **Optimized Condition : 75,000 cells/cm^2, 0.75% methylcellulose**
    a. Into a new **50 mL tube**, add:
    b. 6.24 mL of serum-free DMEM
    c. 7.2 mL of 2% methylcellulose stock
    d. 5.76 mL of 500,000 cells/mL cell solution (Step 2)
        I. Mix gently by pipetting up and down, make sure the solution is well-mixed
4. **Mix gently by pipetting up and down then Aliquot** the optimized condition mix into each well: add **160 µL of cell mix** into each well **(24 wells total)**.
5. Grow spheroids in **37C, 5% CO2 incubator**. Spheroids are split every 3-4 days.

Cell Density (cells/cm^2)	Methylcellulose concentration (%)	Amount of methylcellulose 2% needed (microL) for 1 well	Cell number (cells)	Volume of (500,000 cells/mL) needed (microL) for 1 well	Volume of (500,000 cells/mL) needed (microL) for master mix	Amount of methylcellulose 2% needed (microL) for master mix	Amount of serum-free DMEM (microL) for master mix	Total number of cells needed	Put in cell concentration from CytoFlex (cells/mL)	Volume needed to obtain 4,000,000 cells (step (mL)
75000	0.75	60	24000	48	240	300	260	120000	640300	6.247071

Complete Gamma-H2AX Stain Protocol_4/7/24

Emily Rhine - May 03, 2025, 9:10 PM CDT

Title: Gamma-H2AX Stain Protocol

Date: 4/7 & 4/8
Content By: Carley, Emily, Jayson
Content:

Drug	Solvent	Stock Concentration	Working Concentration
Etoposide	DMSO	16.991mM	4 uM

Materials:

- Fix buffer (Fisher Scientific, BDB557870)
- Permeabilization buffer III (Fisher Scientific, BDB558050)
- Blocking buffer (10% FBS in PBS)
- Primary conjugated yH2AX antibody
- Etoposide

Day 1 Procedure:

1. Thaw Etoposide
2. Seed 6 well plate well plate with 2 million 2D cells per well
3. Spin down volume of 2D cells equal to 6-12 million cells total in 15 mL conical tube
4. Resuspend cells in 6 mL complete DMEM
5. Transfer 1 mL of cells to each 2D well
6. Add 5 mL of complete DMEM to control wells
7. Create Master mix of drug for 2D and 3D
8. master mix (2D):
9. 15 mL complete DMEM + 4.2 uL etoposide
10. master mix (3D):
11. 3.6 mL complete DMEM + 4.2 uL etoposide
12. Add 5 mL of master mix (2D) to 2D drug wells
13. Add 1.2 mL of master mix to 3D drug wells
14. Let incubate at 37C for at least 24 hours.

	Drug	Stock Concentration (mM)	Stock Concentration (uM)	Desired Treatment Concentration (uM)	Individual Well Volume (uL)	Volume of Stock Drug Needed (uL)	Number of Wells	Volume of Drug for Master Mix (uL)	Volume of Media Already in Well (mL)	Volume of Media for Master Mix (mL)
2D	Etoposide	16.991	16991	4	6000	1.4125125066	3	4.2375375199	14.4	3.6
3D	Etoposide	16.991	16991	4	6000	1.4125125066	3	4.2375375199	14.4	3.6

Day 2 Procedure:

Staining:

1. Preheat fix buffer I to 37 degrees Celsius. Cool perm buffer on ice. **(need to locate these and know where to heat up fix buffer)**
2. In TC, prepare cells as though you were planning to dissociate them
3. 2D:
4. Aspirate off old media making sure not to touch the bottom with the pasture pipette
5. Wash cells with 1 mL of PBS/ well
6. Aspirate PBS
7. Add 1 mL of Trypsin/ well
8. Incubate for 5 minutes at 37C
9. Add 3 mL of complete DMEM to neutralie trypsin
10. Transfer each well to seperate 15 mL conical tube

11. 3D - SEE 4/7/25 ACCUTACE DISSOCIATION PROTOCOL
12. Transfer each well to seperate 15 mL conical tube
13. Centrifuge 15 mL conical tubes at 500 x g for 5 min.
14. **Aspirate excess liquid, being careful to avoid disturbing the pellet.**
15. **********all following steps will be done at your bench in non-sterile conditions not in the BSC**********
16. **Resuspend** dissociated cells in fix buffer at a ratio of 10 uL fix buffer per million cells. Fix at 37 degrees Celsius for 10-15 min.
17. **30 uL/ sample**
18. Wash cells with a 500 uL of blocking buffer.
19. ***Note: For all washes, centrifuge at 500 g for 5 min, then aspirate excess liquid, being careful to avoid disturbing the pellet.***
20. Permeabilize by adding 20 uL/million cells of permeabilization buffer to the pellet. **Pipet up and down a few times to ensure the pellet is evenly mixed.** Permeabilize on ice for 30 minutes.
21. **60 uL/ sample**
22. Meanwhile, prepare antibody by making a 1:1000 dilution of antibody in blocking buffer (depending on the brand/type of primary antibody used, this ratio may differ.
23. **Math:** Antibody solution: 12 samples total, go for overage so make for 15. We do 100 uL per every 10^7 cells present which is 10 million cells. Since we have 2-3 million total, we will do 40uL for each sample.
24. 15 samples x 40 uL = 600 uL of blocking buffer
25. To do a 1000x dilution: 600/1000 = **0.6 uL of antibody**
26. After permeabilizing, wash the samples once with blocking buffer.
27. Stain with the antibody dilution at an amount of 100 uL mixture per 10 million cells. Place on a Nutator (shaker plate) at room temperature for 1 hr.
28. (100uL/10 million)*(xuL/3million) = **30uL/ sample tube**
29. Wash with blocking buffer (500 uL of blocking buffer, spin down, and aspirate)
30. **Resuspend** pellet in 150 uL of blocking buffer for flow cytometry.

Flow Cytometry Steps:

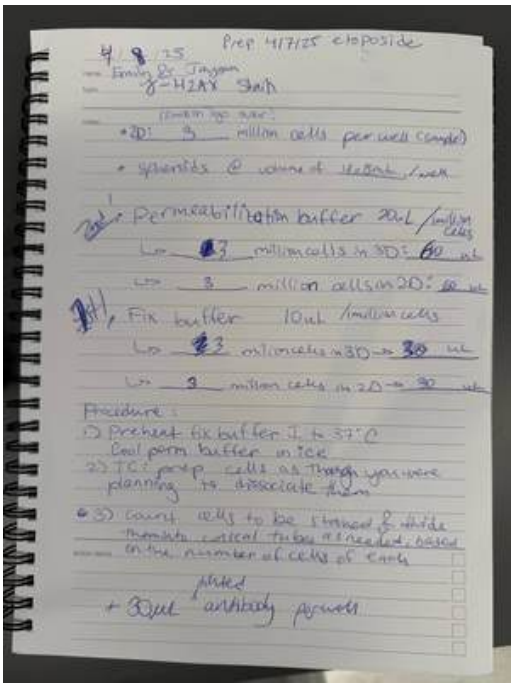
1. Set up fluoresence reading
2. Run QC
3. Settings:
4. Open new experiment and name appropritely
5. Alter stop to **150,000** events
6. Change to fast read
7. Change stop volume to 140uL
8. Run daily clean
9. Load first sample (3D drug - most cell death expected)
10. If after 2 minutes less than 150,000 events have been read, change events to match expected events for the rest of the samples **making sure not to set it less than 50,000 events.**
11. Run the rest of the samples.
12. Save data to Hess Lab drive
13. Run daily clean

Conclusion & Action Items:

- See other photo entries and hand written notes
- Redo stain 4/17 & 4/18
- Update benching and final report with protocol changes

Photos_4/7_4/8

Emily Rhine - Apr 13, 2025, 4:29 PM CDT



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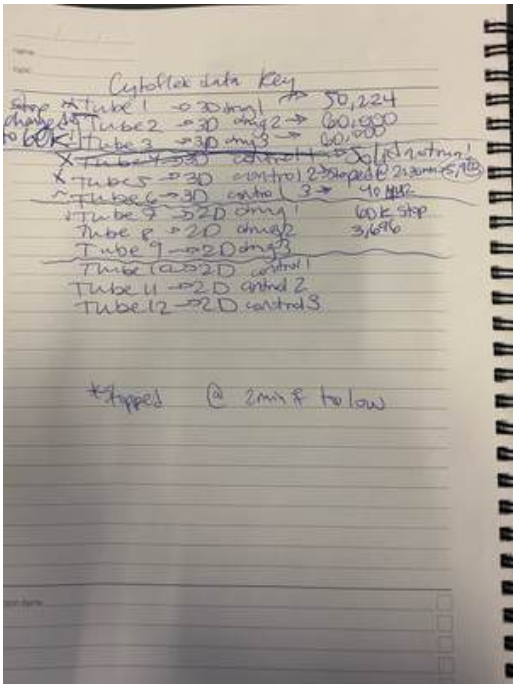

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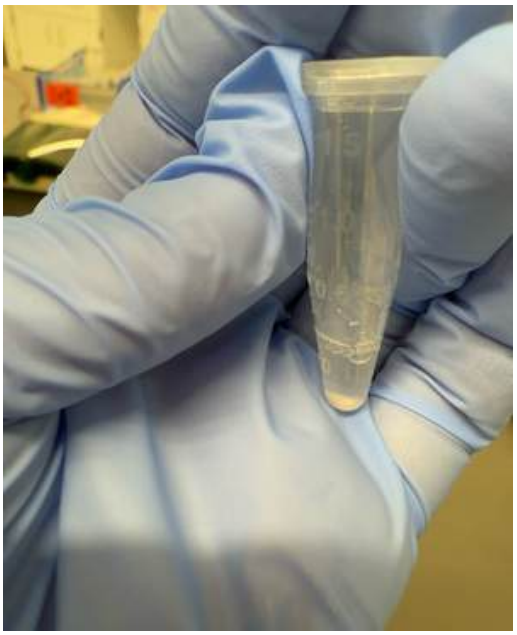

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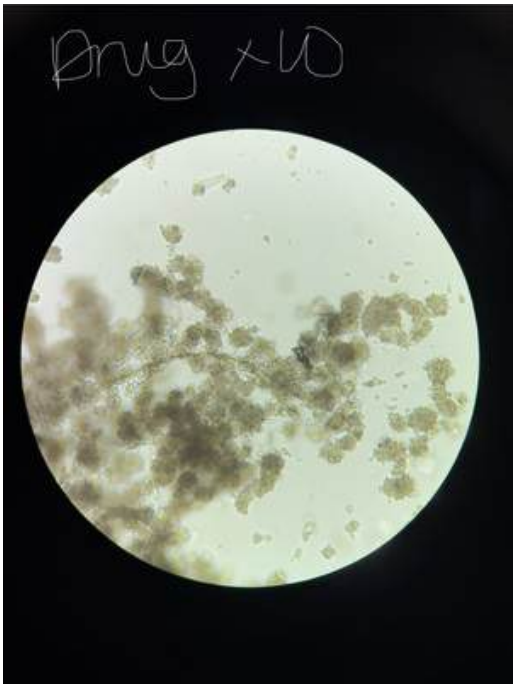

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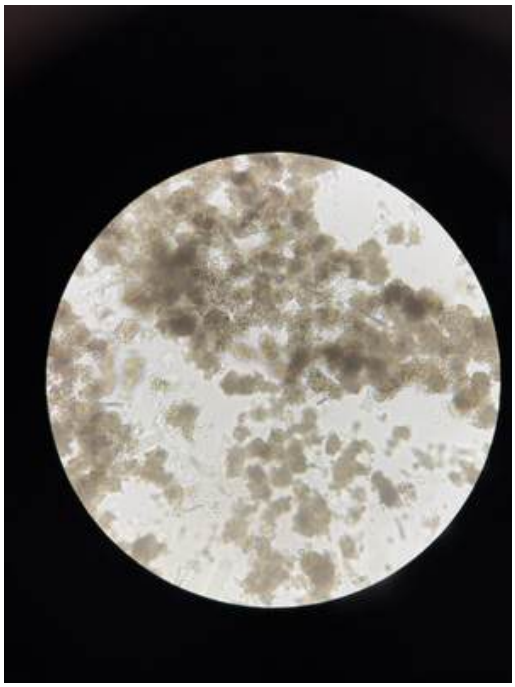

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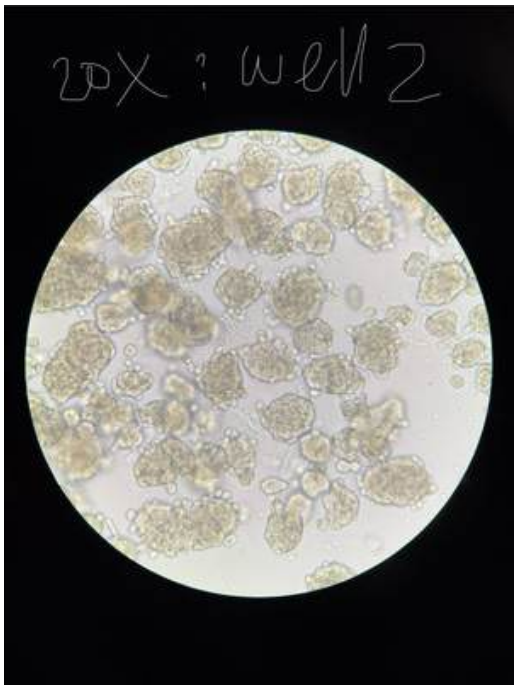
Spheroid imaging_4/18

Emily Rhine - Apr 18, 2025, 1:10 PM CDT



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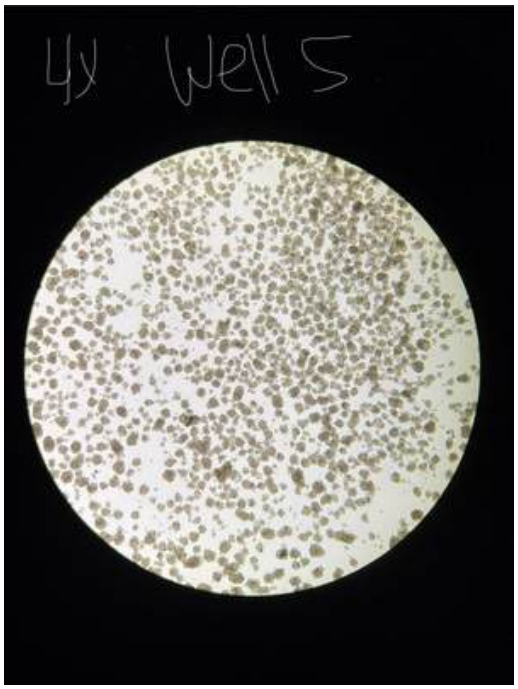
Emily Rhine - Apr 18, 2025, 1:11 PM CDT



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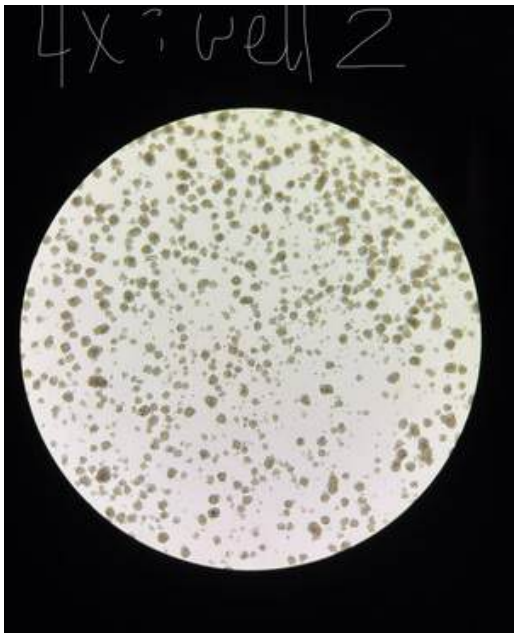
Emily Rhine - Apr 18, 2025, 1:11 PM CDT



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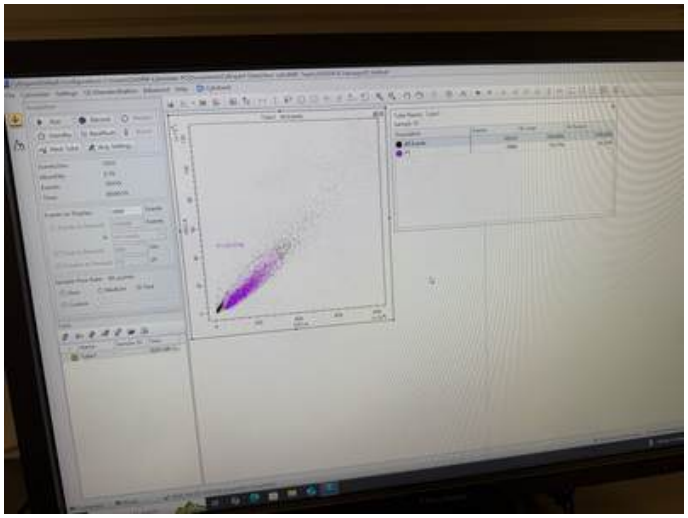
Emily Rhine - Apr 18, 2025, 4:22 PM CDT



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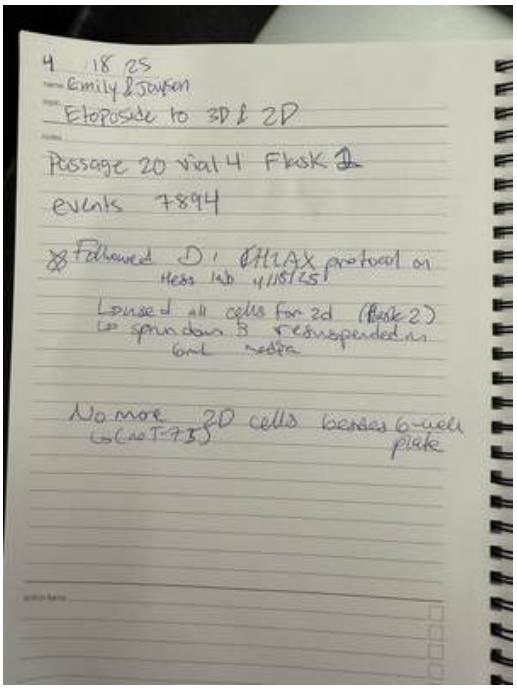
Emily Rhine - Apr 18, 2025, 4:22 PM CDT



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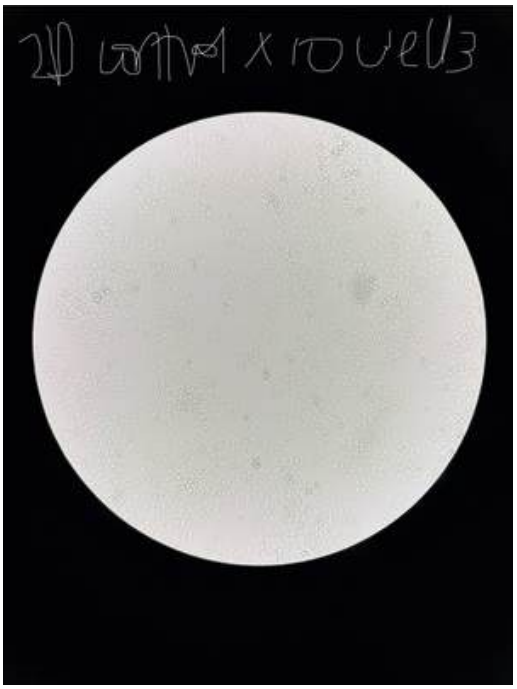
Imaging_4/19

Emily Rhine - Apr 19, 2025, 11:53 AM CDT



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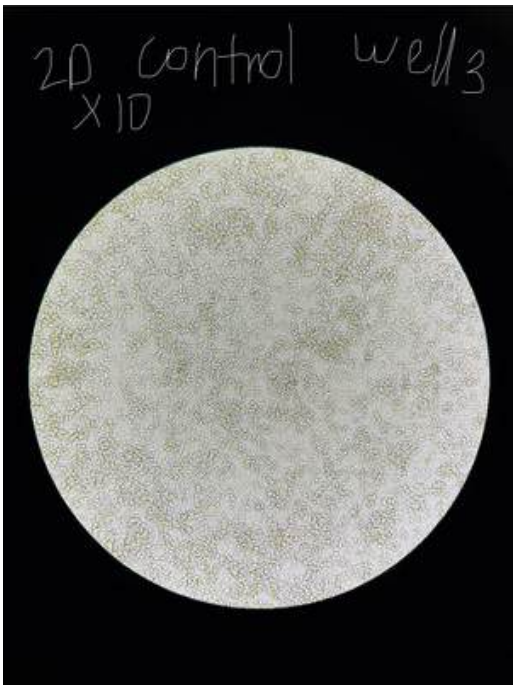
Emily Rhine - Apr 19, 2025, 11:53 AM CDT



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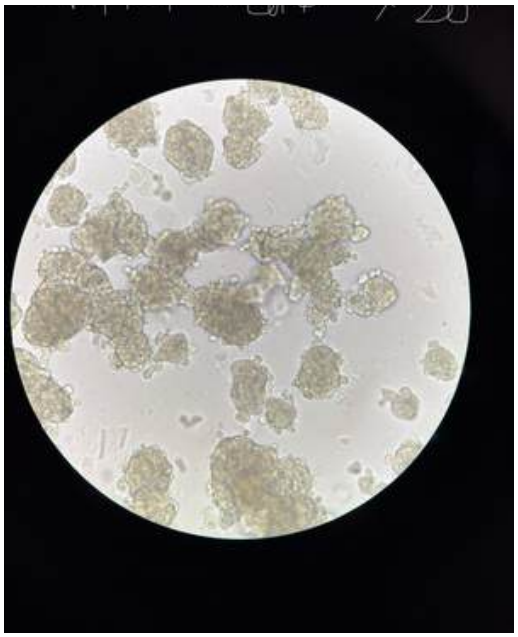
Emily Rhine - Apr 19, 2025, 11:56 AM CDT



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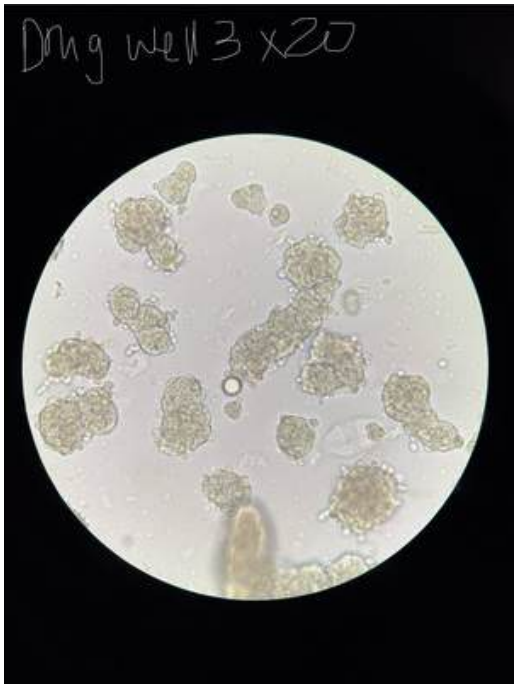
Emily Rhine - Apr 19, 2025, 11:56 AM CDT



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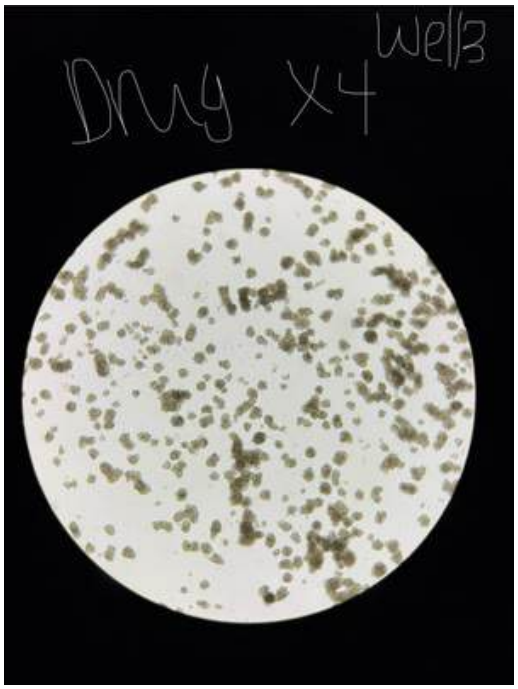
Emily Rhine - Apr 19, 2025, 11:56 AM CDT



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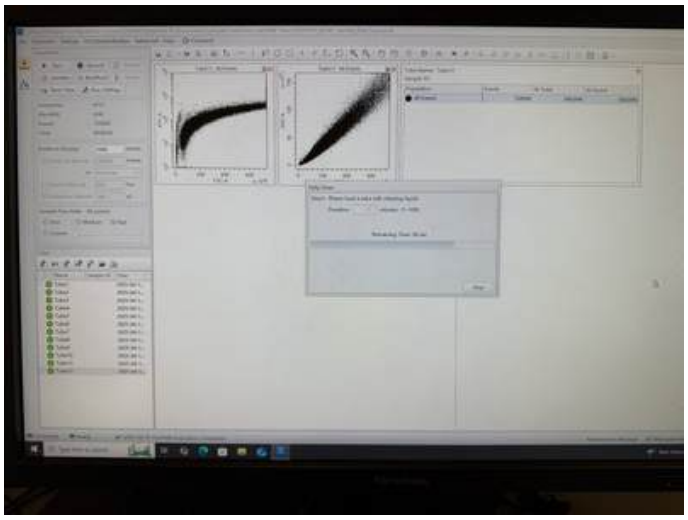
DD6AB391-BB1F-4A4C-9BB3-DA34A9A6DAAE.jpg (362 kB)

Results_4/19

Emily Rhine - Apr 19, 2025, 5:41 PM CDT



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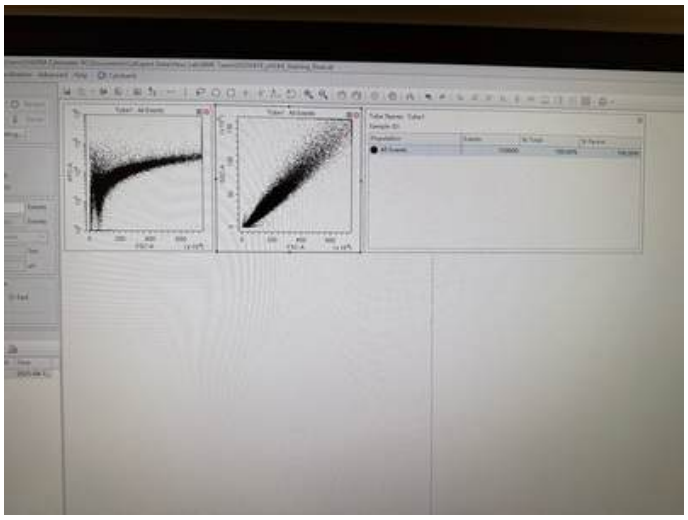
Emily Rhine - Apr 19, 2025, 5:42 PM CDT



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768E8E7F-A51C-4CA9-BC2E-002940F84F7E.jpg (311 kB)

ImageJ Analysis Protocol_11/25/2024

Emily Rhine - Dec 12, 2024, 3:28 PM CST

Title: ImageJ Analysis Protocol (abbreviated)

Date: 11/25/2024

Content by: Julia, Ana, Emily, and Jayson

Content:

Out of 120 images taken, 5 z-stack slices per 6 wells for 4 conditions, take the most in-focus images ("Spheroid Images" entry) complete the following steps:

1. Open ImageJ application and upload desired image
2. Set scale bar using the scale bar on the image
3. Go to image → type → 16 bit
4. Threshold image
5. Go to process → binary → watershed
6. Trace around well circle to isolate spheroids as the only thing being analyzed
7. Go to analyze particles → set interval to 4147-infinity, select show outlines from drop down, check “display results” and “exclude on edges” boxes, clear results boxes, and click OK
    - 4147 μm² taken from measurement of spheroid with ≥20 cells which is deemed the benchmark to accurately model the tumor micro environment [1].
    - See entry "Spheroid Size Threshold_ImageJ Analysis_11/25/2024" in Emily Rhine folder
8. Save measurements to an excel sheet
9. Record data results to seperate excel file by combine measurement averages
10. In excel compiled sheet and run 4 way ANOVA test in excel
11. Generate a suitable plot for this comparison (see example below)

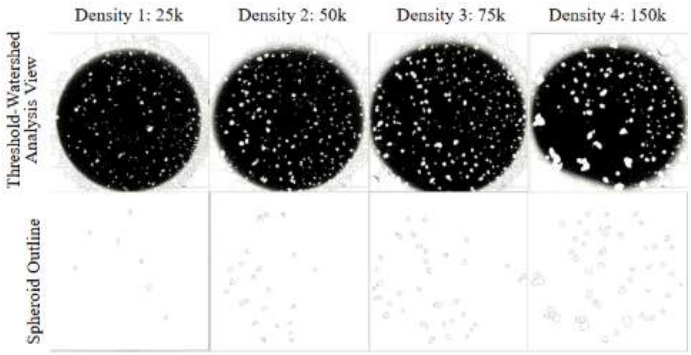


Figure 8: ImageJ analysis of spheroids across seeding densities 1-4 (25k, 50k, 75k, and 150k cells/cm², respectively). Images in top row are in threshold-watershed view and images in bottom row are in spheroid outline view.

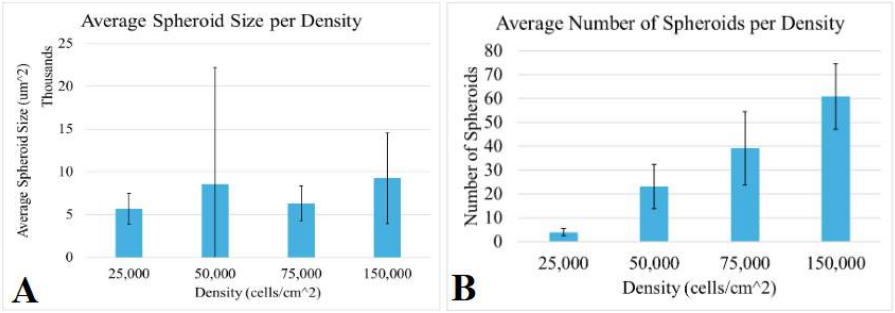


Figure 9: Spheroid analysis across seeding densities 1-4 (25k, 50k, 75k, and 150k cells/cm²). **A:** Average spheroid size (in µm²) across seeding densities (in cells/cm²). **B:** Average number of spheroids across seeding densities (in cells/cm²). Error bars in **A** and **B** display standard deviation.

References

[1] G. Razian, Y. Yu, and M. Ungrin, “Production of Large Numbers of Size-controlled Tumor Spheroids Using Microwell Plates,” J Vis Exp, no. 81, p. 50665, Nov. 2013, doi: 10.3791/50665.

Conclusions/Action Items:

- Add reduced protocol to poster and final report
- Add full protocol to appendix of final report
- Update ImageJ analysis, statistical comparison, and other testing protocols as needed

CellTiter-Glo_2/10/25

Emily Rhine - Feb 09, 2025, 10:38 PM CST

Title: CellTiter-Glo

Date: 2/10/25

Content by: Carley Schwartz & Emily

Content:

Add 1X volume of Cell Titer Glo mix (stored at -30C) to well volume. It's worth volume-checking a single well to ensure correct volumes are used.

- Mix plates for 2 minutes at RT on an orbital shaker. Cover plate to reduce light exposure.
- Incubate plates for 10 minutes at room temperature. Cover plate to reduce light exposure.
- This plate can now be analyzed on a chemiluminescence machine (peak emission wavelength of cell titer glo is 560nm).
- Analyze data: Normalized triplicate cell viability numbers for each drug treatment to vehicle only numbers (column 2). Plot on a log-linear plot and determine approximate IC50 values.

Conclusions & Action Items:

- Discuss protocol alterations to 2D protocol to better adapt it to 3D
- Implement protocol 9:30 am 2/10 with Carley
- See Emily LabArchives entry: "CellTiter-Glo 2D vs 3D_2/7/25"
- Update finalized protoclol with what worked & what didn't after experiment has been run and data has been processed
    - Make the necessary changes to the protocol

Accutase Spheroid Dissociation_6 well_4/8/25

Emily Rhine - Apr 13, 2025, 5:27 PM CDT

Title: Accutase Spheroid Dissociation_6 well

Date: 4/7/25- 4/8/25

Content By: Emily & Althys

Content:

Spheroid Dissociation Protocol

Moved 6-well plate from the incubator set to 37°C and 5% CO_2 to BSC. Labeled 6, 50 mL, conical tubes with proper conditions. Using a single-channel P1000 Eppendorf pipette set to 1000uL, gently pipetted up and down near well walls to mechanically break up spheroids. Transferred 4.8mL of well contents to corresponding 50mL conical tube as gently as possible, to avoid creating bubbles, using a P1000 pipet set to 1000µL. Washed each well twice with 1000 uL of PBS collect any remaining cells and added to the corresponding conical tube. Added 12.4 uL of additional PBS to each 50 mL conical tube to have a 1:3 media to PBS dilution to prepare it for centrifugation (Eppendorf, Centrifuge 5910 Ri, 5943000131). Balanced centrifuge with 3 tubes on each side and pelleted the spheroids at 800g for 15 min at room temperature (22°C). Gently removed the supernatant from one conical tube using pasteur pipette attached to vacuum while ensuring the cell pellet was not disturbed. Resuspended the pellet in 1000 µL Accutase (amount is adjusted for the cell number being dissociated) using a P1000 pipette by pipetting up and down at least 5 times. By completing these two steps concurrently, cell death from dehydration is limited. Repeated for all conical tubes. Incubated tubes at 37°C and 5% CO_2 for 10 min. The Accutase-cell solution is pipetted up and down another 5 times using a P1000 pipette set to 1000 µL. Finally, 3000 µL of complete DMEM was added in a 3:1 ratio to Accutase to neutralize it.

Conclusions & Action Items:

- Use protocol for RT-qPCR and gamma-H2AX
- Update Benchling
  - https://benchling.com/uw_hesslab/f/lib_9oa99j6P-accutase-dissociation/etr_NszVb1zj-spheroid-dissociation_gammah2ax_4825/edit
- Add complete and abridged protocol to final report

Emily Rhine - May 03, 2025, 9:13 PM CDT

1. Prepare 6, 50 mL, conical tubes
2. **Pipette contents of wells up and down using P1000 set to 800uL to break up spheroids and move them away from well walls (do this alongside step 3 for each well)**
    a. Note: Try to make as few bubbles as possible in this step
3. Transfer the media (with cancer spheroids inside) from the wells into the according conical **50 mL tubes using a P1000** pipet tip set to **1000µL.**
    a. Wash well with 1000 uL PBS in each well *twice* to collect any remain cells and add to the corresponding conical tube
    b. Note: Try to make as few bubbles as possible in this step/
4. To each 50 mL conical tube, **add 14.4 mL of PBS**
5. Pellet the spheroids via centrifugation at **800g, 15 min,** then **remove the supernatant using a P1000 pipet**
    a. Remove all liquid from conical tube 50 mL at a time using P1000 pipette making sure not to remove the cell pellet from the conical tube
    b. Note: Try to make as few bubbles as possible in this step
    c. Note: You dont have to change pipette tips as you remove the media unless you think the tip may be contaminated
    d. **Note: do this step concurently with step 6 (the step after)**
6. **Resuspend the pellets in 1000 µL Accutase** using a P1000 pipette.
    a. Thoroughly mix the solution by pipetting up and down 5 times and going against the wall of the tube (or until you can visably see the cell pellet has been broken up a little bit) using a P1000 pipette
    b. **Note: Complete this step concurently with the last step so that the cells do not dry out between the steps.** Once one tube is aspirated add accutase right away.
7. Incubate the tubes in **37°C for 10 -minutes.**
8. Pipette the solution in each tube **up and down 5 times** using a P1000 pipette set to 1000 µL, add **3000 µL of DMEM** so that the final volume reaches 4 mL

RT-qPCR_2/21/25

Emily Rhine - Feb 21, 2025, 8:08 PM CST

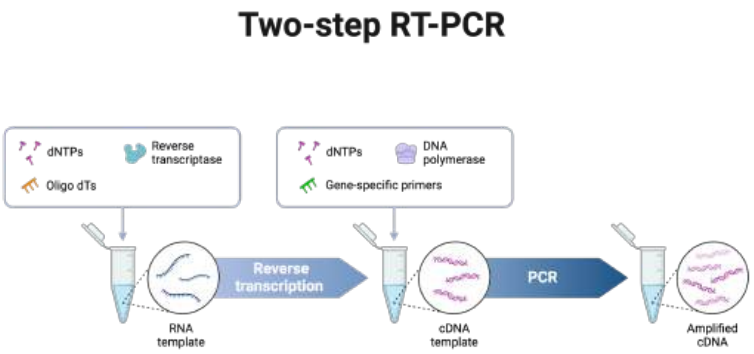
Title: RT-qPCR

Date: 2/21/25

Content By: Carley S.

Present: Carley, Emily, Julia, Ana, Jayson

Content:



Emily Rhine - Feb 21, 2025, 8:11 PM CST

RNA Extraction via Qiagen RNAeasy Kit

1. Clean bench and spray down with RNaseZap
2. Make RLT buffer (350µL per sample) by adding β-mercaptoethanol (10µL for every 1 mL of RLT buffer) into a separate tube inside the fume hood.
3. *Note: β-mercaptoethanol should be added freshly before each set of preparations.*
4. Prepare 1-5 million cells and spin down at 500g for 5 minutes. Aspirate supernatant and resuspend cells in 350µL of prepared RLT buffer.
5. Pipet cell solution onto a QiaShredder column and spin for 2 minutes at 14000 rpm. ***Discard column, not flow-through!***
6. Add 350µL of 70% ethanol to flowthrough. Transfer mixture to RNAeasy spin column and spin for 30s at 10000rpm. Discard flow-through.
7. Prepare Dnase solutions by adding 10µL of DNase I stock solution to 70µL of RDD buffer for each column. Mix well.
8. DNase in the resuspended form is stored in the -30 in the box with reagents for cDNA synthesis
9. Add 80µL of DNase I + RDD mix onto the column and let sit at room temperature for 15 min.
10. Add 350µL of RW1 buffer to column and spin 30s at 10000rpm. Discard flowthrough.
11. Add 500µL of RPE buffer to column and spin 30s at 10000rpm. Discard flowthrough.
12. Add 500µL of RPE buffer to column and spin 2 minutes at 14000rpm. Discard flowthrough.
13. Place Column into new 1.5mL tube and elute with 30-100µL of RNase-free water. Let sit for >5 minutes and spin down for 1 minute at 10000 rpm. Nanodrop to determine concentration.

14. Purified RNA can be stored at -80 C for 6 months, -20 C for 1 month.

cDNA Synthesis: RNAeasy purified RNA

1. This protocol is for 20µL/2µg of RNA protocol. Prepare in PCR tubes:
    1. RNA (2µg) x
    2. Oligo dT (0.5µg/µL) 2
    3. H2O 8-x
    4. *Note: If using a transcript specific primer, Oligo concentration should be 0.5-1 µM in final RT reaction. I usually use 2µL of 10µM stock.*
2. Heat sample at 70°C for 5 minutes. Place heated sample on ice for 5 min.
3. Add 10µL of following mix:
    1. 5X AMV RT Buffer 4
    2. 10mM dNTPs 1
    3. RNAsin 0.25
    4. AMV RT 0.75
    5. H2O 4
4. Incubate at 42°C for 2 hours.
5. If going into a PCR, add 50 uL of Milli-Q-H2O and proceed to set up qPCR reaction

Setting up qPCR: TaqMan method:

- Note: Check out more information on TaqMan method here: https://tools.thermofisher.com/content/sfs/manuals/cms_041280.pdf
- **Designing the experiment**: For these experiments, you require a housekeeping gene as a loading control in addition to your genes of interest. We generally order these conjugated to VIC and our target gene conjugated to FAM.

There are a number of ways to set up this experiment. You can use the $\Delta\Delta C_T$ calculation. This works since you the primer sets have been properly optimized.

Alternatively, you can set up the experiment as described in the for SYBR Green. You will need the following samples. where you will need the following controls. (You will have each sample in quadruplicate (this is a requirement).

Experimental sample is measured at 1:10 dilution in **quadruplicate** with gene expression assay and house keeping gene (both are in a single well)

Note: these are only if you are making a standard curve:

Primer curve of control template to be done (1 well each dilution) for each primer set consisting of:

- *No dilution*
- *1:5 dilution*
- *1:25 dilution*
- *1:125 dilution*
- *1:625 dilution*
- *1:3125 dilution*
- *Empty sample*

Setup the Following Master Mix

Reagent	1X	5X
20X Taq Man Gene Expression Assay Target Gene	1	5
20X Taq Man Gene Expression Assay Housekeeping Gene	1	5
2x Taq Gene Expression Master Mix	10	50
cDNA Template (1:10 dilution)	4	20
RNAse Free water	4	20
	20	100

Emily Rhine - Feb 21, 2025, 8:11 PM CST

Analysis: Use the relative quantification (ΔΔCt) method to analyze results. The general guidelines for analysis include:

• View the amplification plot; then, if needed:

– Adjust the baseline and threshold values.

– Remove outliers from the analysis.

• In the well table or results table, view the Ct values for each well and for each replicate group.

Perform additional data analysis using any of the following software:

Relative Quantification application thermofisher.com/connect

ExpressionSuite™ Software[1] thermofisher.com/expressionsuite

Complete Gamma-H2AX Stain Protocol_4/7/24

Emily Rhine - May 03, 2025, 9:11 PM CDT

Title: Gamma-H2AX Stain Protocol

Date: 4/7 & 4/8
Content By: Carley, Emily, Jayson
Content:

Drug	Solvent	Stock Concentration	Working Concentration
Etoposide	DMSO	16.991mM	4 uM

Materials:

- Fix buffer (Fisher Scientific, BDB557870)
- Permeabilization buffer III (Fisher Scientific, BDB558050)
- Blocking buffer (10% FBS in PBS)
- Primary conjugated yH2AX antibody
- Etoposide

Day 1 Procedure:

1. Thaw Etoposide
2. Seed 6 well plate well plate with 2 million 2D cells per well
3. Spin down volume of 2D cells equal to 6-12 million cells total in 15 mL conical tube
4. Resuspend cells in 6 mL complete DMEM
5. Transfer 1 mL of cells to each 2D well
6. Add 5 mL of complete DMEM to control wells
7. Create Master mix of drug for 2D and 3D
8. master mix (2D):
9. 15 mL complete DMEM + 4.2 uL etoposide
10. master mix (3D):
11. 3.6 mL complete DMEM + 4.2 uL etoposide
12. Add 5 mL of master mix (2D) to 2D drug wells
13. Add 1.2 mL of master mix to 3D drug wells
14. Let incubate at 37C for at least 24 hours.

	Drug	Stock Concentration (mM)	Stock Concentration (uM)	Desired Treatment Concentration (uM)	Individual Well Volume (uL)	Volume of Stock Drug Needed (uL)	Number of Wells	Volume of Drug for Master Mix (uL)	Volume of Media Already in Well (mL)	Volume of Media for Master Mix (mL)
2D	Etoposide	16.991	16991	4	6000	1.4125125066	3	4.2375375199	14.4	3.6
3D	Etoposide	16.991	16991	4	6000	1.4125125066	3	4.2375375199	14.4	3.6

Day 2 Procedure:

Staining:

1. Preheat fix buffer I to 37 degrees Celsius. Cool perm buffer on ice. **(need to locate these and know where to heat up fix buffer)**
2. In TC, prepare cells as though you were planning to dissociate them
3. 2D:
4. Aspirate off old media making sure not to touch the bottom with the pasture pipette
5. Wash cells with 1 mL of PBS/ well
6. Aspirate PBS
7. Add 1 mL of Trypsin/ well
8. Incubate for 5 minutes at 37C
9. Add 3 mL of complete DMEM to neutralie trypsin
10. Transfer each well to seperate 15 mL conical tube

11. 3D - SEE 4/7/25 ACCUTACE DISSOCIATION PROTOCOL
12. Transfer each well to seperate 15 mL conical tube
13. Centrifuge 15 mL conical tubes at 500 x g for 5 min.
14. **Aspirate excess liquid, being careful to avoid disturbing the pellet.**
15. **********all following steps will be done at your bench in non-sterile conditions not in the BSC**********
16. **Resuspend** dissociated cells in fix buffer at a ratio of 10 uL fix buffer per million cells. Fix at 37 degrees Celsius for 10-15 min.
17. **30 uL/ sample**
18. Wash cells with a 500 uL of blocking buffer.
19. ***Note: For all washes, centrifuge at 500 g for 5 min, then aspirate excess liquid, being careful to avoid disturbing the pellet.***
20. Permeabilize by adding 20 uL/million cells of permeabilization buffer to the pellet. **Pipet up and down a few times to ensure the pellet is evenly mixed.** Permeabilize on ice for 30 minutes.
21. **60 uL/ sample**
22. Meanwhile, prepare antibody by making a 1:1000 dilution of antibody in blocking buffer (depending on the brand/type of primary antibody used, this ratio may differ.
23. **Math:** Antibody solution: 12 samples total, go for overage so make for 15. We do 100 uL per every 10^7 cells present which is 10 million cells. Since we have 2-3 million total, we will do 40uL for each sample.
24. 15 samples x 40 uL = 600 uL of blocking buffer
25. To do a 1000x dilution: 600/1000 = **0.6 uL of antibody**
26. After permeabilizing, wash the samples once with blocking buffer.
27. Stain with the antibody dilution at an amount of 100 uL mixture per 10 million cells. Place on a Nutator (shaker plate) at room temperature for 1 hr.
28. (100uL/10 million)*(xuL/3million) = **30uL/ sample tube**
29. Wash with blocking buffer (500 uL of blocking buffer, spin down, and aspirate)
30. **Resuspend** pellet in 150 uL of blocking buffer for flow cytometry.

Flow Cytometry Steps:

1. Set up fluoresence reading
2. Run QC
3. Settings:
4. Open new experiment and name appropritely
5. Alter stop to **150,000** events
6. Change to fast read
7. Change stop volume to 140uL
8. Run daily clean
9. Load first sample (3D drug - most cell death expected)
10. If after 2 minutes less than 150,000 events have been read, change events to match expected events for the rest of the samples **making sure not to set it less than 50,000 events.**
11. Run the rest of the samples.
12. Save data to Hess Lab drive
13. Run daily clean

Conclusion & Action Items:

- See other photo entries and hand written notes
- Redo stain 4/17 & 4/18
- Update benching and final report with protocol changes

SOX2 and GAPDH RT-qPCR_4/7/25-4/17/25

Althys Cao - May 05, 2025, 7:50 PM CDT

Title: Gamma-H2AX Stain Protocol

Date: 4/7 - 4/17
Content By: Althys, Ana, Julia
Content:

4/7 - 2D cell passaging & 3D spheroid dissociation & Step 1: RNA Extraction

3D spheroid dissociation (6-well plate)

Note: have 6 wells, combine 2 wells for 1 replicate of cells in 3D cells for qPCR (6 wells --> 3 replicates)
Prepare 3 eppendorf 50 mL tubes
Pipette contents of wells up and down using P1000 set to 800uL to break up spheroids and move them away from well walls (do this alongside step 3 for each well)
Note: Try to make as few bubbles as possible in this step
Transfer the media (with cancer spheroids inside) from the wells into the according conical **50 mL tubes using a P1000** pipet tip set to **800µL.**
Combine content of 2 wells in the 6-well plate into 1 tube
Wash well with 1000 uL PBS in each well *twice* to collect any remain cells and add to the corresponding conical tube (NOTE: After washing all 2 wells for each replicate, each tube will have additional 4000 uL PBS into the 15 mL conical tube)
Note: Try to make as few bubbles as possible in this step
To each 50 mL conical tube, **add 24.8 mL of PBS** (28.8 mL total with the wash step - step 3)
Pellet the spheroids via centrifugation at **800g, 15 min,** then **remove the supernatant using a P1000 pipet**
Remove all liquid from conical tube 50 mL at a time using P1000 pipette making sure not to remove the cell pellet from the conical tube
Note: Try to make as few bubbles as possible in this step
Note: You dont have to change pipette tips as you remove the media unless you think the tip may be contaminated
Note: do this step concurently with step 6 (the step after)
Resuspend the pellets in 1000 µL Accutase using a P1000 pipette.
Thoroughly mix the solution by pipetting up and down 5 times and going against the wall of the tube (or until you can visably see the cell pellet has been broken up a little bit) using a P1000 pipette set to 750 µL.
Note: Complete this step concurently with the last step so that the cells do not dry out between the steps. Once one tube is aspirated add accutase right away.
Incubate the tubes in **37°C for 10 minutes.**
Pipette the solution in each tube **up and down 5 times** using a P1000 pipette set to 1000 µL, add **3000 µL of DMEM so that the final volume reaches 4 mL,** and transfer 900uL of mixed cell solution to a 1.5mL eppendorf tube.
Repeat untill all samples have been prepared for the cytoflex
Run cytoflex cell counting protocol
Calculate doubling time
https://www.cellseeker.org/cellcalc/growth-rate-and-doubling-time/

Tube	Cytoflex number	Cell conc (cells / mL)	Cell number		
1		#VALUE!	#VALUE!		
2		#VALUE!	#VALUE!		
3		#VALUE!	#VALUE!		

qPCR Step 1 - RNA Extraction

Note: will do 6 sets of experiments: 3 x (2 mil 2D cells) & 3 x (2 mil cells in 3D spheroids)

Reagents & stuff needed:

- RNaseZap (bench 3, main lab space)
- RLT buffer (in RNeasy Mini Kit, this kit it on the shelves on top of the sink near the group's lab bench)
- β-mercaptoethanol (in chemical room, VERY smelly, change gloves and throw out tips and tubes asap)
- 6 x QiaShredder columns
- 70% ethanol (for experiments) (ethanol in flammable cabinet in chemical room, add molecular grade water in chemical room to dilute to 60%)
- 6 x RNAeasy spin columns
- DNase solution: DNase I stock solution & RDD buffer (in fridge, Carley can. get them)
- RW1 buffer (in RNeasy kit)
- RPE buffer (in RNeasy kit)
- RNase-free water (molecular grade water, on shelf on lab bench, bench 3)
- 6 x 1.7 mL tubes

RNA Extraction via Qiagen RNAeasy Kit (adapted for protocol)

Clean bench and spray down with RNaseZap

Prepare 6 tubes of cells:

3 x (2 mil cells in 2D)

3 x (2 mil cells in 3D)

Spin the tubes down at 500 g, 5 mins

While the tubes are spinning down, prepare a new 15 mL conical tube, add 3 mL of RLT buffer and 30-µL of β-mercaptoethanol

While the tubes are spinning down, prepare 6 x QiaShredder columns, label accordingly (2D/3D)

After tubes are done spinning, aspirate supernatant and resuspend cells in 350µL of prepared RLT buffer in each tube by pipetting upand down

Pipet cell solution onto a QiaShredder column and spin for 2 minutes at 14000 rpm. ***Discard column, not flow-through!***

While the tubes are spinning down, prepare 6 x RNAeasy spin columns, label accordingly (2D/3D)

Add 350µL of 70% ethanol to flowthrough. Transfer mixture to RNAeasy spin column and spin for 30s at 10000rpm. **Discard flow-through.**

While the tubes are spinning down, prepare a new 1.7 mL microtubes (in the same cabinet as the TC cabinet), add 80µL of DNase I stock solution to 560µL of RDD buffer and mix well

DNase in the resuspended form is stored in the -30 in the box with reagents for cDNA synthesis

Make sure the flowthrough is discarded (see step 6)

Add 80µL of DNase I + RDD mix onto the column and let sit at room temperature for 15 min.

Add 350µL of RW1 buffer to column and spin 30s at 10000rpm. **Discard flowthrough**.

Add 500µL of RPE buffer to column and spin 30s at 10000rpm. **Discard flowthrough.**

Add 500µL of RPE buffer to column and spin 2 minutes at 14000rpm. **Discard flowthrough.**

While stuff is spinning down, prepare 6 x 1.7 mL tubes, label accordingly (2D/3D)

Place Column into new 1.7mL tube and elute with 50µL of RNase-free water. Let sit for 6 minutes and spin down for 1 minute at 10000 rpm. Use 2uL to nanodrop to determine concentration (hit end experiment at the end)., conc in ng/uL

Purified RNA can be stored at -80 C for 6 months, -20 C for 1 month. *(stored at -20C freezer at the group's bench)*

Sample name	Concentration (ng/uL)	A260/A280	A260/A230
2D cells - 1	356.4	2.06	1.77
2D cells - 2	470.1	2.08	1.79

2D cells - 3	506.5	2.06	1.99
Dissociated 3D cells - 1	163.4	2.05	0.88
Dissociated 3D cells - 2	386.9	2.07	1.31
Dissociated 3D cells - 3	336.4	2.07	2.16

4/9 - Step 2: cDNA Synthesis

Stuff needed:

- PCR tubes
- Oligo dT (in -30, thaw on ice)
- Molecular grade water
- 5X AMV RT Buffer (in -30. thaw on ice)
- 10mM dNTPs (in -30, thaw on ice)
- RNAsin (in -30, thaw on ice)
- AMV RT (in -30, thaw on ice)
- Ice bucket

Sample Name	Concentration (ng/uL)	Volume of RNA to Use in cDNA Reaction (uL)	Volume of water to add (uL)
2D cells - 1	356.4	2.8058361392	5.1941638608
2D cells - 2	470.1	2.1272069772	5.8727930228
2D cells - 3	506.5	1.9743336624	6.0256663376
Dissociated 3D cells - 1	163.4	6.1199510404	1.8800489596
Dissociated 3D cells - 2	386.9	2.5846471957	5.4153528043
Dissociated 3D cells - 3	336.4	2.9726516052	5.0273483948

This protocol is for **20µL**/1µg of RNA protocol. Prepare in PCR tubes:
RNA (2µg) x (see column C)
Oligo dT (0.5µg/µL) 2
H2O 8-x (see column D)
Note: If using a transcript specific primer, Oligo concentration should be 0.5-1 µM in final RT reaction. I usually use 2µL of 10µM stock.
Note: it is ideal if 2ug of RNA is used, however, because of the low concentration of RNA, 1ug is used instead
Heat sample at 70°C for 5 minutes. Place heated sample on ice for 5 min.
While waiting for the sample, prepare the master mix (scale up to 8):
5X AMV RT Buffer: 32 uL (buffer for the transcriptase)
10mM dNTPs: 8 uL
RNAsin: 2 uL
AMV RT: 6 uL (reverse transcriptase)
H2O: 32 uL
Add 10µL of the master mix into each sample
Incubate at 42°C for 2 hours (lid temperature: 105*C). Carley can pick them up and store them in freezer box
If going into a PCR, add 50 uL of Milli-Q-H2O and proceed to set up qPCR reaction (once water is in, store them in -20)

4/17 - Step 3: qPCR

Note: Check out more information on TaqMan method here:
https://tools.thermofisher.com/content/sfs/manuals/cms_041280.pdf

Designing the experiment: For these experiments, you require a housekeeping gene as a loading control in addition to your genes of interest. We generally order these conjugated to VIC and our target gene conjugated to FAM.

Name	Cat No.
ACTB_VIC	
GAPDH_VIC	

There are a number of ways to set up this experiment. You can use the $\Delta\Delta C_T$ calculation. This works since you the primer sets have been properly optimized. *Alternatively, you can set up the experiment as described in the for SYBR Green*. You will need the following samples. where you will need the following controls. (You will have each sample in quadruplicate (this is a requirement).

Experimental sample is measured at 1:10 dilution in **quadruplicate** with gene expression assay and house keeping gene (both are in a single well)

Note: these are only if you are making a standard curve:

Primer curve of control template to be done (1 well each dilution) for each primer set consisting of:

No dilution

1:5 dilution

1:25 dilution

1:125 dilution

1:625 dilution

1:3125 dilution

Empty sample

Question: do I need to set up a standard curve.

No, you just need to dilute your experimental samples at a 1:10 dilution.

Setup the Following Master Mix

Note: 6 samples, 4 replicates/sample --> total of 24 reactions --> scale up to 30

Question about cDNA dilution and amount: from step 2, we have 20 uL of 1ug of cDNA. We will add 50 uL of H2O before qPCR reaction (bringing it up to 70 uL of 1 ug of cDNA). We will use 4 uL of this 70 uL solution, meaning in each reaction we are using ~57 ng of cDNA. From the TaqMan method this amount of cDNA is enough (we need 1-100 ng), but is this the correct interpretation of the protocol?

Alternatively: if we want 100 ng, then add 20 uL of H2O before qPCR reaction then use 4 uL of cDNA for each reaction

So you will end up with the 70 uL of your cDNA this is correct. You will only need 3 pg to 100 ng in your reaction, this is a super low amount required. You want to save some of your cDNA incase you want to use it later so we will plan on y'all make a 1:10 dilution for each sample. The table below outlines this. This 50 uL of 1:10 dilution will then each be aliquoted out into your replicates. Each replicate will get 4 uL of your diluted cDNA so for the 4 reps this will be 16 uL total leaving you with overage.

Sample Name	Total Concentrated cDNA (1 ug)	Dilution of cDNA	Total Dilution Volume (uL)	Volume of cDNA (uL)	Volume of H2O for Dilution (uL)
2D cells - 1	70	1:10	50	5	45
2D cells - 2	70	1:10	50	5	45
2D cells - 3	70	1:10	50	5	45

Dissociated 3D cells - 1	70	1:10	50	5	45
Dissociated 3D cells - 2	70	1:10	50	5	45
Dissociated 3D cells - 3	70	1:10	50	5	45

Another Question: are we doing both SOX2 and GADPH in the same well

So I think this is perhaps an area where you guys might want to review the basic principals of how Taqman works that we went over in a previous meeting. As a reminder, taqman qPCR works by having specific primers and probes for target gene and control with specific fluorescences on each allowing you to assay both a target gene and a control in the same replicate.

Y'all have 6 samples total, with 4 replicates, this is 24 tubes in total. Doing 30x sounds good with me. In a tube you will make you first master mix (M1) which will be close the column D you have below. You will add the Sox2 gene expression assay, your control (GADPH) gene expression assay, Taqman master mix, and water. This master mix will not get cDNA. Then you will do a secondary mastermix (M2) which is for each of your samples. You know that each reaction totals 20 uL, you will have 4 reps but currently don't have cDNA present, thus each reaction from the master mix is 16 uL. 16 x 4 = 64, you have 6 samples in total so you will make 6 M2s that will recieve 64 uL of your M1. You will have left over M1, that is okay. You will then add in your cDNA to your M2s, this will be 4 for each reaction is 4 x 4 = 16 uL total. After you have made all of your master mixes now you can aliquot out each of the reps into the 96 well plate.

Reagent	**1X**	**5X**	**30X**	**35X**	
20X Taq Man Gene Expression Assay Target Gene (SOX2)	1	5	30	35	
20X Taq Man Gene Expression Assay Housekeeping Gene (GADPH)	1	5	30	35	
2x Taq Gene Expression Master Mix	10	50	300	350	
cDNA Template (1:10 dilution) (different for each	4	20	120	140	
RNAse Free water	4	20	120	140	
	20	100	600	700	

- If have not yet from Step 2, add 50 uL of molecular grade H2O into the 20 uL of cDNA from step 2
- Dilute cDNA solution 1:10: take 5 uL of cDNA solution into a different PCR tube and add 45 uL of molecular grade H2O
- Set up Master Mix 1 (everything but cDNA, 30X):
  - 30 uL of 20X Taq Man Gene Expression Assay Target Gene (SOX2)
  - 30 uL of 20X Taq Man Gene Expression Assay Housekeeping Gene (GADPH)
  - 300 uL of 2x Taq Gene Expression Master Mix
  - 120 uL of RNAse Free water
- Aliquot 64 uL pf Master Mix 1 into each PCR tube. We will have 6 tubes in total pertaining to 6 samples. This is to make Master Mix 2
- In each tube, add 16 uL of cDNA (diluted 1:10) relative to each sample. We will have a total of 80 uL of Master Mix 2, pertaining to 4 replicates/reactions per sample (4X of 20uL/reaction)
- Add 4 uL of cDNA into each well
- Setup the following protocol:
  - 95ºC 10min
  - 95ºC 15s
  - 60ºC 60s
  - plate read
  - Go to 2 39 times

Analysis: Use the relative quantification (ΔΔCt) method to analyze results. The general guidelines for analysis include:
• View the amplification plot; then, if needed:
– Adjust the baseline and threshold values.
– Remove outliers from the analysis.
• In the well table or results table, view the Ct values for each well and for each replicate group.
Perform additional data analysis using any of the following software:
Relative Quantification application thermofisher.com/connect
ExpressionSuite™ Software[1] thermofisher.com/expressionsuite

I woud make a note of what fluoresnce is the sox2 you ordered and what fluorescence is our control GADPH.

2D Sample 1	2D Sample 2	2D Sample 3	3D Sample 1	3D Sample 2	3D Sample 3						
Replicate 1	Replicate 1	Replicate 1	Replicate 1	Replicate 1	Replicate 1						
Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2						
Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3						
Replicate 4	Replicate 4	Replicate 4	Replicate 4	Replicate 4	Replicate 4						

4/21 - Step 3: qPCR (redo)

Reagent	**1X**	**5X**	**30X**	**35X**
20X Taq Man Gene Expression Assay Target Gene (SOX2)	1	5	30	35
20X Taq Man Gene Expression Assay Housekeeping Gene (GADPH)	1	5	30	35
2x Taq Gene Expression Master Mix	10	50	300	350
cDNA Template (1:10 dilution) (different for each	4	20	120	140
RNAse Free water	4	20	120	140
	20	100	600	700

- Dilute cDNA solution 1:4 take 10 uL of cDNA solution into a different PCR tube and add 30 uL of molecular grade H2O
- Set up Master Mix 1 (everything but cDNA, 35X):
    - 35 uL of 20X Taq Man Gene Expression Assay Target Gene (SOX2)
    - 35 uL of 20X Taq Man Gene Expression Assay Housekeeping Gene (GADPH)
    - 350 uL of 2x Taq Gene Expression Master Mix
    - 140 uL of RNAse Free water
  - Aliquot 80 uL pf Master Mix 1 into each PCR tube. We will have 6 tubes in total pertaining to 6 samples. This is to make Master Mix 2
  - In each tube, add 20 uL of cDNA (diluted 1:4) relative to each sample. We will have a total of 80 uL of Master Mix 2, pertaining to 4 replicates/reactions per sample (5X of 20uL/reaction)
  - Aliquot 20uL of Master Mix 2 into each well
  - Setup the following protocol:
    - 95ºC 10min
    - 95ºC 15s
    - 60ºC 60s

- plate read
- Go to 2 39 times

Althys Cao - May 05, 2025, 7:50 PM CDT

Results

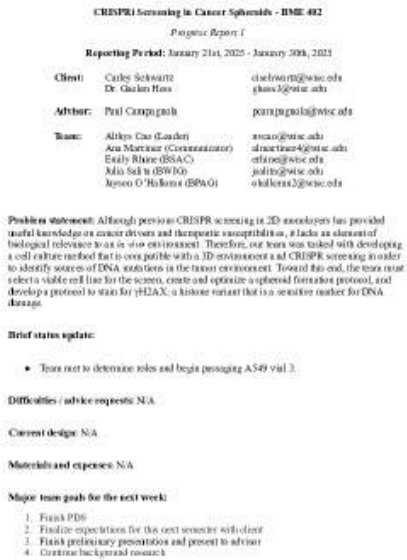
Althys Cao - May 05, 2025, 7:51 PM CDT

Download

BME_SOX2_GAPDH_Trail2_2025-04-21-9944_20250421_093408_Results_20250421_110626.xls (29.7 kB)

Progress Reports

Emily Rhine - Feb 09, 2025, 11:25 PM CST



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 1

Reporting Period: January 21st, 2025 - January 30th, 2025

Client:	Carley Schwartz	cschwartz@wisc.edu
	Dr. Gaolin Hou	ghou3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader)	xcao@wisc.edu
	Ana Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Julia Salkin (BWIG)	jsalkin@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an in vivo environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Team met to determine roles and begin passaging A549 vial 3.

Difficulties / advice requests: N/A

Current design: N/A

Materials and expenses: N/A

Major team goals for the next week:

1. Finish PDS
2. Finalize expectations for this next semester with client
3. Finish preliminary presentation and present to advisor
4. Continue background research

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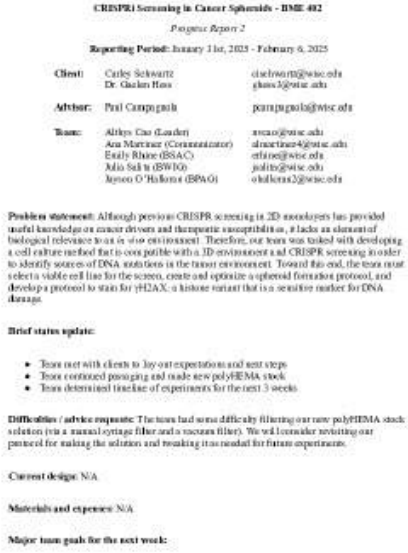
Progress_Report_1_-_1_30_2025.pdf (175 kB)

Emily Rhine - Feb 09, 2025, 11:25 PM CST



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 2

Reporting Period: January 31st, 2025 - February 6, 2025

Client:	Carley Schwartz	cschwartz@wisc.edu
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	Julia Salkin (BWIG)	jsalkin@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an in vivo environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Team met with clients to lay out expectations and next steps
- Team continued passaging and made new polyHEMA stock
- Team determined timeline of experiments for the next 3 weeks

Difficulties / advice requests: The team had some difficulty filtering our new polyHEMA stock solution (via a manual syringe filter and a vacuum filter). We will reconsider revisiting our protocol for making the solution and tweaking it as needed for future experiments.

Current design: N/A

Materials and expenses: N/A

Major team goals for the next week:

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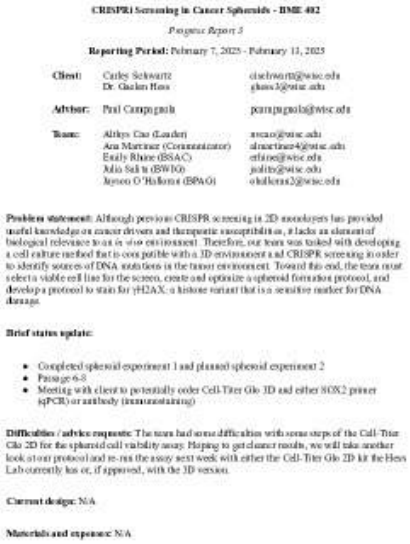
Progress_Report_2_2_6_2025.pdf (120 kB)

Emily Rhine - Feb 16, 2025, 2:26 PM CST



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 3

Reporting Period: February 7, 2025 - February 13, 2025

Client:	Carles Schwartz Dr. Charles Hess	cschwartz@wisc.edu chess3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader) Ana Martinez (Communicator) Emily Rhine (BSAC) Julia Salkin (BWIG) Jayson O'Halloran (BPAG)	avcao@wisc.edu almartinez4@wisc.edu erhine@wisc.edu jsalkin@wisc.edu ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Completed spheroid experiment 1 and planned spheroid experiment 2
- Passage 6-8
- Meeting with client to potentially order Cell-Titer Glo 3D and either SOX2 primer (qPCR) or antibody (immunostaining)

Difficulties / advice requests: The team had some difficulties with some steps of the Cell-Titer Glo 2D for the spheroid cell viability assay. Hoping to get clearer results, we will take another look at our protocol and re-run the assay next week with either the Cell-Titer Glo 2D kit the Hess Lab currently has or, if approved, with the 3D version.

Current design: N/A

Materials and expenses: N/A

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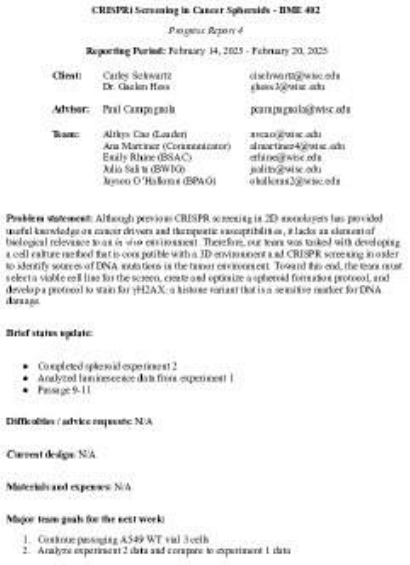
Progress_Report_3_2_13_2025.pdf (122 kB)

Emily Rhine - Mar 11, 2025, 9:13 PM CDT



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 4

Reporting Period: February 14, 2025 - February 20, 2025

Client:	Carles Schwartz Dr. Charles Hess	cschwartz@wisc.edu chess3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader) Ana Martinez (Communicator) Emily Rhine (BSAC) Julia Salkin (BWIG) Jayson O'Halloran (BPAG)	avcao@wisc.edu almartinez4@wisc.edu erhine@wisc.edu jsalkin@wisc.edu ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Completed spheroid experiment 2
- Analyzed luminescence data from experiment 1
- Passage 9-11

Difficulties / advice requests: N/A

Current design: N/A

Materials and expenses: N/A

Major team goals for the next week:

1. Continue passaging A549 WT vial 3 cells
2. Analyze experiment 2 data and compare to experiment 1 data

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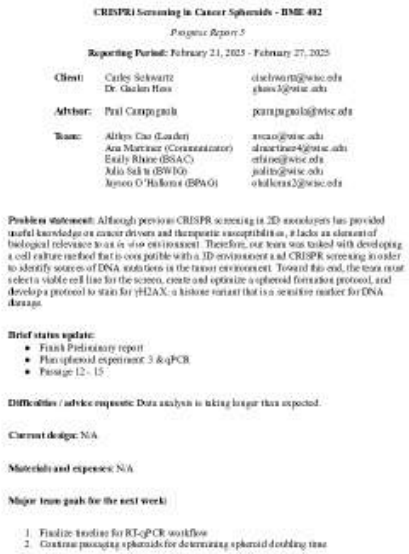
Progress_Report_4_2_20_2025.pdf (119 kB)

Emily Rhine - Mar 11, 2025, 9:13 PM CDT



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 5

Reporting Period: February 21, 2025 - February 27, 2025

Client:	Carles Schwartz	cschwartz@wisc.edu
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	Julia Salkin (BWIG)	jsalkin@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Finish Preliminary report
- Plan spheroid experiment 3 &qPCR
- Passage 12 - 15

Difficulties / advice requests: Data analysis is taking longer than expected.

Current design: N/A

Materials and expenses: N/A

Major team goals for the next week:

1. Finalize timeline for RT-qPCR workflow
2. Continue passaging spheroids for determining spheroid doubling time

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Progress_Report_5_2_27_2025.pdf (118 kB)

Emily Rhine - Mar 11, 2025, 9:13 PM CDT

CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 6

Reporting Period: February 28, 2025 - March 6, 2025

Client:	Carles Schwartz	cschwartz@wisc.edu
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Team:	Alihys Cao (Leader)	acao@wisc.edu
	Ana Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Julia Salkin (BWIG)	jsalkin@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Passage 0-2
- Spheroid passaging
- First trial of spheroid dissociation & determined ways to improve current protocol
- Seeded spheroids for next trial of determining doubling time and to prep for qPCR
- Determined timeline for upcoming 10 days

Difficulties / advice requests: Optimization of accutase spheroid dissociation in order to establish spheroid doubling time.

Current design: N/A

Materials and expenses: N/A

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Progress_Report_6_3_6_2025.pdf (140 kB)

Emily Rhine - Mar 14, 2025, 12:26 PM CDT

CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 7

Reporting Period: March 7, 2025 - March 13, 2025

Client:	Carles Schwartz Dr. Ghaslan Huss	cschwartz@wisc.edu ghuss3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alihys Cao (Leader) Ana Martinez (Communicator) Emily Rhine (BSAC) Julia Salit (BWIG) Jayson O'Halloran (BPAG)	acao2@wisc.edu almartinez4@wisc.edu erhine@wisc.edu jsalitz@wisc.edu ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- Passage 3-5
- Spheroid passaging
- Second trial of spheroid dissociation & determined ways to improve current protocol
  - Evaluate spheroid doubling time
- Seeded spheroids for next trial of determining doubling time and to prep for qPCR

Difficulties / advice requests: Optimization of accutase spheroid dissociation in order to establish spheroid doubling time. We find that we keep losing a significant amount of cells during the process such that our doubling time is inaccurately low. New PolyHema stock solution may be affecting experimental results (improper filtering), so we also plan to make a new batch with a 30-min UV sterilization of plates instead of vacuum filtration [1].

[1] "Do poly(HEMA coated plates need to be sterilized for use in embryoid body formation?," ResearchGate. Accessed: Mar. 13, 2025. [Online]. Available:

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Progress_Report_7_3_13_2025.pdf (1.07 MB)

Emily Rhine - Apr 13, 2025, 4:20 PM CDT

CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 8

Reporting Period: March 14, 2025 - March 20, 2025

Client:	Carles Schwartz Dr. Ghaslan Huss	cschwartz@wisc.edu ghuss3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alihys Cao (Leader) Ana Martinez (Communicator) Emily Rhine (BSAC) Julia Salit (BWIG) Jayson O'Halloran (BPAG)	acao2@wisc.edu almartinez4@wisc.edu erhine@wisc.edu jsalitz@wisc.edu ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- 2D cell passaging
- Spheroid passaging
- Third and fourth trial of spheroid dissociation & determined ways to improve current protocol
  - Evaluated spheroid doubling time/ confluency
- We still needed spheroids for next trial of determining doubling time and to prep for qPCR after spring break

Difficulties / advice requests: Optimization of accutase spheroid dissociation in order to establish spheroid doubling time.

Current design: Cells seeded in 24 well plate at 75k cells/cm^2 with 0.75% methylcellulose in full DMEM (10% FBS, P/s p/s).

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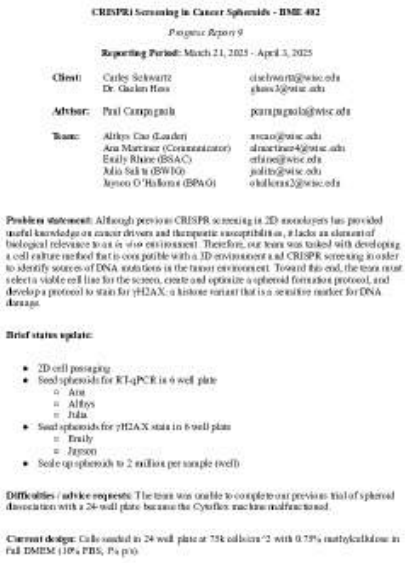
Progress_Report_8_3_20_2025_1_.pdf (413 kB)

Emily Rhine - Apr 13, 2025, 4:20 PM CDT



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 9

Reporting Period: March 21, 2025 - April 3, 2025

Client:	Carles Schwartz	cschwartz@wisc.edu
	Dr. Charles Hess	ghess3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader)	xcao@wisc.edu
	Ara Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Julia Salkin (BWIG)	jsalkin@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- 2D cell passaging
- Seed spheroids for RT-qPCR in 6 well plate
  - Ara
  - Alikys
  - Julia
- Seed spheroids for γH2AX stain in 6 well plate
  - Emily
  - Jayson
- Scale up spheroids to 2 million per sample (well)

Difficulties / advice requests: The team was unable to complete our previous trial of spheroid dissociation with a 24-well plate because the Cytoflex machine malfunctioned.

Current design: Cells seeded in 24 well plate at 75k cells/cm^2 with 0.75% methylcellulose in full DMEM (10% FBS, Pen pen)

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Progress_Report_9_4_3_2025.pdf (219 kB)

Emily Rhine - Apr 13, 2025, 4:20 PM CDT

CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 10

Reporting Period: April 3, 2025 - April 10, 2025

Client:	Carles Schwartz	cschwartz@wisc.edu
	Dr. Charles Hess	ghess3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader)	xcao@wisc.edu
	Ara Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Julia Salkin (BWIG)	jsalkin@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- 2D cell passaging
- RT-qPCR step 1 (RNA extraction) and step 2 (cDNA synthesis) protocols
  - Ara
  - Alikys
  - Julia
- γH2AX staining protocol
  - Emily
  - Jayson

Difficulties / advice requests: We struggled with not having enough cells for gamma-H2AX staining due to the cell pellets not being secure enough such that frequent aspiration led to us loosing a significant amount of cells

Current design: Cells seeded in 6 well plate at 75k cells/cm^2 with 0.75% methylcellulose in full DMEM (10% FBS, Pen pen)

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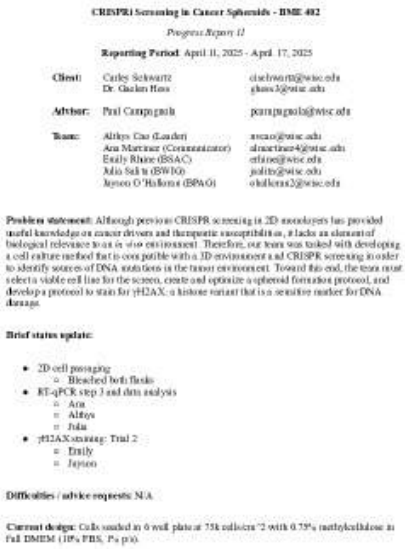
Progress_Report_10_4_10_2025.pdf (154 kB)

Emily Rhine - Apr 17, 2025, 8:20 PM CDT



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 11

Reporting Period: April 11, 2025 - April 17, 2025

Client:	Carles Schwartz	cschwartz@wisc.edu
	Dr. Charles Hess	chess3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader)	xcao@wisc.edu
	Ana Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Julia Sulit (BWIG)	jsulit@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an in vivo environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- 2D cell passaging
  - Bleached both flasks
- RT-qPCR step 3 and data analysis
  - Ana
  - Alikys
  - Julia
- γH2AX staining: Trial 2
  - Emily
  - Jayson

Difficulties / advice requests: N/A

Current design: Cells seeded in 6 well plate at 75k cells/cm^2 with 0.75% methylcellulose in Full DMEM (10% FBS, P/s p/s).

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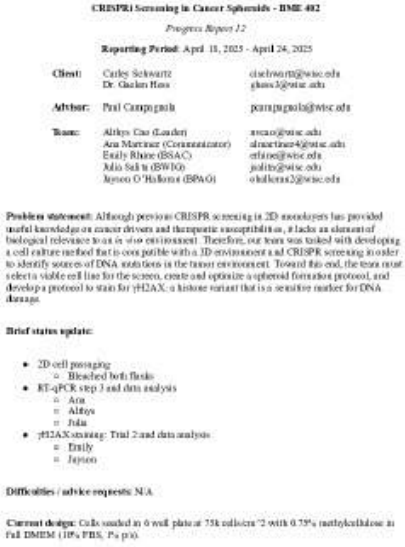
Progress_Report_11_4_17_2025.pdf (151 kB)

Emily Rhine - May 05, 2025, 9:30 PM CDT



CRISPRi Screening in Cancer Spheroids - BME 402

Progress Report 12

Reporting Period: April 18, 2025 - April 24, 2025

Client:	Carles Schwartz	cschwartz@wisc.edu
	Dr. Charles Hess	chess3@wisc.edu
Advisor:	Paul Campagnola	pcampagnola@wisc.edu
Team:	Alikys Cao (Leader)	xcao@wisc.edu
	Ana Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Julia Sulit (BWIG)	jsulit@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Problem statement: Although previous CRISPR screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an in vivo environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPR screening in order to identify sources of DNA mutations in the tumor environment. Toward this end, the team must select a viable cell line for the screen, create and optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX, a histone variant that is a sensitive marker for DNA damage.

Brief status update:

- 2D cell passaging
  - Bleached both flasks
- RT-qPCR step 3 and data analysis
  - Ana
  - Alikys
  - Julia
- γH2AX staining: Trial 2 and data analysis
  - Emily
  - Jayson

Difficulties / advice requests: N/A

Current design: Cells seeded in 6 well plate at 75k cells/cm^2 with 0.75% methylcellulose in Full DMEM (10% FBS, P/s p/s).

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Progress_Report_12_4_24_2025.pdf (151 kB)

Updated PDS

Emily Rhine - May 05, 2025, 9:14 PM CDT

CRISPRi Screening in Cancer Spheroids to Investigate Factors in Genome Stability

BME 400

January 31, 2025

Section 305

Product Design Specifications

Client:	Catley Schwartz	cjschwartz@wisc.edu
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Advisor:	Paul Campagnola	pcampagnola@wisc.edu
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	Ana Martinez (Communicator)	almartinez4@wisc.edu
	Emily Rhine (BSAC)	erhine@wisc.edu
	Mila Sulka (BWIG)	jsulka@wisc.edu
	Jayson O'Halloran (BPAG)	ohalloran2@wisc.edu

Function: Although previous CRISPRi screening in 2D monolayers has provided useful knowledge on cancer drivers and therapeutic susceptibilities, it lacks an element of biological relevance to an *in vivo* environment. Therefore, our team was tasked with developing a cell culture method that is compatible with a 3D environment and CRISPRi screening in order to identify sources of DNA mutations in the tumor environment. On a high scale, the team must use the selected cell line, A549, to optimize a spheroid formation protocol, and develop a protocol to stain for γH2AX: a histone variant that acts as a sensitive marker for DNA damage.

Client requirements:

1. The team must choose an appropriate human cell line for the project, ensuring to maximize cell spheroid viability.
2. The team must develop a spheroid formation protocol for the chosen cell type or line that optimizes seeding density and viscosity.
3. The team must optimize the cancer spheroid characteristics and their formation protocols for CRISPR screens.
4. By May 2025, the team must develop a protocol to stain for γH2AX: an indicator for the location of maximal damage due to DNA double-strand breaks (DSBs).
5. By May 2025, the team must perform high throughput genome-wide CRISPR screening to check for the effects of different genes on the amount of γH2AX detected.
6. Future steps post-design project include performing an analysis to determine the

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Unlinked_CRISPRi_Screening_in_Cancer_Spheroids_Product-Design-Specifications__1_31_25.pdf (210 kB)

Timeline_2/7/25

Emily Rhine - Feb 07, 2025, 12:32 PM CST

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"); la_iframe_1746634863385.contentDocument.close();

Preliminary Design Presentation_2/7/25

Emily Rhine - Feb 07, 2025, 12:32 PM CST

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CRISPRi_Screening_in_Cancer_Spheroids_Preliminary_Presentation_2_7_2025.pdf (865 kB)

Preliminary Report_3/3/25

Emily Rhine - Mar 03, 2025, 9:08 PM CST

Non-Small Cell Lung Cancer Spheroids to Investigate Factors in Genome Stability

DEPARTMENT OF
Biomedical Engineering
UNIVERSITY OF WISCONSIN–MADISON

Preliminary Report

BME 402: Biomedical Engineering Capstone Design

March 3, 2025

Alihyn Cao, Emily Rhine, Ana Martinez, Julia Salvia, Jayson O'Halloran, Ms. Carley Schwartz, Dr. Gaelen Hess (Department of Biomolecular Chemistry and Center for Human Genomics and Precision Medicine), and Dr. Paul Campagnola (Department of Biomedical Engineering, Peter Tong Department Chair)

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CRISPRi_Screening_in_Cancer_Spheroids_Preliminary_Report_3_3_25.pdf (815 kB)

Executive Summary

Emily Rhine - Apr 17, 2025, 8:33 PM CDT

402-Excellence-8-CRISPRi screening- Executive Summary

CRISPRi screening in cancer spheroids to investigate factors in genome stability

Alleyn Cao, Ana Martinez, Emily Rhine, Julia Salin, Jayson O'Halloran
Advisor: Dr. Paul Campagnola
Client: Dr. Gaelen Hess, Ms. Carley Schwartz

Background. Non-small cell lung cancer (NSCLC) has a 28% 5-year survival rate and limited treatment options according to the American Cancer Society (2025). A major obstacle in treating NSCLC is the continuous evolution of cancer genomes, which leads to frequent mutations, clonal diversity, and ultimately, resistance to therapeutic treatment. Although 2D cancer cell cultures provide in vitro data for BME applications like early cancer drug validation, 3D cell models, known as spheroids, provide a more accurate recapitulation of the in vivo environment. Spheroids can be used for studying cancer progression and therapeutic responses in vitro without the disadvantages of animal models and with more biological relevance than 2D culture. Increasing the scale of the spheroid model is necessary for analyzing the significance of 2D and 3D differences through RT-qPCR and γH2AX staining to assess DNA damage through double strand breaks (DSBs).

Protocol Optimization. First, the passaging schedule of A549 cells and dependence of doubling time on cell density was established. A549 spheroids were formed using ultra-low attachment plates (an inexpensive and common method). Tissue culture plates were coated with a hydrophilic polyHEMA film that causes cells to self-aggregate into spheroids 2–5 days after seeding. Various conditions, methylcellulose concentration and seeding density, were repeatedly tested to find out the ideal condition. Spheroid count, size, and cell viability were optimized at 0.75% methylcellulose and 75k cells/cm^2. Then, spheroids were dissociated into single cells to determine the doubling time of A549 cells in 2D culture; this protocol was needed for the subsequent RT-qPCR and γH2AX staining protocols. The accutase dissociation protocol, originally written by Honeder et al. (2021), was optimized using available Hess lab equipment to ensure minimal cell loss.

Testing and Results. For spheroid formation, size, and abundance verification testing, the team imaged a 96 well plate with varying methylcellulose concentrations and seeding densities using BioTek Cytation. Next, the CellTiter-Glo® Luminescent Cell Viability Assay was used to compare cell survival in various spheroid conditions. Then, a γH2AX stain was completed to compare prevalence of DSBs in 2D WT A549s, etoposide (common cancer drug) treated 2D WT A549s, spheroids, and etoposide treated spheroid populations. These results confirm the hypothesis that there is an increase in DSBs in etoposide versus control and 3D versus 2D. RT-qPCR provides statistically significant evidence that there are key differences in genetic expression between the 2D and 3D populations.

Future Work. Hess lab will make A549 CRISPR interference (CRISPRi) cell line spheroids and run additional experiments. In addition to RT-qPCR and γH2AX stain data, these protocols will be used for a genome-wide CRISPRi screen to identify tumor-specific factors that regulate genome stability in tumor formation and metastasis.

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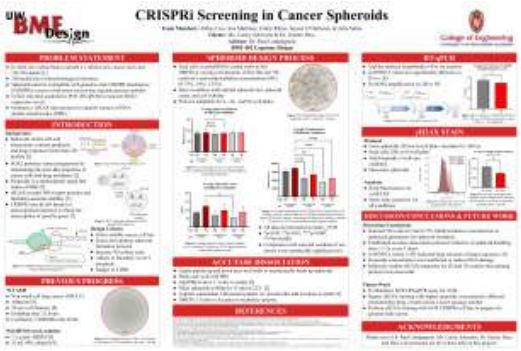
402-Excellence-8-CRISPRi_screening-_Executive_Summary.pdf (83.5 kB)

Final Poster

Emily Rhine - Apr 23, 2025, 11:17 PM CDT



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https://docs.google.com/presentation/d/1ktSSRkvVpee7XVRIr5U5pElhNJEy7P4j/edit?slide=id.p1#slide=id.p1

Final Report

Emily Rhine - May 05, 2025, 9:20 PM CDT

Non-Small Cell Lung Cancer Spheroids to Investigate Factors in Genome Stability

DEPARTMENT OF
Biomedical Engineering
UNIVERSITY OF WISCONSIN-MADISON

Final Report

BME 402: Biomedical Engineering Capstone Design

May 5, 2025

Alihyn Cao, Emily Rhine, Ana Martinez, Julia Salvia, Jayson O'Halloran, Ms. Carley Schwartz,
Dr. Gaelen Hess (Department of Biomolecular Chemistry and Center for Human Genomics and
Precision Medicine), and Dr. Paul Campagnola (Department of Biomedical Engineering, Peter
Tong Department Chair)

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CRISPRi_Screening_in_Cancer_Spheroids_Final_Report_5_5_25.pdf (10.8 MB)

SOX2_Stemness Gene_12/17/2024

Emily Rhine - Dec 17, 2024, 7:26 PM CST

Title: SOX2_Stemness Gene

Date: 12/17/2024

Content By: Emily

Content:

Link: SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine

Summary:

SOX-2

- "**In poly-HEMA culture, A549 cells** formed substratum-detached **spheroids** with characteristics of intermediate epithelial to mesenchymal transition (EMT) and **exhibited greater expression of SOX2 than did control 2D cells**." [1]
- "**Knockdown of SOX2 markedly suppressed the growth of A549 cell aggregates in poly-HEMA culture conditions** and furthermore increased their sensitivity to the anticancer drug vinblastine with concomitant downregulation of the activity of the anti-apoptotic AKT kinase." [1]
- "Interestingly, a small molecule, RepSox, which replaces SOX2, stimulated A549 cell growth in poly-HEMA 3D culture condition." [1]
- "SOX2 may therefore be an invaluable therapeutic target of NSCLC." [1]

Application:

- Since the expression of SOX2 is shown to increase in 3D versus 2D environment, we can use this expected outcome to monitor the health and functionality of our spheroids.
- A qPCR test for SOX2 gene expression would be a great way for us to confirm these results.
    - For exact qPCR/real-time qPCR protocol see original article
- SOX2 is deemed important for A549 tumor microenvironment recapitulation due to its ability to be targeted by cancer drugs such as vinblastine. [1]

References

[1] C. Choe, H. Kim, S. Min, S. Park, J. Seo, and S. Roh, “SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine,” OncoTargets and therapy, vol. 11, p. 6197, Sep. 2018, doi: 10.2147/OTT.S175810.

Conclusions/Action items:

- In the final report Jayson stated:
    - "For instance, SOX2 is a transcription factor associated with stemness and tumor progression in non-small cell lung cancer (NSCLC). It regulates self-renewal, differentiation, and pluripotency, making it a key

driver of cancer stem cell (CSC) properties. SOX2 is frequently co-expressed with surface markers such as CD133 and CD44, which are commonly used to identify CSCs. CD133, a marker of tumorigenicity, is often upregulated in SOX2-expressing cells and is linked to enhanced self-renewal and tumor initiation [1]. Similarly, CD44, which plays a role in cell adhesion and migration, is associated with SOX2 in promoting metastasis and resistance to therapies [1]. These surface markers, in conjunction with SOX2, delineate a subpopulation of CSCs that contribute to lung cancer progression, recurrence, and therapeutic resistance, making them a target for gene therapies in NSCLC.

- Review this paper as necessary for qPCR testing and biological relevance/ motivation for selecting SOX2.
- Finish and submit final report

GAPDH for qPCR in Organoids_2/2/2025

Emily Rhine - Feb 07, 2025, 5:19 PM CST

Title: GAPDH for qPCR in Organoids

Date: 2/2/2025

Goals: Better understand the scientific field overlap between organoids and cancer therapies.

Content:

- Link: Organoids as a new model for improving regenerative medicine and cancer personalized therapy in renal diseases
- Excerpts:
  - "Ethical issues related to the use of embryonic stem cells, has fueled research on adult, patient-specific pluripotent stem cells as a model for discovery and therapeutic development, but to date, normal and cancerous renal experimental models are lacking. Several research groups are focusing on the development of organoid cultures. Since organoids mimic the original tissue architecture in vitro, they represent an excellent model for tissue engineering studies and cancer therapy testing." [1]
  - "Nonetheless, similarly to normal organoids, detection of a SOX2 positive population as well as of CK8-18 positive cells at the periphery of spheroid structures, pinpointed the presence of stem-like and differentiated compartments, respectively (Fig. 5a). " [1]
  - **"GAPDH protein was used as internal control" [1]**
- Summary: There is a section on how to stain and image spheroids/organoids with drug treatment that may be useful in the future. However, for future qPCR results GAPDH can be used as a good comparison "housekeeping" gene.

References

[1] L. Grassi et al., “Organoids as a new model for improving regenerative medicine and cancer personalized therapy in renal diseases,” Cell Death Dis, vol. 10, no. 3, pp. 1–15, Feb. 2019, doi: 10.1038/s41419-019-1453-0.

Conclusions & Action Items:

- Continue research on RNA primers before 2/6 meeting
- GAPDH is a good housekeeping gene for spheroids/organoids
- Continue research on cancer drugs and their interaction with lung cancer cells.

A549_Housekeeping Gene_2/7/25

Emily Rhine - Feb 07, 2025, 5:18 PM CST

Title: Housekeeping Genes for A549

Date: 2/7/25

Goals: Determine whether Beta-Actin or GAPDH is a better housekeeping gene for qPCR for an adherent Non-small cell lung cancer line.

Content:

Link: [Identification of suitable reference genes for gene expression studies using quantitative polymerase chain reaction in lung cancer in vitro]

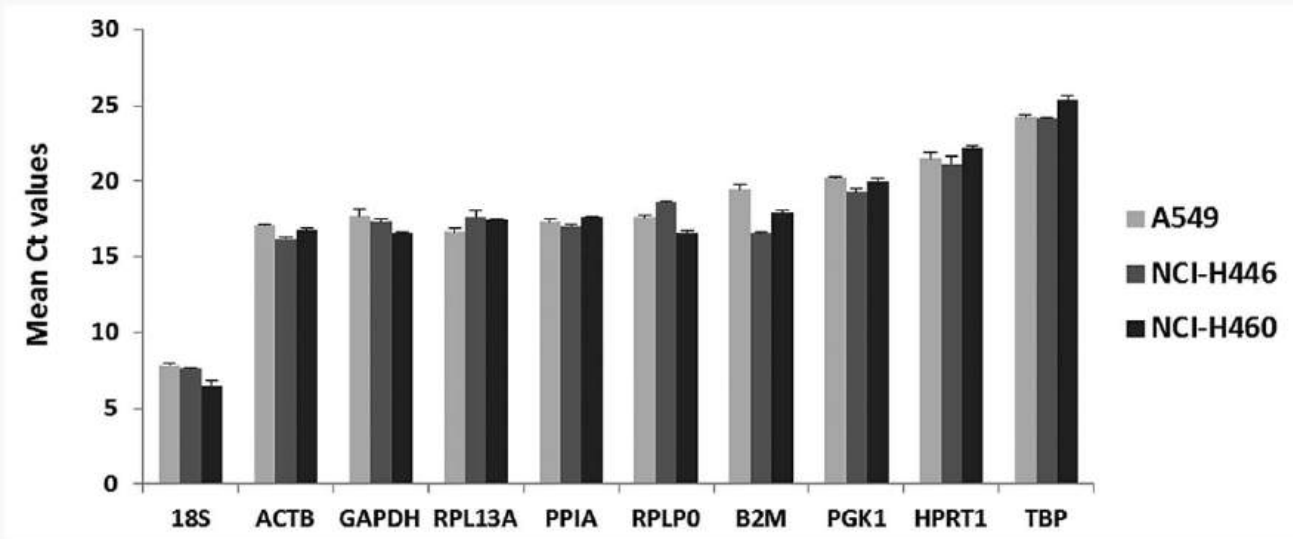


Figure 1 - Mean Ct values of the candidate reference genes among experimental samples. Ct, threshold cycle; ACTB, β-actin; GADPH, glyceraldehyde-3-phosphate dehydrogenase; RPL13A, ribosomal protein LI3a; PPIA, peptidylprolyl isomerase A; RPLP0, ribosomal protein large P0; B2M, β-2-microglobulin; PGK1, phosphoglycerate kinase-1; HPRT1, hypoxanthine phosphoribosyl transferase-1; TBP, TATA box binding protein.

[1]

- "By contrast, B2M and RPLP0 were the least stable. BestKeeper analysis revealed that GAPDH, 18S and B2M were the most stable and RPLP0, PPIA and HPRT1 were the least stable reference genes. This was consistent with previous studies demonstrating that GADPH and 18S were the most stable reference genes in NSCLC (31,37)" [1]
  - (31) https://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-6-200
  - (37) https://publications.ersnet.org/content/erj/26/6/1002

References

[1] H. Ali et al., “Identification of suitable reference genes for gene expression studies using quantitative polymerase chain reaction in lung cancer in vitro,” Molecular Medicine Reports, vol. 11, no. 5, pp. 3767–3773, May 2015, doi: 10.3892/mmr.2015.3159.

Conclusions/Action Items:

- Beta-Actin and GAPDH are both good (roughly equivalent) housekeeping genes for qPCR for in an adherent non-small cell lung cancer line

- Carley and Hess lab generally use Beta-Actin
- Will we still use qPCR or is immunostaining better for our purposes?

A549 Protein Denaturation_3/10/25

Emily Rhine - Mar 10, 2025, 8:16 PM CDT

Title: A549 Protein Denaturation

Date: 3/10/25

Content:

- 3.2. *Protein denaturation*

  Direct evidence for the denaturation of proteins in mammalian cells is reviewed by Lepock[1]. The thermal stability of proteins is usually expressed in terms of the transition temperature for denaturation T_m. Since this is the temperature at which half the protein denatures, and denaturation is both time and temperature dependent, considerable denaturation occurs at lower temperatures. Proteins appear to have transition widths of 10–12°C[18], implying that denaturation first occurs at 5–6°C below T_m. A summary of some important results concerning protein denaturation in cells follows (more details are given in Lepock[1]):

  - A number of mammalian cell lines (the rodent lines CHL V79, CHO, L929, and 3T3 and human lines HeLa and A549) have five major transitions with the T_m of the lowest transition equal to 50°C. The important parameter is the onset temperature of denaturation which is ~40°C in rodent cells and 41–42°C in human cells when both types of cells are grown at 37°C. This is the lowest temperature of detectable protein denaturation using differential scanning calorimetry (DSC). [1]

References

Lepock. J. (2002) "Cellular effects of hyperthermia: relevance to the minimum dose for thermal damage"

Conclusions & Action Items:

- A549s are fairly sensitive to protein denaturation.
  - 40-50C
- See PDF of source below

CRISPR screen on growth in spheroids_9_10_2024

Emily Rhine - Sep 15, 2024, 7:41 PM CDT

Title: CRISPR screen on growth in spheroids

Date: 9/10/2024

Content by/Present: Emily

Goals: Review media provided by the client to better understand the project and its motivation.

Content:

Link: CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities

Facts and Information of Interest:

- "**CRISPR phenotypes in 3D more accurately recapitulated those of *in vivo* tumors**, and genes with differential sensitivities between 2D and 3D conditions were highly enriched for genes that are mutated in lung cancers." [1]
- "Notably, we found that carboxypeptidase D is responsible for removal of a C-terminal RKRR motif2 from the α-chain of the insulin-like growth factor 1 receptor that is critical for receptor activity. Carboxypeptidase D expression correlates with patient outcomes in patients with lung cancer, and **loss of carboxypeptidase D reduced tumor growth**." [1]
- Motivation: "Despite the large increase in the catalogue of mutations observed across diverse cancer types (the ‘long tail’)1, it is **frequently unclear which mutations are functional cancer drivers.** Therefore, it is a central challenge to scalably investigate these genes in relevant cancer models to assign causality and identify cancer-specific vulnerabilities." [1]
- **Competing Methods:**
  - "Genetically engineered mouse models recapitulate tumor growth and microenvironment, but are limited by scalability, time and cost." [1]
  - "Xenograft-based models are limited in scale, and can be difficult to manipulate in vitro." [1]
  - "Genome-scale investigation of cancer growth and drug sensitivity has largely relied on in vitro 2D cell culture, which lacks many features of disease, such as hypoxia, altered cell–cell contacts and rewired metabolism." [1]
  - "In vitro organoid models alleviate some of these concerns, but are much less scalable." [1]
- These researchers used **"non-small-cell lung carcinoma"** for their cell lines. [1]
- Methods
  - "These 10 cell lines were transduced with a spCas9 lentiviral vector with a blasticidin selection marker (Addgene no. 52962), and selected with blasticidin (10 μg ml−1). Single-cell clones of these selected cell lines were individually tested for their Cas9-cutting efficiency by lentiviral infection with pMCB30639, a self-GFP-cutting reporter that has both GFP and an sgRNA against GFP on the same backbone. Single clones with high Cas9-cutting efficiency were established and used in the CRISPR screens and other biological assays." [1]
- "To culture lung cancer cells as 3D spheroids at genome scale, we used either pre-treated ultra-low attachment plates (Corning, no. 3261) or polyhema (Sigma, no. P3932) coated tissue culture plates. " [1]

References

[1] K. Han et al., “CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities,” Nature, vol. 580, no. 7801, pp. 136–141, Apr. 2020, doi: 10.1038/s41586-020-2099-x.

Conclusions/action items:

Article Summary:

This article was incredibly helpful in understanding what our team will be doing for the next year. Most notably, the description of how CRISPR-Cas9 was used in conjunction with these lung cancer spheroids in the methods section was enlightening.

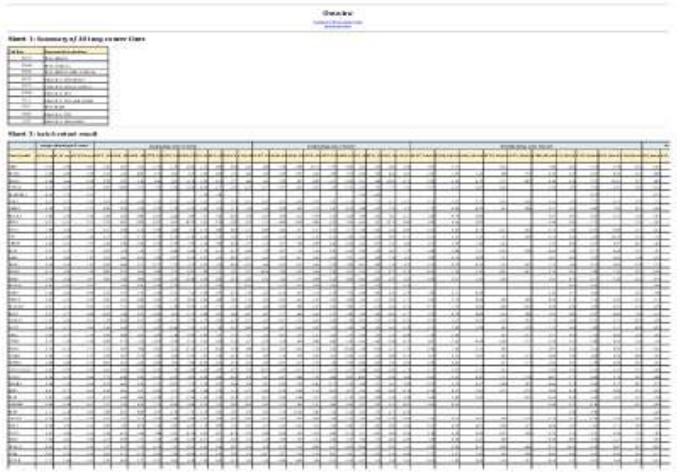
Action Items:

- Add "Competing Methods" bullet point to PDS document and add link for future reference.
- Continue reviewing the literature provided by the client to better understand the background and motivation for this project.
- Ask the client if they use the same method as outlined in the paper CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities they gave us for reference.

Emily Rhine - Sep 19, 2024, 7:31 PM CDT

Cell lines	Representative mutations
H1437	TP53, CDKN2A
H1568	TP53, SMARCA4
H1650	TP53, CDKN2A, EGFR, SMARCA4
H1792	KRAS G12C, TP53, KEAP1
H1975	EGFR, TP53, PIK3CA, CDKN2A
H2009	KRAS G12A, TP53
H23	KRAS G12C, TP53, LKB1, KEAP1
H322	TP53, KEAP1
H358	KRAS G12C, TP53
A549	KRAS G12S, LKB1, KEAP1

Emily Rhine - Sep 19, 2024, 7:31 PM CDT



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41586_2020_2099_MOESM7_ESM.xlsx (473 kB)

CellTiter-Glo 2D vs 3D_2/7/25

Emily Rhine - Feb 09, 2025, 11:48 PM CST

Title: CellTiter-Glo 2D vs 3D_2/7/25

Date: 2/7/25

Content:

Because we will be using CellTiter-Glo in a viability assay for our spheroids Monday (2/10) it is essential that we understand how to adapt the protocol and measurements from 2D to 3D rather than having to buy a new 3D Promega kit.

- Background
  - "The CellTiter-Glo® 3D Cell Viability Assay(a) is a homogeneous method to determine the number of viable cells in 3D cell culture based on quantitation of the ATP present, which is a marker for the presence of metabolically active cells." [1]
  - "This ready-to-use reagent is based on the original CellTiter-Glo® Luminescent Cell Viability Assay chemistry and **eliminates the need to combine buffer with lyophilized substrate when preparing reagent.** The CellTiter-Glo® 3D Cell Viability Assay is formulated with more robust lytic capacity and is designed for use with microtissues produced in 3D cell culture, **although it is similar in performance to the classic reagent when assaying monolayers of cells produced in 2D cell culture. This assay is compatible with multiwell-plate formats, making it ideal for automated high-throughput screening (HTS) using cell proliferation and cytotoxicity assays.** The homogeneous assay procedure (Figure 1) involves addition of a single reagent (CellTiter-Glo® 3D Reagent) directly to cells cultured in serum-supplemented medium. **Cell washing, removal of medium and multiple pipetting steps are not required**." [1]
    - We will need to still do this because we do not have the CellTiter-Glo® 3D Reagent.

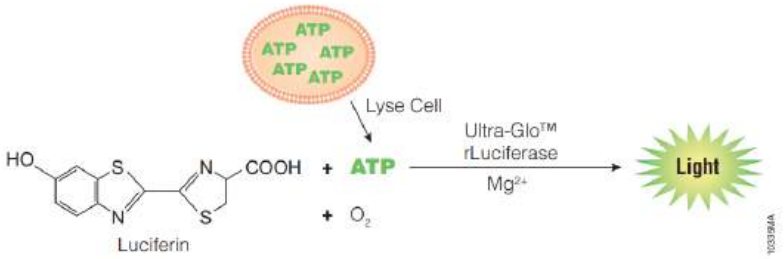
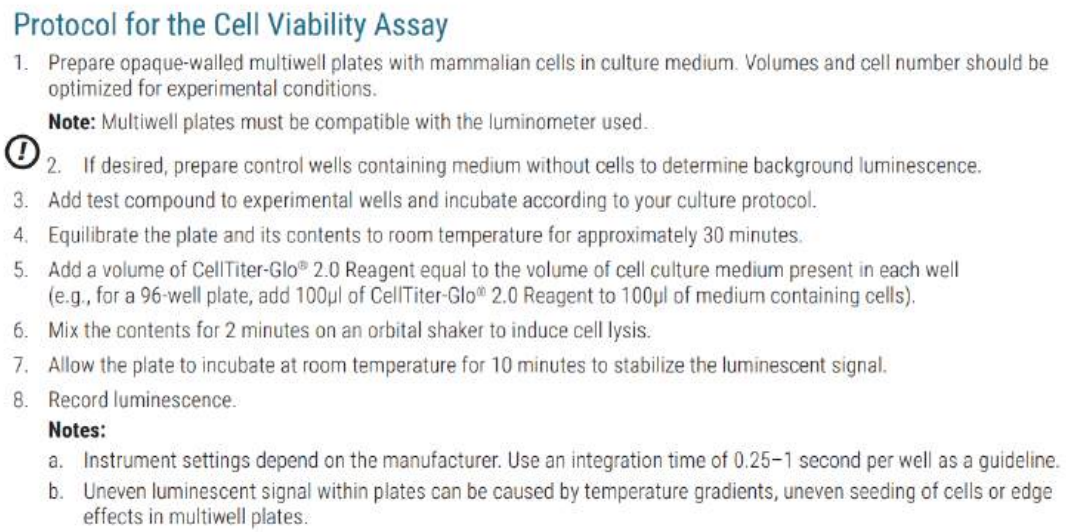


Figure 2. CellTiter-Glo® 3D Cell Viability Assay principle.

  - [1]
- Results:
  - "The linear correlation can be easily demonstrated with cells in 2D cell culture (Figure 3, Panel B). For 3D cell culture, the relationship between seeded cell number and luminescent output after several days in culture is often curvilinear due to the effects of contact inhibition on cell proliferation, as well as reduced metabolic activity and/or necrosis in the central region of large microtissues (Figure 3, Panel C)" [1]
  - See page 4 of PDF
- Experiment Supplies needed: [1]
  - 100µl/assay in 96-well plates
  - 22°C water bath
  - 96-well plates
  - Pipette
  - Plate shaker (5 minutes)

- luminometer, CCD camera or imaging device capable of reading luminescence in multiwell plate

- Steps: [1]
  1. Thaw the CellTiter-Glo® 3D Reagent at 4°C overnight.
  2. Place the reagent in a 22°C water bath prior to use for approximately 30 minutes.
  3. Mix gently by inverting the contents to obtain a homogeneous solution.
  4. Add test compound to experimental wells, and incubate according to your culture protocol. Be sure that the volume of the sample plus test compounds is low enough to allow addition of an equal volume of reagent, and subsequent mixing without well-to-well contamination.
  5. Equilibrate the plate and its contents to room temperature (22–25°C) for approximately 30 minutes
  6. add 100µl/well in 96-well plate
  7. Mix the contents vigorously for 5 minutes to induce cell lysis.
  8. Allow the plate to incubate at room temperature for an additional 25 minutes to stabilize the luminescent signal.
  9. Record luminescence.
  10. See PDF for "Protocol for Generating an ATP Standard Curve"

- **Additional Warnings:**
  - "Different cell types produce different amounts of ATP, and values reported for the ATP level in a particular cell type vary considerably (1,4,5,11,12). Moreover, there is typically a gradient of decreasing ATP concentration
    from the viable cell layer on the exterior to the nonviable cells at the center of microtissues, and the relative change in this
    gradient can vary considerably with cell type (13). It is possible with some 3D cell culture methods to generate large
    amounts of biomass in a single well that can impact the performance of the assay. This typically occurs around 10µM
    ATP, which is the upper limit of the assay linearity. Performing an ATP standard curve (Section 3.C) can confirm whether
    the luminescence produced by a given 3D cell culture method is under the 10µM limit." [1]
  - "Extracellular or intercellular ATP concentration is reported to be very low (1–5 µM) in normal healthy tissues; however, it is significantly increased **(to >100 µM) in the tumor microenvironment** (26)." [2]
  - "The protein levels were normalized to β-actin" [2]
  - 

    Protocol for the Cell Viability Assay

    1. Prepare opaque-walled multiwell plates with mammalian cells in culture medium. Volumes and cell number should be optimized for experimental conditions.

       Note: Multiwell plates must be compatible with the luminometer used.

    2. If desired, prepare control wells containing medium without cells to determine background luminescence.
    3. Add test compound to experimental wells and incubate according to your culture protocol.
    4. Equilibrate the plate and its contents to room temperature for approximately 30 minutes.
    5. Add a volume of CellTiter-Glo® 2.0 Reagent equal to the volume of cell culture medium present in each well (e.g., for a 96-well plate, add 100µl of CellTiter-Glo® 2.0 Reagent to 100µl of medium containing cells).
    6. Mix the contents for 2 minutes on an orbital shaker to induce cell lysis.
    7. Allow the plate to incubate at room temperature for 10 minutes to stabilize the luminescent signal.
    8. Record luminescence.

       Notes:

       a. Instrument settings depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline.
       b. Uneven luminescent signal within plates can be caused by temperature gradients, uneven seeding of cells or edge effects in multiwell plates.

    [2]

Reference:

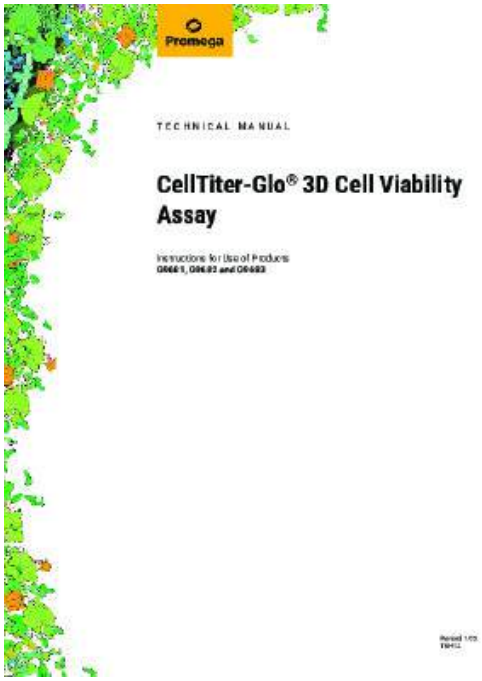
[1] “CellTiter-Glo ® 3D Cell Viability Assay Instructions for Use of Products G9681, G9682 and G9683.” Accessed: Feb. 07, 2025. [Online]. Available: https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/celltiter-glo-3d-cell-viability-assay-protocol.pdf?rev=88083aa3f7284e898ff0f218aa3c6b59&sc_lang=en

[2] S. Song et al., “ATP promotes cell survival via regulation of cytosolic [Ca2+] and Bcl-2/Bax ratio in lung cancer cells,” Am J Physiol Cell Physiol, vol. 310, no. 2, pp. C99–C114, Jan. 2016, doi: 10.1152/ajpcell.00092.2015.

Conclusions & Action Items:

- I followed the reference rabbit trail to see if I could find the exact base level amount of ATP in A549, but I could not after 3 articles.
  - The best I could find was ">100µM"
- Compare to 2D "CellTiter-Glo® 2.0 Assay Technical Manual"
  - Note differences & changes that must be made

Emily Rhine - Feb 07, 2025, 5:21 PM CST



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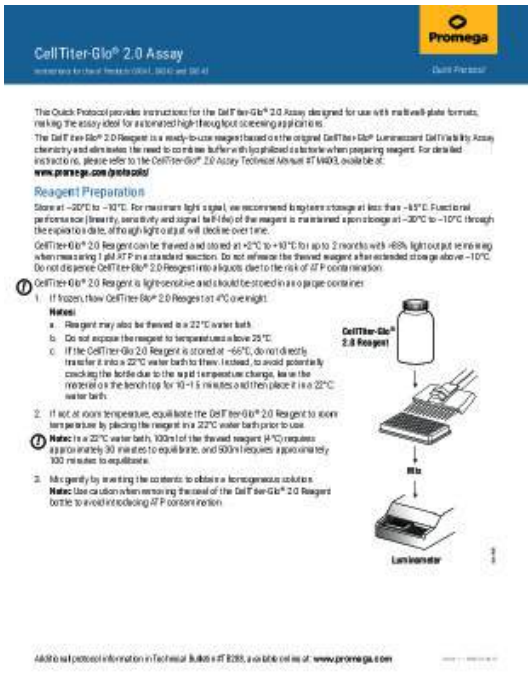
celltiter-glo-3d-cell-viability-assay-protocol.pdf (1.79 MB)

Emily Rhine - Feb 07, 2025, 7:49 PM CST



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CellTiter-Glo_2.0_Assay_Quick_Protocol_FB257.pdf (390 kB)

Emily Rhine - Feb 16, 2025, 3:53 PM CST

Title: Cell-Titer Glo Protocol Review

Date: 2/16/25

Content By: Emily

Content:

After a close review of the CellTiter-Glo protocol "CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin," the Cell-Titer Glo reagent should be added immediately after adding fresh media to your cells. Since the assay measures the current level of ATP in the cells, the data is most accurate right after the media change [1], [2].

2. Prepare control wells containing medium without cells to obtain a value for background luminescence.
3. Add the test compound to experimental wells, and incubate according to culture protocol.
4. Equilibrate the plate and its contents at room temperature for approximately 30 minutes. [1]

- "The homogeneous assay procedure involves adding the single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum-supplemented medium. Cell washing, removal of medium and multiple pipetting steps are not required. The system detects as few as 15 cells/well in a 384-well format in 10 minutes after adding reagent and mixing." [1]

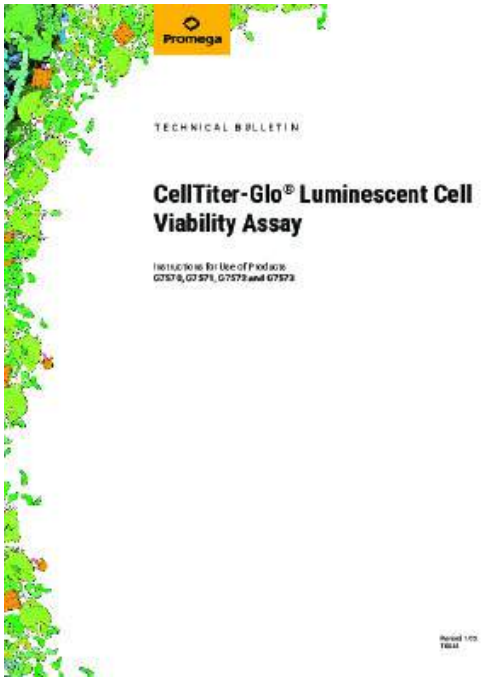
References

[1] “CellTiter-Glo® Luminescent Cell Viability Assay.” Accessed: Feb. 16, 2025. [Online]. Available: https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-luminescent-cell-viability-assay/

Conclusions & Action Items:

- Update team, advisor, and client with new information/resources
- Redo Cell-Titer Glo Assay if the client still deems a redo important
- Otherwise, simply analyze the data gathered (2/10)

Emily Rhine - Feb 16, 2025, 3:49 PM CST



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celltiter-glo-luminescent-cell-viability-assay-protocol.pdf (1.15 MB)

Resuspension of Spheroids_2/9/25

Emily Rhine - Feb 09, 2025, 10:56 PM CST

Title: Resuspension of Spheroids

Date: 2/9/25

Content By: Emily

Content:

A protocol taken from CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities to passage cells may be adapted to resuspend the spheroids in a different amount of media in order to apply the CellTiter-Glow protocol. This Glo protocol which requires a 1:1 ratio of media to CellTiter-Glo Buffer/lysate which is impossible with the current volume of media (160uL) and the size constaints of a 96-well plate (200-300uL). However, as I suggested in our 2/7 client meeting, it is possible to centrifuge the spheroids and resuspend them in 100uL of media and 100uL of CellTiter-Glo Buffer/lysate.

1. **Cancer spheroids were collected in methylcellulose media and diluted with PBS (~3 medium volumes) to reduce viscosity of the medium before centrifugation.**
2. **Spheroids were then centrifuged at 800g for 15 min and medium and PBS was removed from the spheroid pellets.**
3. **Accutase was added to the pellets to dissociate the spheroids into single cells.**
    - **10 ml of accutase used per 100 million cells in spheroids**
    - **Incubated them for 30 min until spheroids are fully dissociated into single cells**
4. **The single cells were then reseeded at the starting density (50,000 cells per cm2, 500 µl growth medium per cm2).**

Excerpt:

"The number of dead cells was estimated similarly by dividing total integrated Sytox Green intensities of spheroids by the average integrated Sytox Green intensity of a single dead cell. We chose a cell density (50,000 cells per cm2) that showed about 30% peak cell death rate within 24 h after initial seeding. For all subsequent experiments, cells were initially seeded at 50,000 cells per cm2 density in 500 µl of RPMI 1640 medium containing 0.75% methylcellulose. Spheroids were then split every 3–4 days. To passage cells, cancer spheroids were collected in methylcellulose media and diluted with PBS (~3 medium volumes) to reduce viscosity of the medium before centrifugation. Spheroids were then centrifuged at 800g for 15 min and medium and PBS was removed from the spheroid pellets. Accutase (Innovative Cell Technologies, no. AT104) was added to the pellets to dissociate the spheroids into single cells. We used 10 ml of accutase per 100 million cells in spheroids and incubated them for about 30 min until spheroids were fully dissociated into single cells. The single cells were then reseeded at the starting density (50,000 cells per cm2, 500 µl growth medium per cm2)." [1]

References

[1] K. Han et al., “CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities,” Nature, vol. 580, no. 7801, pp. 136–141, Apr. 2020, doi: 10.1038/s41586-020-2099-x.

Conclusions & Action Items:

- Present protocol to Carley 2/10/25

- Alter protocol as needed before applying protocol in conjunction with CellTiter-Glo protocol
- **The main steps to consider seem to be 1) Spheroids were then centrifuged at 800g for 15 min and medium and PBS was removed from the spheroid pellets. 2) Make sure not to aspirate off the cell/spheroid pellet!**

Spheroid Formation 3_2/26/25

Emily Rhine - Feb 26, 2025, 10:09 AM CST

Cell Seeding Protocol (2/26/2025)

WEDNESDAY, 2/26/2025

1. Collect cells from that day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL)**.
2. Make a **2 mL cell solution of 500,000 cells/mL (750,000 cells in total)**
    a. **Volume needed to obtain 750,000 cells from the day's cell passage: 750,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)=**
        I. Can also put in **cell contration (cells/mL) from Step 1** into **Cell J2** from the table below, the volume needed for step 2a will be in **Cell K2.**
    b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**.
    c. Spin down the the tube at **200g for 5 minutes**, then **remove supernatant** to collect cells using vacuum filter
    d. Resuspend cells in **6 mL of serum-free DMEM**.
3. **Optimized Condition : 75,000 cells/cm^2, 0.75% methylcellulose**
    a. Into a new 50 mL tube, add:
    b. 1.56 mL of serum-free DMEM
    c. 1.8 mL of 2% methylcellulose stock
    d. 1.44 mL of 500,000 cells/mL cell solution (Step 2)
        I. Mix gently by pipetting up and down, make sure the solution is well-mixed
4. **Mix gently by pipetting up and down then Aliquot** the optimized condition mix into each well: add **160 µL of cell mix** into each well **(24 wells total)**.
5. Grow spheroids in **37C, 5% CO2 incubator**. Spheroids are split every 3-4 days.

Cell Number at the beginning of the growth phase (N_0) [cells]

1550000

Cell Number at the end of the growth phase (N_t) [cells]

7336000

Time the cells were growing (Δt) [hours]

46

The growth rate (µ) of your cells is [1/day] :

0.81

The population doubling time of your cells is [hours] :

20.5

For most updated version see: https://benchling.com/uw_hesslab/f/lib_wSFKaFAF-spheroid-formation/etr_XKpgunXw-cell-seeding-protocol-2262025/edit

Spheroid Drug Response Model_Alternate Protocols_3/4/2025

Emily Rhine - Mar 05, 2025, 1:22 PM CST

Title: Spheroid Drug Response Model

Date: 3/4/25

Content:

Link: Establishing an In Vitro 3D Spheroid Model to Study Medulloblastoma Drug Response and Tumor Dissemination

- Lists all important, relevant protocols that parallel our project:
  - **Basic Protocol 1**: Generation and maintenance of 3D medulloblastoma (3D-MB) spheroids
  - **Support Protocol 1**: Measuring spheroid size for coefficient-of-variation analysis
  - **Basic Protocol 2**: Assessing drug response in 3D-MB spheroids
  - **Support Protocol 2**: 384-well 3D-MB spheroid generation
  - **Basic Protocol 3**: Immunohistochemical staining of 3D-MB spheroids
  - **Basic Protocol 4**: Modeling metastatic dissemination using 3D-MB migration models
  - **Support Protocol 3**: RNA extraction from 3D-MB spheroids

Timing: Every 2 to 3 days following initial 4-day incubation period

17. Manually inspect ULA plate under the microscope to assess spheroid health.

Representative examples of spheroids from each cell line are shown in Table 1. Figure 2A shows a healthy ONS76 spheroid imaged over a period of 21 days.

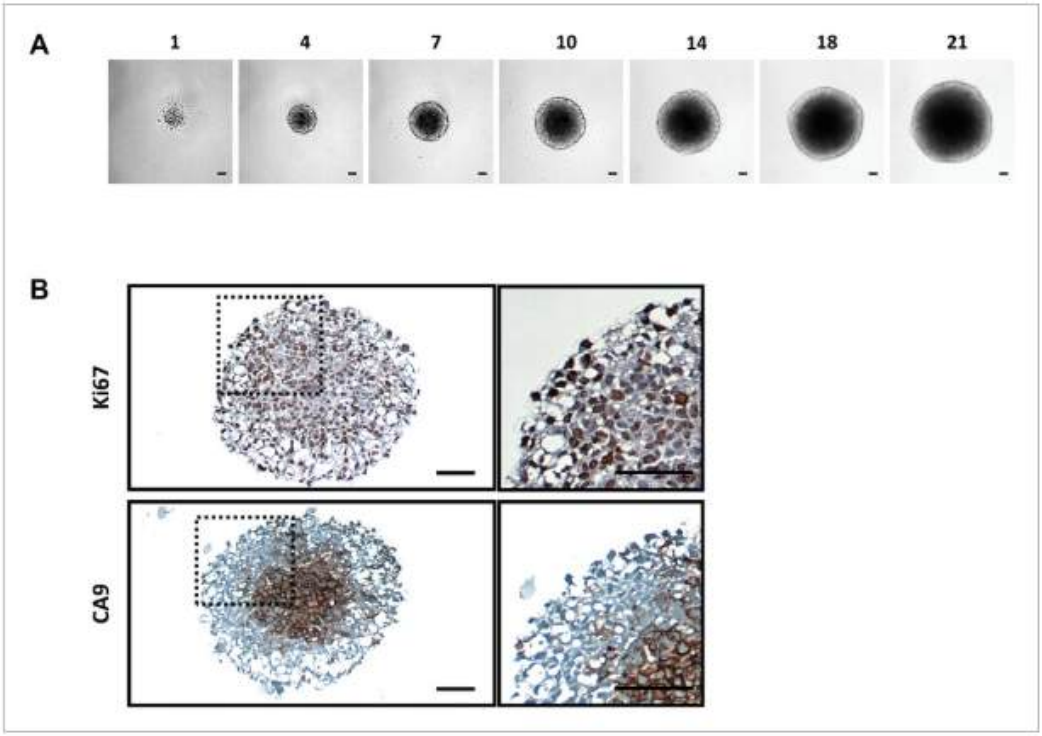


Figure 2 Open in figure viewer | PowerPoint

3D-MB spheroid growth characteristics over a 21-day period. (**A**) Representative images of ONS76 3D-MB spheroids over a 21-day period (scale bar: 100 μm). (**B**) Immunohistochemical staining of ONS76 3D-MB spheroids on day 21 for markers of proliferation (Ki67) and hypoxia (CA9) (scale bar: 100 μm).

18. Gently remove 100 μl neurosphere medium from each well using a multichannel pipet.

Ensure that the spheroids remain undisturbed by placing the pipet at a 45° angle on the wall of the well.

19. Pour fresh neurosphere medium into a reagent reservoir.

20. Add 100 μl fresh neurosphere medium to each well using a multichannel pipet.

Use the same technique of placing the pipet on an angle as in step 18 to avoid disturbing the spheroids.

21. If needed, place plate back in the incubator and continue to maintain spheroids by performing medium changes every 2 to 3 days following steps 17 to 20.

Support Protocol 1: MEASURING SPHEROID SIZE FOR COEFFICIENT-OF-VARIATION ANALYSIS

In this support protocol, we describe how to perform CV analysis by manually imaging each well (containing an individual spheroid) and measuring spheroid size using the macro developed by Ivanov et al. (2014) and the open-access software ImageJ. CV analysis is a useful method of determining assay uniformity by calculating variations in spheroid size within a plate. CV is commonly used to assess the reproducibility of in vitro models, and CV scores of <20% variation are considered acceptable (Sittampalam, Grossman, Brimacombe, Arkin, & Auld, 2004).

We advise that this protocol be performed on day 4 of spheroid formation (see Basic Protocol 1, step 16), when spheroids should have reached the optimal size of 250 to 350 µm in diameter, before moving on to downstream applications (Basic Protocols 2 to 4).

Materials

3D-MB spheroids (see Basic Protocol 1)

Imaging apparatus [e.g., brightfield microscope (Canon, DS126431, or equivalent) with attached camera (Olympus, CKX41) or automated imaging system]
Calibration slide (AmScope, A36CALM7-3PL)
Computer
ImageJ version 1.53n (**https://imagej.nih.gov/ij/**)

Timing: ≤1 hr

1. Image 3D-MB spheroids at 10× magnification with an imaging apparatus (there should be one spheroid per well). Use a calibration slide to determine scale of the images.
2. Transfer images to a computer.
3. Use ImageJ macro developed by Ivanov et al. (2014) and open-access software ImageJ to analyze spheroid size:
   a. Download ImageJ and start program.
   b. Using the image of the calibration slide from step 1, measure scale of images (number of pixels equal to 100 µm) at 10× magnification.
   c. Download macro provided by Ivanov et al. (**https://figshare.com/s/32f81784ee28e3fde015**) and enter scale value after "*distance*=."
   d. Go to Plugins > Macros > Install and select amended macro text file.
   e. Go to Plugins > Macros and select installed macro (typically listed at the bottom).
   f. Select input folder containing the original spheroid images.
   g. Select output folder (an empty folder that will include any processed images with the spheroid outlined).
   h. Run macro.
      This macro automatically calculates spheroid area and draws a blue outline of the detected spheroid.
   i. Wait for macro to finish processing before analyzing the images (see steps 4 to 8).
4. Drag and drop output folder into ImageJ and assess blue outline of the detected spheroids. If the macro has failed to detect the correct spheroid circumference, manually outline spheroid and go to Analyze > Measure (or Ctrl + M) to calculate area.
5. Export spheroid measurements from ImageJ into a spreadsheet.
6. Using the area values, calculate spheroid diameter using the following equations:

$$Radius\,(r) = \sqrt{\frac{A}{\pi}}$$

$$Diameter\,(d) = 2r$$

7. Calculate mean and standard deviation of the diameter measurements for each plate (or by cell line if multiple lines have been seeded within the same plate).
8. Calculate CV for each plate/cell line using the following equation:

$$CV\,(\%) = \frac{Standard\ deviation\ of\ spheroid\ diameter}{Mean\ spheroid\ diameter} \times 100$$

9. Proceed with downstream protocols (Basic Protocols 2 to 4) if CV is <20%.
   CV values >20% indicate that there were inconsistencies in cell seeding.

Conclusion/ Action items:

- Ensure proper use of pipette when aspirating off media to not disturb cell pellet
- Attempt to dye with Trypan blue (Gibco, 15250061) to gain accurate 3D imaging spheroid data?
    - Hemocytometer (or automated cell-counting system) - does the client have this?
- Look into new ImageJ protocol: **(https://figshare.com/s/32f81784ee28e3fde015)**
- **Send article to team and client for their review**

Emily Rhine - Mar 05, 2025, 2:29 PM CST

Reference

[1] S. J. Roper and B. Coyle, “Establishing an In Vitro 3D Spheroid Model to Study Medulloblastoma Drug Response and Tumor Dissemination,” Current Protocols, vol. 2, no. 1, p. e357, 2022, doi: 10.1002/cpz1.357.

Emily Rhine - Mar 05, 2025, 1:21 PM CST

Establishing an In Vitro 3D Spheroid Model to Study Medulloblastoma Drug Response and Tumor Dissemination

Sophie J. Roper and Beth Coyle

Roper and Coyle

1 of 29

CURRENT PROTOCOLS

Download

Current_Protocols_-_2022_-_Roper_-_Establishing_an_In_Vitro_3D_Spheroid_Model_to_Study_Medulloblastoma_Drug_Response_and.pdf (1.53 MB)

Alternate Spheroid Formation Protocols_3/4/2025

Emily Rhine - Mar 05, 2025, 2:28 PM CST

Title: Alternate Spheroid Formation Protocols

Date: 3/4/25

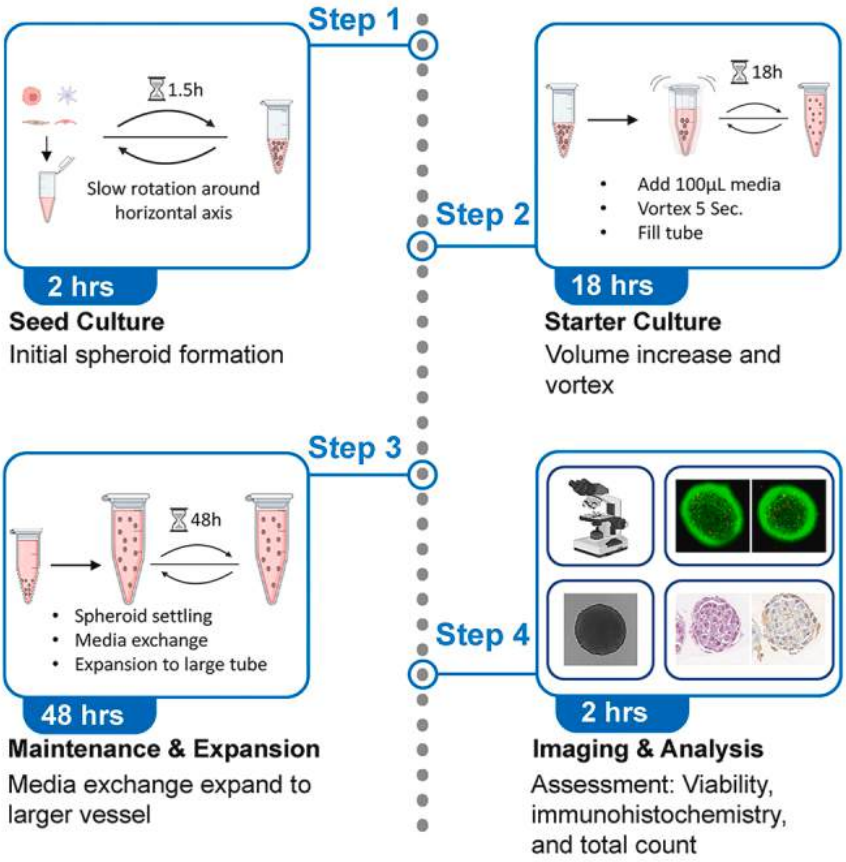
Content:

Link: [Protocol for generation of multicellular spheroids through reduced gravity]

- See also other star methods for spheroid formation.
- Spheroid decision matrix:

Methods for spheroid generation

	Labor required	Reagent need	Need for special equipment	Ease of media change	Ease of development tracking/imaging	Ease of retrieval	Potential for use with low adherence cell lines	Potential for long term culture (14+ days)	Potential for high throughput production	Consistency of spheroids
Current method	+	+	+++	+++	+++	++	+++	+++	+++	++
Hanging drop	+++	+	++	-	+	++	+++	-	+	+++
Low adherence plates	++	++	++	+	+++	++	+	+	++	++
Spinner flask	+	+++	+++	+++	+	++	-	+++	+++	-
Rotating wall vessel	+	+++	+++	+++	++	++	-	+++	+++	-
Microfluidic devices	++	+++	+++	++	+	+	+++	++	++	+++
Scaffold/Matrix	++	++	+	++	-	+	+++	+	++	+++
Magnetic levitation	++	++	+++	+++	+	++	+++	++	++	+++



References

[1] S. J. Roper and B. Coyle, “Establishing an In Vitro 3D Spheroid Model to Study Medulloblastoma Drug Response and Tumor Dissemination”, doi: 10.1002/cpz1.357.

Conclusions/Action Items:

- This matrix seems to match our matrix from last semester well
- Look at how to make our own figures on BioRender

Alternate_Dissociation of Spheroids _3/11/25

Emily Rhine - Mar 11, 2025, 2:07 PM CDT

Title: Alternate Spheroid Formation Protocols

Date: 3/11/25

Content:

Link: Dissociation of mono- and co-culture spheroids into single cells for subsequent flow cytometric analysis

- "We established protocols for dissociation of mono- and co-culture spheroids consisting of human fibroblasts and human endothelial cells. Cell recovery rate and viability after dissociation were evaluated with hemocytometer and by flow cytometry. The diameter of cells and the amount of cell aggregates were quantified by Casy®-technology and the cellular composition was analyzed by flow cytometry." [1]
- "Smaller spheroids (10,000 cells) could be dissociated with Accutase®, whereas larger spheroids (50,000 cells) required more stringent dissociation conditions." [1]
    1. "After sedimentation of the spheroids, the supernatants were removed and the spheroids were incubated in 300 µl Accutase"
    2. "The incubation was done at 37 °C under continuous shaking in a Thermomixer® comfort."
    3. "The spheroids were thoroughly resuspended ten times with a 200 µl pipette with low-retention tips, incubated for 10 min in the Thermomixer® and resuspended again ten times (one dissociation cycle)."
    4. "The occurring shear forces support the enzymatic digestion of the spheroids. The success of the dissociation was monitored by microscopic analysis."
        - "If the vast majority of cells formerly organized in a spheroid were single cells, the dissociation was considered to be successful. In case of many persisting greater cell aggregates, the dissociation was considered to be incomplete and the dissociation cycle was repeated. The maximum incubation time was 40 min correlating with four dissociation cycles." [1]
- In order to obtain a sufficient number of cells for the flow cytometric analysis in case of the smaller spheroids, 20 spheroids consisting of 10,000 cells each were dissociated in a doubled volume of enzyme solution."
- Analyzed dissociated spheroid cells using fluorescence staining and Casy®-technology

References

[1] U. Grässer, M. Bubel, D. Sossong, M. Oberringer, T. Pohlemann, and W. Metzger, “Dissociation of mono- and co-culture spheroids into single cells for subsequent flow cytometric analysis,” Annals of Anatomy - Anatomischer Anzeiger, vol. 216, pp. 1–8, Mar. 2018, doi: 10.1016/j.aanat.2017.10.002.

Conclusions & Action Items:

- No need for centrifugation?
- Increase Accutase amount ?
- Pitch alternate dissociation protocol at team meeting 3/11

γ-H2AX_12/17/2024

Emily Rhine - Dec 17, 2024, 8:18 PM CST

Title: γ-H2AX

Date: 12/17/2024

Content:

Google query: "gamma h2ax staining protocol"

Link: Quantitative γ-H2AX immunofluorescence method for DNA double-strand break analysis in testis and liver after intravenous administration of InCl3

Summary:

- "It is well known that a severe cell injury after exposure to ionizing radiation is the induction of DNA double-strand breaks (DSBs). After exposure, an early response to DSBs is the phosphorylation of the histone H2AX molecule regions adjacent to the DSBs, referred to as γ-H2AX foci. The γ-H2AX assay after external exposure is a good tool for investigating the link between the absorbed dose and biological effect. However, less is known about DNA DSBs and γ-H2AX foci within the tissue microarchitecture after internal irradiation from radiopharmaceuticals." [1]
- "H2AX phosphorylation and the formation of γ-H2AX foci in the testis are not restricted to radiation effects. The formation of haploid germ cells requires the formation and repair of meiosis-specific DNA DSBs through programmed changes in the chromatin structure [35, 36]. The phosphorylation and dephosphorylation of H2AX throughout the different stages of the spermatogenic cycle creates specific staining patterns correlated to germ cell development" [1]

γ-H2AX immunofluorescence labeling

The specimens were rinsed three times for 5 s each in phosphate buffer saline (PBS 0.1 M, pH 7.4) and dehydrated in a graded alcohol (EtOH) series (70–100%). They were then further immersed in equal volumes of ethanol (100%) and xylene (100%), followed by xylene (100%). The testes and the liver lobes were then infiltrated with 100% paraffin for 1 h at 58 °C, followed by immersion in fresh (100%) paraffin at 58 °C overnight. From the paraffin blocks, consecutive sections were made on a rotation microtome (Rotary Microtome, HM 360, Microm International GmbH, Waldorf, Germany). Five micrometer-thick sections of the testis and liver tissues were collected on SuperFrost Plus microscope slides (G Menzel, Braunschweig, Germany) and dried for 16–18 h at 37 °C. The sections were then deparaffinized, starting with immersion in 100% xylene, followed by immersion in a graded alcohol series down to 70% EtOH. Antigen retrieval was performed by immersing the slides in citrate buffer (pH 6.0) containing a detergent (0.5% Tween 20) that was heated to 90 °C for 10 min. The slides were allowed to cool to room temperature (RT, around 20 °C), incubated in acetone (100%, about 5 s), and then rinsed in PBS three times for 5 min each.

For γ-H2AX immunofluorescence labeling, two different primary antibodies against γ-H2AX were used: one mouse monoclonal anti-phospho-histone H2A.X, (clone JBW301, Merck Millipore, Darmstadt, Germany) and one rabbit polyclonal anti-γ-H2AX (Thermo Scientific Art no. PA1-25001). First, the sections were encircled with a silicon pen and immersed three times for 5 min each in a washing solution of PBSTX (0.1%) (phosphate buffer saline 0.1 M, pH 7.5, 0.1% Triton X-100) at RT. The slides were incubated with a blocking solution of PBSTX (0.1%) containing 1% bovine serum albumin (BSA) for 60 min at RT. The primary anti-γ-H2AX antibodies were diluted in 1% BSA/PBSTX (0.05%) to a concentration of 1 µg/mL for the monoclonal and 1.25 µg/ml for the polyclonal. Sections were incubated with the γ-H2AX antibody solution in a moisture chamber for 16–18 h at 4 °C. Randomly selected sections from non-exposed and exposed animals were incubated without the primary antibodies, i.e., they were used as specificity controls of the γ-H2AX labeling and secondary antibody binding. Sections were then rinsed in PBS three times for 5 min each under gentle shaking.

To visualize the primary antibody binding sites, sections were incubated with secondary goat antibodies against mouse IgG conjugated with Alexa Fluor 488 (Jackson Immunoresearch, Baltimore, MD, USA) or against rabbit IgG conjugated with Alexa Fluor 568 (Invitrogen. Art no. A11036). Secondary antibodies were diluted 1:150 (13.3 µg/mL) in 1% BSA/PBS, and sections were incubated for 60 min at RT. Sections were then rinsed in PBS three times for 5 min each and were incubated with 4′,6-diamidino-2-phenylindole (DAPI, nuclear labeling, Invitrogen, USA) at a concentration of 0.1 µM for 20 min at RT. Sections were then rinsed with PBS for 5 min before being mounted and coverslipped using DAKO fluorescent mounting medium (Carpenteria, USA). [1]

References

[1] A. Stenvall, E. Larsson, B. Holmqvist, S.-E. Strand, and B.-A. Jönsson, “Quantitative γ-H2AX immunofluorescence method for DNA double-strand break analysis in testis and liver after intravenous administration of 111InCl3,” EJNMMI Res, vol. 10, p. 22, Mar. 2020, doi: 10.1186/s13550-020-0604-8.

Conclusions/Action Items:

- Review protocol next semester
- Find alternate protocol to compare to
- Condense protocol and create materials list from it

γH2AX stain on A549s using Etoposide_5/3

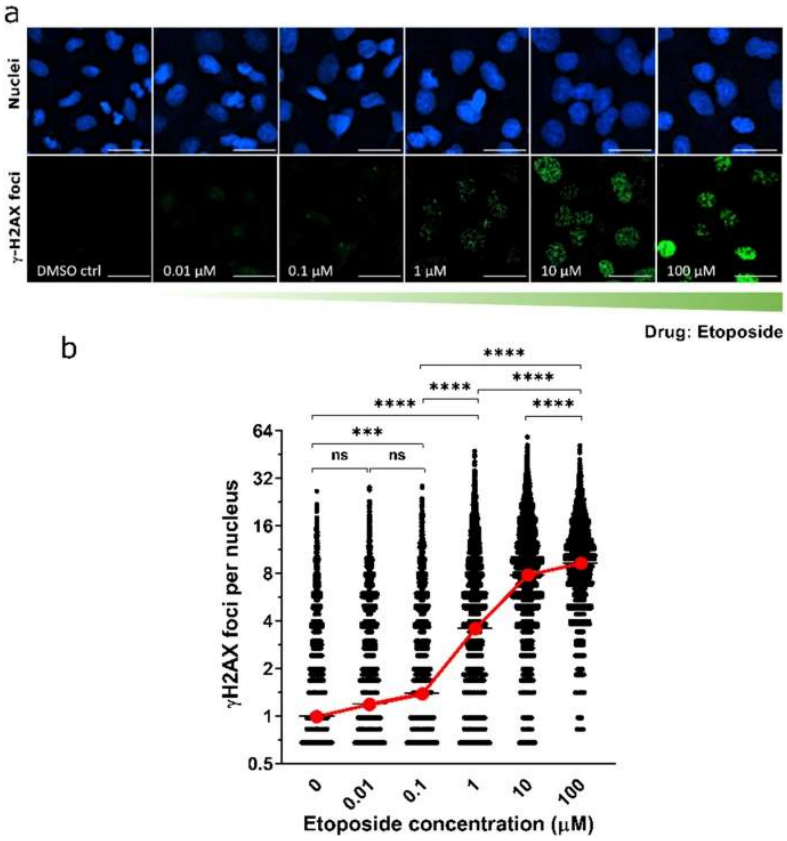
Emily Rhine - May 03, 2025, 8:00 PM CDT

Title: γH2AX stain on A549s using Etoposide

Date: 5/3/25

Contents:

- This is a better version of what we did
- We diluted the 16uM of Etoposide in our protocol, but these researchers warned that "there is a threshold of fluorescence intensity required to detect a signal. This fluorescence intensity threshold appears to correlate with cell density and the amount of DNA damage induced." [1]



[1]

γ-H2AX foci of double-strand breaks quantified by automated spot counting algorithm. (a) Representative images of γ-H2AX foci in A549 cells treated with etoposide (1.5 h, 0–100 μM); γ-H2AX (green) and nuclear DNA stained with DAPI (blue). Scale bar is 20 μm. (b) Data summarizing Spot Count analysis and quantitation of individual γ-H2AX foci per nucleus (black) and average γ-H2AX foci per nucleus (red). The data are presented as mean ± standard deviation of 1500 nuclei per sample with four biological replicates, **P < 0.01. ***P < 0.001, ****P < 0.0001, one-way ANOVA. Average γ-H2AX for each concentration reveals a dose–response relationship (red line). [1]

References

[1] F. K. Noubissi, A. A. McBride, H. G. Leppert, L. J. Millet, X. Wang, and S. M. Davern, “Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay,” Sci Rep, vol. 11, no. 1, p. 8945, Apr. 2021, doi: 10.1038/s41598-021-88296-3.

Conclusions & Action Items:

- Finish and submit final report
- See complete PDF attached below
- Use this optimized γH2AX stain protocol for future work on this project

Emily Rhine - May 03, 2025, 7:52 PM CDT

scientific reports

OPEN Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay

Download

s41598-021-88296-3.pdf (2.37 MB)

Stock PolyHEMA Protocol_2/9/25

Emily Rhine - Feb 09, 2025, 11:45 PM CST

Title: Stock PolyHEMA Protocol

Original Date:10/23/2024

Current Date: 2/9/25

Content By: Emily

Content:

Poly-HEMA hydrogel coating: [2]

Link: SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine

A total 1.3 g of poly-HEMA (Sigma-Aldrich Co.) was dissolved in 33 mL of 99% ethanol, and the solution was mixed overnight at 37°C. Fifty microliters or 3.2 mL of the poly-HEMA stock solution was added to 96-well plates and 10 cm dishes, respectively, in the tissue culture hood, and plates and dishes were swirled using a plate rotator for 10 minutes. Plates were left to dry overnight and then washed with PBS immediately before use. [1]

Alternate Protocol: https://pmc.ncbi.nlm.nih.gov/articles/PMC9006308/#S6 [2]

- Materials:
  -
  - Poly(2-hydroxyethyl methacrylate) (PolyHEMA) (2g) (Sigma, P3932)
  - 95% (v/v) ethanol
  - Vacuum desiccator (VWR, cat. no. 24987–004)
  - 0.22 μm cellulose acetate membrane with bottle top vacuum filter system (Corning, cat. no. 430756)
  - Hot plate and magnetic stirrer
  - Magnetic stir bar
  - 200 mL beaker
  - Parafilm (Millipore Sigma, cat. no. P7793)
  - Glass thermometer (Thomas Scientific, cat. no. 9313A27)
  - Aluminum foil
  - Biosafety Class II tissue culture hood
- Steps (4 hours) [2]
  1.
  2. Weigh and add 2g PolyHEMA to 100 mL 95% ethanol in a glass beaker.
  3. Add a magnetic stir bar to the beaker and place the beaker on a hot plate with a magnetic stirrer and stir at 65°C until fully dissolved (typically ≥3 hours). Cover first with aluminum foil, followed by generous amounts of Parafilm to prevent evaporation.
     Poke a glass thermometer through the top of the aluminum foil and Parafilm to ensure that the temperature of the PolyHEMA solution is as close to 65°C as possible.
  4. Allow the PolyHEMA solution to cool to room temperature.
  5. In a tissue culture hood, filter the PolyHEMA solution through a 0.22 μm filter using the vacuum filter system.

6. In a tissue culture hood, place the bottle of filtered PolyHEMA solution (with cap off) in a vacuum desiccator for 30 minutes to degas the solution.
7. In a tissue culture hood, after the solution is degassed, replace the screw cap and use Parafilm to seal the cap-bottle junction on the outside of the bottle, as an extra measure to prevent unwanted gas exchange.

This protocol describes how to make the PolyHEMA solution used to coat glass-bottom dishes in Basic Protocol 1. PolyHEMA solution should be kept under tight seal at room temperature when not in use, and after it is sterile filtered it should only be opened in a Biosafety Class II tissue culture hood using aseptic technique. We suggest using a hot plate with a feedback system or probe to monitor the actual temperature of this solution as it is being mixed. Otherwise, take great care to monitor and adjust the hot plate temperature so that the PolyHEMA solution reaches 65°C for 3 hours while stirring, or until all PolyHEMA crystals are dissolved. Undissolved PolyHEMA interferes with organoid imaging. Once this reagent is made, it can be stored for up to 3 months at room temperature. We caution against leaving coated plates with 1x PBS in the incubator for more than 3 weeks. The coatings may degrade by this time. Always visually check coating integrity before using it for culture. [2]

References

[1] C. Choe, H. Kim, S. Min, S. Park, J. Seo, and S. Roh, “SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine,” OncoTargets and therapy, vol. 11, p. 6197, Sep. 2018, doi: 10.2147/OTT.S175810.

[2] H. M. Cambra, N. P. Tallapragada, P. Mannam, D. T. Breault, and A. M. Klein, “Triple-decker sandwich cultures of intestinal organoids for long-term live imaging, uniform perturbation and statistical sampling,” Curr Protoc, vol. 2, no. 1, p. e330, Jan. 2022, doi: 10.1002/cpz1.330.

Conclusions/Action items:

- I could not find the Kreeger Labs PolyHema Stock Recipe Protocol on the Lab Drive, so I found an alternate version posted by NIH.
  - I can reasonable trust this protocol since it is also being used to image organoids.
- Recommend this protocol for future PolyHema stock creation.
- Update and alter protocol as needed to optimize for our needed quantity/volume

SOX2_Antibody_2/7/25

Emily Rhine - Feb 07, 2025, 9:03 PM CST

Title: SOX2_Antibody

Date: 2/7/25

Content:

Link: Anti-SOX2 antibody:

Product Sort by	Star rating	Images	Publications	Target	Application	Reactive species
ab97959 Anti-SOX2 antibody	★★★★☆ 74 reviews	9	737	SOX2	ICC/IF, WB, IHC-P	Mouse, Rat, Human

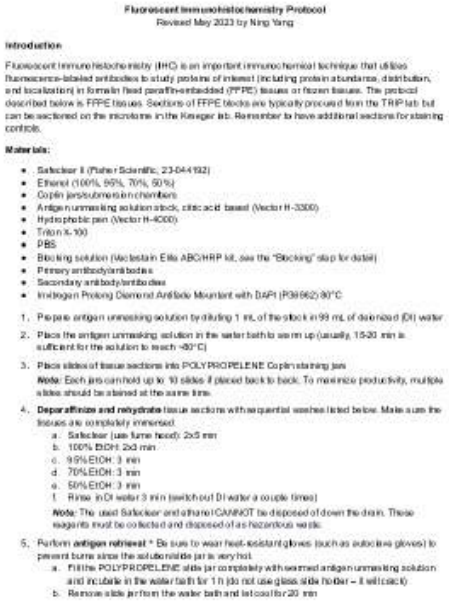
- Anti-SOX2 antibody ab97959 is a rabbit polyclonal antibody that is used in SOX2 western blotting, IHC and immunofluorescence. Suitable for human, mouse and rat samples.
- 74: reviews of this antibody
  - "Interaction between the estrogen receptor and fibroblast growth factor receptor pathways in non-small cell lung cancer"
  - "Expression of OCT-4 and SOX-2 in Bone Marrow-Derived Human Mesenchymal Stem Cells during Osteogenic Differentiation"
- 45: 5 star reviews with comments about it working well for IHC

Conclusions & Action Items:

- Recommend this antibody for SOX2 to team and Carley
- Continue follow up research for how this antibody has been used for spheroids and A549 cells
  - "Interaction between the estrogen receptor and fibroblast growth factor receptor pathways in non-small cell lung cancer"

Kreeger Lab IHC_2/9/25

Emily Rhine - Feb 09, 2025, 11:30 PM CST



Fluorescent Immunohistochemistry Protocol
Revised May 2023 by Ning Yang

Introduction

Fluorescent immunohistochemistry (IHC) is an important immunochemical technique that utilizes fluorescence-labeled antibodies to study proteins of interest (including protein abundance, distribution, and localization) in formalin fixed paraffin-embedded (FFPE) tissues or frozen tissues. The protocol described below is FFPE tissues. Sections of FFPE blocks are typically procured from the TRIP lab but can be sectioned on the microtome in the Kreeger lab. Remember to have additional sections for staining controls.

Materials:

- Safeclear II (Fisher Scientific, 23-044192)
- Ethanol (100%, 95%, 70%, 50%)
- Coplin jars/submersion chambers
- Antigen unmasking solution stock, citric acid based (Vector H-3300)
- Hydrophobic pen (Vector H-4000)
- Triton X-100
- PBS
- Blocking solution (Vectastain Elite ABC/HRP kit, see the "Blocking" step for detail)
- Primary antibody/antibodies
- Secondary antibody/antibodies
- Invitrogen ProLong Diamond Antifade Mountant with DAPI (P36962) 80°C

1. Prepare antigen unmasking solution by diluting 1 mL of the stock in 99 mL of deionized (DI) water
2. Place the antigen unmasking solution in the water bath to warm up (usually, 15-20 min is sufficient for the solution to reach ~80°C)
3. Place slides of tissue sections into POLYPROPELENE Coplin staining jars
   Note: Each jars can hold up to 10 slides if placed back to back. To maximize productivity, multiple slides should be stained at the same time.
4. **Deparaffinize and rehydrate** tissue sections with sequential washes listed below. Make sure the tissues are completely immersed
   a. Safeclear (use fume hood): 2x5 min
   b. 100% EtOH: 2x3 min
   c. 95% EtOH: 3 min
   d. 70% EtOH: 3 min
   e. 50% EtOH: 3 min
   f. Rinse in DI water 3 min (switch out DI water a couple times)
   Note: The used Safeclear and ethanol CANNOT be disposed of down the drain. These reagents must be collected and disposed of as hazardous waste.
5. Perform **antigen retrieval** * Be sure to wear heat-resistant gloves (such as autoclave gloves) to prevent burns since the solution/slide jar is very hot.
   a. Fill the POLYPROPELENE slide jar completely with warmed antigen unmasking solution and incubate in the water bath for 1 h (do not use glass slide holder – it will crack)
   b. Remove slide jar from the water bath and let cool for 20 min

Download

Fluorescent_IHC_Protocol_for_tissue_2023.docx.pdf (209 kB)

qPCR Background Information_2/18/25

Emily Rhine - Feb 18, 2025, 1:57 PM CST

Title: qPCR Background Information

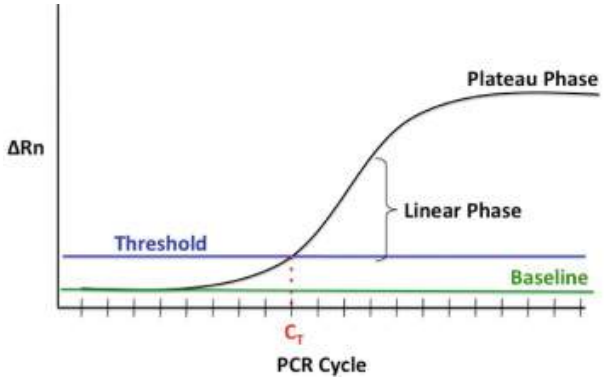
Date: 2/18/25

Goal: Better understand qPCR before our next client meeting.

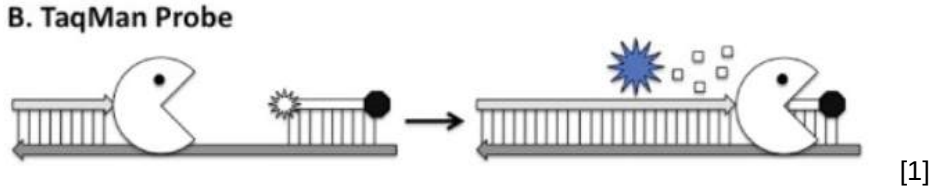
Content:

Link: Chapter Twenty Three - Explanatory Chapter: Quantitative PCR

- Takeaways:
  - q-PCR measures the amount of a specific target DNA sequence.
    - "Baseline: During cycles 1–15 of the qPCR, fluorescence is below the limits of detection, but it is increasing." [1]
    - "CT: The amplification cycle at which the ΔRn exceeds the threshold. The CT is a quantitative description of the starting concentration of template." [1]
  - The team has selected SOX2 as a good stemness marker to measure with β-actin used as a housekeeping gene ("control gene")
  - Plan 1-2 days to complete full qPCR protocol
  - For further information about TaqMan probes see link above



[1]



[1]

- Excerpt: "Quantitative PCR (qPCR), also called real-time PCR or quantitative real-time PCR, is a PCR-based technique that couples amplification of a target DNA sequence with quantification of the concentration of that DNA species in the reaction. This method enables calculation of the starting template concentration and is therefore a frequently used analytical tool in evaluating DNA copy number, viral load, SNP detection, and allelic discrimination. When preceded by reverse-transcription PCR, qPCR is a powerful tool to measure mRNA expression and is the gold standard for microarray gene expression data confirmation. Given the broad applications of qPCR and the many technical variations that have been developed, a brief survey of qPCR, including technical background, available chemistries, and data analysis techniques will provide a framework for both experimental design and evaluation." [1]

References

[1] J. S. Dymond, “Chapter Twenty Three - Explanatory Chapter: Quantitative PCR,” in Methods in Enzymology, vol. 529, J. Lorsch, Ed., in Laboratory Methods in Enzymology: DNA, vol. 529. , Academic Press, 2013, pp. 279–289. doi: 10.1016/B978-0-12-418687-3.00023-9.

Conclusions & Action Items:

- Prepare for client meeting on qPCR
- With team: come up with a few options of primers to pitch to the clients at our meeting (2/20)

Background Research_1/21/25

Emily Rhine - Jan 29, 2025, 1:30 PM CST

Title: Background Research Topics
Date: 1/21/24
Content By: Emily
Goals: Establish a need for more research in key areas. Follow up on these research ideas when possible.
Content:

- Research role of methylcellulose concentration in spheroid formation
  - Minispheroids as a Tool for Ligament Tissue Engineering: Do the Self-Assembly Techniques and Spheroid Dimensions Influence the Cruciate Ligamentocyte Phenotype?
- Research role of seeding density on spheroid size/health
  - A549 Spheroid Formation - how to product small and defined spheroids?
- Research percent cell viability assay
- Calculate whether, using the ideal seeding density, it is possible/realistic to scale up spheroid formation to the 50 million cells necessary for the genome wide screen
  - Predict time, materials necessary, and complexity
- gamma-H2AX staining in spheroids
  - When to dissociate cells from the spheroid to keep them alive with 3D characteristics?
  - Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay
  - Linking hypoxia, DNA damage and proliferation in multicellular tumor spheroids
  - Phosphorylated histone H2AX in spheroids, tumors, and tissues of mice exposed to etoposide and 3-amino-1,2,4-benzotriazine-1,3-dioxide
    - "phosphorylated histone H2AX (serine 139 phosphorylated histone H2AX; gammaH2AX)"
      "**For spheroids, γH2AX intensity predicted clonogenic cell survival for cells recovered 90 min after drug injection, regardless of position of the cells within the spheroid. Similar results were obtained for etoposide in tumors; however, the γH2AX signal for tirapazamine was smaller than expected for the observed amount of cell killing.** Frozen sections of tumors confirmed the greater intensity of γH2AX staining in cells close to blood vessels of tumors soon after treatment with etoposide and the opposite pattern for tumors exposed to tirapazamine. Analysis of cells or frozen sections from mouse spleen and kidney suggests that information can also be obtained on initial damage in normal tissues. These results support the possibility of using γH2AX antibody staining as a method to aid in prediction of tumor and normal tissue response to treatment."
      Flow Cytometry for γH2AX.

      Cells that were fixed in 70% ethanol were kept at −20°C for up to 2 weeks before analysis. Before antibody labeling, samples were rehydrated and incubated with mouse monoclonal anti-phospho-histone H2AX antibody (Upstate Biotechnology) as described previously **(18)**. After 2 h at room temperature, cells were rinsed and incubated with 200 μl of secondary antibody [Alexa 488 goat-antimouse IgG (H + L)F(ab′)2 fragment conjugate; Molecular Probes] for 1 h at room temperature. Cells were rinsed and resuspended in 400 μl of cold Tris buffer containing 1 μg/ml ml 4′,6-diamidino-2-phenylindole (Sigma). Samples were analyzed for DNA content and γH2AX antibody binding using
    - a Coulter Elite flow cytometer.

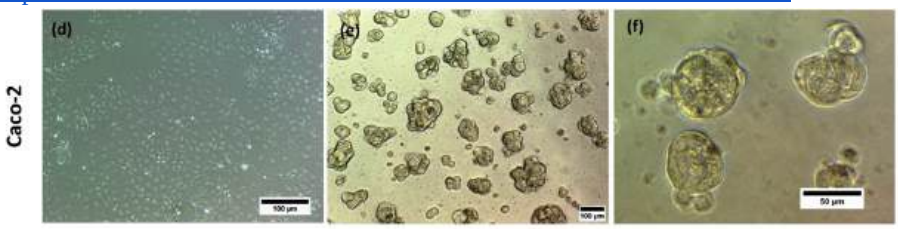
Image Cytometry for γH2AX in Sections.

Frozen sections (5-μm thick) prepared from SiHa tumors and normal tissues were placed on slides, air-dried for no more than 1 min, and fixed in 2% freshly prepared paraformaldehyde for 15 min. Samples were then incubated for 30 min with anti-phospho-histone H2AX monoclonal antibody (Upstate Biotechnology) followed by rinsing and incubation for 15 min with Alexa 488 goat antimouse IgG (Molecular Probes). Slides were dipped in paraformaldehyde, mounted with coverslips using 10 μl of Fluorogard (Bio-Rad), and sealed. Slides were viewed using a Zeiss Axioplan 2 fluorescence microscope, and images were acquired under constant light exposure conditions for each wavelength using a ×10 or ×100 Neofluor objective and a Q-Imaging 1350 EX digital camera. Images were captured and analyzed using Northern Eclipse and ImageJ software.

- Paper 1
  - Fixed (not dissociated) Gold–palladium stain done last step before SEM

    Quantitative real-time PCR analysis

    The following genes were selected and examined by real-time PCR: stemness genes; *KLF4*, *OCT4*, *SOX2*, *NANOG* and *C-MYC*, EMT genes; *Vimentin*, *SNAIL1*,*TWIST1*, *N-cadherin*, *E-cadherin* and *ZEB1*, ABC transporter genes; *ABCB1*, *ABCC1* and *ABCG2*. Spheroids were harvested a day before structural disintegration (day 10 for HT-29 and day 4 for Caco-2 spheroids). The total RNAs were then extracted from parental and spheroid cells using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. After measurement of RNA quantity and quality by Nanodrop (ThermoFisher Scientific, USA), cDNA were synthesized with 1 μg of total RNA using cDNA synthesis kit (GeneAll, Korea). Real-time polymerase chain reaction (RT-qPCR) was performed using the SYBR Premix Ex Taq II real-time PCR kit (TaKaRa, Japan) on the Rotor-Gene Q LightCycler (Qiagene, Germany) with the following conditions: 40 two-step amplification cycles of 95 °C for 5 s and 60 °C for 30 s. The relative expression values of target genes were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as the internal reference gene, by using the $2^{-\Delta CT}$ method. Real-time PCR primers are listed in Table 1.
  - Flow cytometry
    - "The parental and spheroid cells from each cell line were dissociated with trypsin/EDTA and were washed with PBS twice. The dissociated cells were counted using Trypan blue exclusion assay, and if cell viability was more than 95%, they were evaluated for CSC markers expression."
  - https://cancerci.biomedcentral.com/articles/10.1186/s12935-021-01898-9



Morphology of HT-29 and Caco-2 parental adherent monolayer cells and their derived spheroids. Representative phase contrast images from cell lines and their derived spheroids. **a** Parental HT-29 cells grew as an adherent monolayer, **b**, **c** HT-29 derived spheroids cultured at nonadherent and serum free condition showed well-round shape and compact morphology. **d** Caco-2 parental cells as monolayer and, **e**, **f** Caco-2 derived spheroids displayed small and round shape morphology

Conclusions & Action Items:

- Add specific interesting and helpful information into separate entries in each specialized research folder.
- Continue brainstorming research that will need to be done for future experiments.
- Narrow down specific experimental conditions and procedures to bring up to the team and/or the client

Hess Lab Training Requirements_9/10/2024

Emily Rhine - Oct 12, 2024, 4:41 PM CDT

Lab Safety

Required Training

Before starting any work in the lab, you must complete the following sets of training. These can be found on Canvas https://it.wisc.edu/services/canvas/ or https://ehs.wisc.edu/training/

General:

2020-21 HIPAA Privacy & Security Training
SMPH: SMPH: Creating a Respectful and Welcoming Learning Environment (2020)
Preventing Sexual Harassment and Sexual Violence at UW-Madison
Cybersecurity Training (https://it.wisc.edu/it-projects/cybersecurity-awareness-training/#started)

Research-Based:

-Biosafety Required Training
-Chemical Safety: The OSHA Lab Standard
-Chemical Safety: Cryogen Safety Training
-Chemical Safety: Fume Hood Safety Training
-Chemical Safety: Hazard Communication
-Disposing of Hazardous Chemicals
-Biosafety 102: Bloodborne Pathogens for Laboratory and Research
-Biosafety 205: Bio HazMat Shipping Training (Both Campus Transport of Biologicals & Dry Ice Training Path and Dangerous Goods Training Paths)
-Biosafety 105: Biosafety Cabinet Use
-Biosafety 106: Autoclave Use
-Biosafety 107: Centrifuge Safety

Emily Rhine - Sep 27, 2024, 3:56 PM CDT

https://compliance.research.wisc.edu/TILT/Details/8785095

Emily Rhine - Sep 10, 2024, 2:40 PM CDT

Course	Assignment	Completion	Expiration
2022-23 HIPAA Privacy & Security Training	HIPAA Training Quiz	1/25/2023	
2023-24 HIPAA Privacy & Security Training	HIPAA Attestation	10/28/2023	
Biosafety 102: Bloodborne Pathogens for Laboratory and Research	Biosafety 102: Bloodborne Pathogens Safety in Research Quiz 2024	8/15/2024	8/15/2025
Biosafety 105: Biosafety Cabinet Use	Biosafety 105: Biosafety Cabinet Use Quiz	8/31/2023	No Expiration
Biosafety 106: Autoclave Use	Biosafety 106: Autoclave Use: Safety and Efficacy - Verification Quiz	8/31/2023	No Expiration
Biosafety 107: Centrifuge Safety	Biosafety 107: Centrifuge Safety Verification Quiz	8/31/2023	No Expiration
Biosafety Required Training	Biosafety Required Training Quiz 2023	3/12/2023	3/12/2028
Chemical Safety: Hazard Communication - Identifying Chemical Hazards	Final Quiz	8/31/2023	8/31/2028
Chemical Safety: The OSHA Lab Standard	Final Quiz	3/12/2023	

Emily Rhine - Oct 12, 2024, 4:44 PM CDT

Name	Due	Submitted	Status	Score	
Shared Guidelines for Professional Conduct 2023 Assignments		Sep 8 at 9:18pm			
Assignments				100%	100.00 / 100.00
Total				**100%**	100.00 / 100.00

Emily Rhine - Oct 12, 2024, 4:48 PM CDT

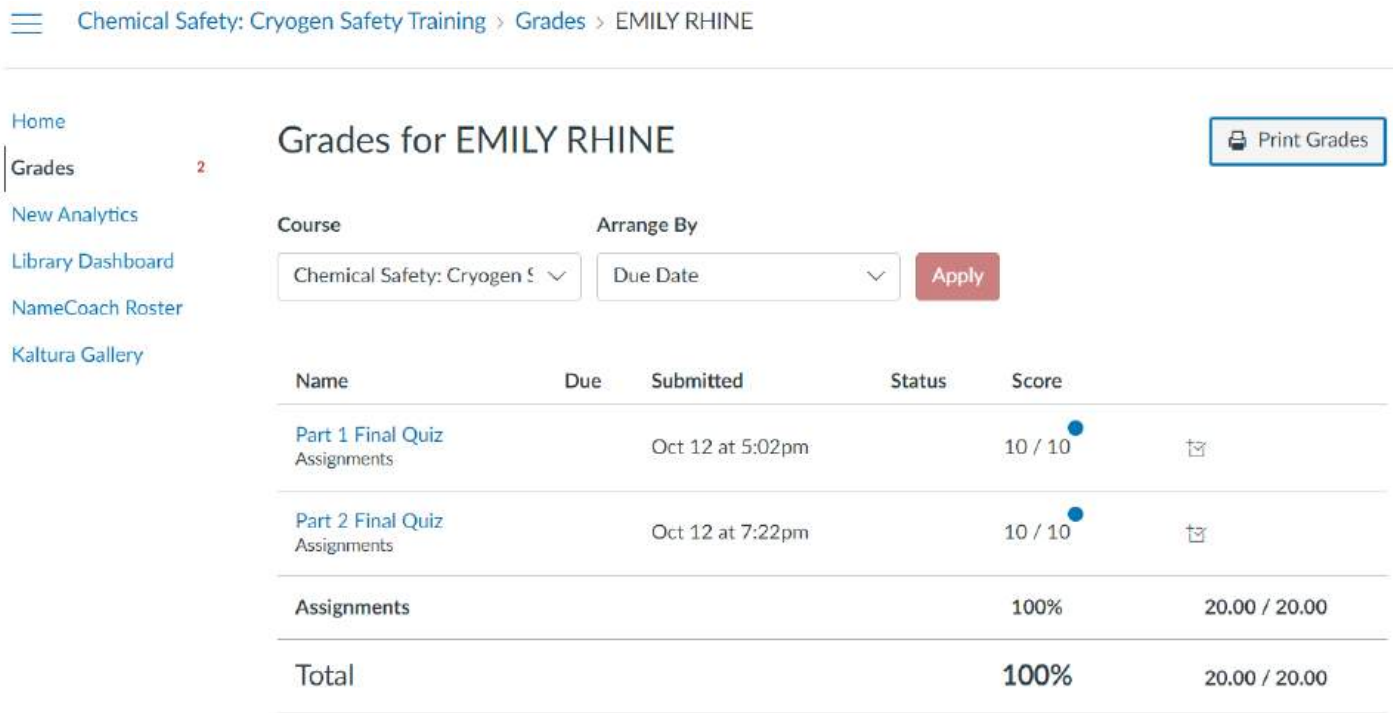
Substituted "ListenWIse" program for "Preventing Sexual Harassment and Sexual Violence at UW-Madison"

4. Certify Completion of GetWIse@Home (Required for completion of the GetWIse@Home Annual Refresher course) GetWIse Requirement Completion	Sep 3 at 9:27pm	1 / 1

Emily Rhine - Oct 12, 2024, 4:48 PM CDT

Biosafety 205 no longer offered

Emily Rhine - Oct 12, 2024, 7:23 PM CDT



Emily Rhine - Apr 10, 2025, 1:38 PM CDT

Grades for Emily Rhine

Print Grades

Course: Dual Use Research of Conc

Arrange By: Due Date

Apply

Name	Due	Submitted	Status	Score	
DURC & PEPP Training 2025 Assignments				-	
DURC/PEPP Quiz Assignments		Apr 10 at 1:36pm		15 / 15	
Assignments				**100%**	**15.00 / 15.00**
Total				**100%**	**15.00 / 15.00**

HIPAA 24-25 Training

Emily Rhine - Nov 19, 2024, 1:39 PM CST

Grades for EMILY RHINE

Print Grades

Course: 2024-2025 HIPAA Privacy

Arrange By: Due Date

Apply

Name	Due	Submitted	Status	Score	
2024-2025 HIPAA Privacy & Security Training Assignments		Nov 19 at 1:37pm		100 / 100	
Assignments				100%	100.00 / 100.00
Total				**100%**	100.00 / 100.00

Spring Experiment Planning 2025_1/31/25

Emily Rhine - Jan 31, 2025, 12:16 PM CST

Title: Goals and Experiments for Spring 2025

Date: 12/17/2024 & 1/31/2025

Content By: Emily & Althys

Content

1. Thaw a new vial of A549s
    a. Freeze 3 new vials of cells too after 1-2 passages
2. Redo spheroid experiment with 50k and 75k seeding densities to confirm results
    a. Alter methylcellulose levels to confirm optimization
    b. Test spheroid dissociation with Accutase protocol
    c. Test percent cell viability
        i. Question for client: are we still doing live/dead staining (ideal)
    d. Select ideal seeding density based on data gathered
3. Calculate whether, using the ideal seeding density, it is possible/realistic to scale up spheroid formation to the 50 million cells necessary for the genome wide screen
    a. Calculate time, materials necessary, and complexity
4. Use qPCR to determine whether or not expression of SOX2 increases in our spheroids to confirm ideal cellular conditions and similarity to tumor environment
    a. "A549 with CRISRPi to add gRNA and look for specific mutation --> See loss in staining if specific proteins are selected to be removed with gRNA" -Gaelen/Carley
5. Lentiviral transduce cells with γH2AX, a biomarker for DNA double strand breaks (dsbs), so the team can understand the effects of gene knockdown via lentivirus.
    a. See "Spheroid Formation Protocols_10/25/2024" entry excerpt below
    b. See if methylcellulose levels alter γH2AX presentation - Carley S.
        i. Question for client: So we are seeding cells at different methylcellulose concentrations (but same density to save time I assume), do CRISPRi, and γH2AX staining to check if the methylcellulose concentrations affect γH2AX presentation?
6. ~~CRISPRi genome-wide screen~~ (Technically not a goal for us to complete according to the client(s))
    a. Will still need to do a faux CRISPRi to check if the spheroid formation protocol actually is compatible with it, and to check whether γH2AX also works

γ-H2AX staining process (basic procedure - as if don't have spheroids) ***Takes 4-4.5 hours

1. Infect cells with lentivirus
    a. Will introduce plasmid with guideRNA, which will target some gene that is involved in gamma-H2AX/DNA damage
        i. The plasmid also has fluorescence: mCherry (positive)
            1. Therefore, associate mCherry+ cells with guideRNA infection into cell

b. Will not get 100% infection → ideal so we can have controls (with no plasmid aka no vector and no mCherry - negative)

c. Wait 3-5 days so that DNA damage caused by lentivirus infection itself will be "healed" → only downstream data and can be ignored

2. Gather population of cells (3-5 million)
3. Fix cells
    a. Immobilize and kill cells
    b. This allows us to stain, image, sort, and other downstream steps
    c. Dissociate cells from spheroid here?
4. Permeabilize cells
    a. Makes holes in cells so "stuff" can get in them (because we are doing an intracellular staining)
    b. Dissociate cells from spheroid here?
5. Antibody 1
    a. In our case, this will be gamma-H2AX
    b. We actually have a primary-conjugated antibody (already has fluorescence so do not need a secondary antibody)
        i. Our fluorophore will be APC - Alexa fluorophore 647
    c. Dissociate cells from spheroid here?
6. Flow Cytometry
    a. Will have graph: mCherry vs. FSC
    b. In each population, will have some cells positive for mCherry and some negative for mCherry (control, not lentivirally infected)
    c. Will "gate" mCherry+ cells and average their gamma-H2AX expression
        i. Two more subplots:
            1. mCherry+ only with APC gamma-H2AX
                a. Expected to see increase in APC gamma-H2AX
                b. On plot, "peak" will be shifted higher/to the right on x-axis (APC gamma-H2AX)
            2. mCherry- only with APC gamma-H2AX
                a. Expect to see decrease in APC-gamma-H2AX (because no lentiviral infection = gene was not knocked down = no increase in DNA damage)
                b. On plot, "peak" will be shifted lower/to the left on x-axis (APC gamma-H2AX)

NOTE: will need to refer to current spheroid CRISPR screening gamma-H2AX staining protocols to see the process (do we dissociate spheroids before fixing, after fixing, or after permeabilizing?)

- **Possible: if earlier dissociation, may be losing some of DNA markers)**
- **Our job: find a "starting point" for when to dissociate based on literature, then test staining at alternative disassociation points and compare whether there are differences in results**

Conclusions & Action Items:

- Discuss plan with Advisor and Client
- Add timeline plan to preliminary presentation
- Update timeline as needed

Preliminary Report_2/21/25

Emily Rhine - Feb 21, 2025, 7:55 PM CST

BioTek Cytation Imaging

In summary, open BioTek Gen5 3.14 application [4], place plate in BioTek with A1 well in on the bottom right of the holder, create new experiment and new protocol making sure that the experiments and photos are saved in the same location and transferred together otherwise both will be lost, set protocol and imaging settings, and click run. The protocol procedure was set as follows: temperature at 37°C; middle imaging; deselect auto exposure; select plate type; and set imaging settings to 4x magnification, Brightfield, FVOW, set z-stack, and distance. Then, run the experiment trial.

Cell-Titer Glo

For full protocol see CellTiter-Glo® Luminescent Cell Viability Assay user manual [3]. Briefly, remove CellTiter-Glo reagent from -30°C freezer storage and let it acclimate to room temperature. Make sure the CellTiter-Glo remains covered in aluminum foil to reduce light exposure. Add 100 uL of 1X of CellTiter-Glo reagent to 96 well plate prepared with 100 uL of cell solution media and cover the wells with aluminum foil. Insert 96 well plate with lid into BioTek with A1 well in on the bottom right of the holder and create a new experiment. Input input se premade protocol: 1) Mix plates for 2 minutes at RT on an orbital shaker, 2) Incubate plates for 10 minutes at room temperature 3) Analyze plate with chemiluminescence setting on BioTek. Analyze data by normalizing replicate cell viability numbers for each condition to vehicle only numbers. Then, plot on a log-linear plot and determine approximate IC50 values.

ImageJ Analysis of BioTek Cytation Images

To begin, choose the most in-focus image (figure 8a) for the desired well and upload it to an image analysis tool named ImageJ. Measure the scale bar in the corner of the image using the line tool and measurement feature. Set the scale under ‘Analyze’ → ‘Set Scale’ by setting the ‘distance in pixels’ to the measured amount of pixels from the images scale bar length. Then set the known length to the scale bar length in µm, and changing the ‘Unit of length’ to read um in order to output results in micrometers. Select the ‘Global’ box to retain the scale bar incase of needing to restart the analysis process with an image, then select ‘OK’.

Starting image analysis, change the image type to a 16-bit under ‘Image’ → ‘Type’ → ‘16-bit’ in order to use the ‘Threshold’ feature. Next, select ‘Image’ → ‘Adjust’ → ‘Threshold’. Uncheck the box titled ‘Dark background’ if selected, then move the ranges until the desired section shows the spheroids highlighted in red, as seen in figure 8b, then hit ‘Apply’. Threshold recognizes pixels containing cells, or in this case spheroids. Next, go to ‘Process’ → ‘Binary’ → ‘Watershed’, which separates any

spheroids that were close together but were recognized as one by the ‘Threshold’ feature. In order to block out any background noise from the edges of the well use the ellipse tool to encircle only the center of the well being analyzed (Figure 8c).

To analyze the highlighted spheroids click ‘Analyze’ → ‘Analyze Particles’. Set the size range to 4147-infinity, as to ensure only spheroids of 20 or more cells were being analyzed. In the dropdown menu under ‘Show:’ select ‘Outlines’ then underneath select the ‘Display results’, ‘Clear results’, and ‘Exclude on edges’ boxes before hitting ‘OK’ (Figure 8d).

To save the results one of two windows will pop up. 1. A window asking to save measurements or 2. A results window. Should option 1 appear, select save measurements, but if option 2 appears, select ‘File’ → ‘Save As’ then save results as an excel file to a secure and known location. Combine the excel files into one master document for data analysis.

Emily Rhine - Feb 21, 2025, 7:56 PM CST

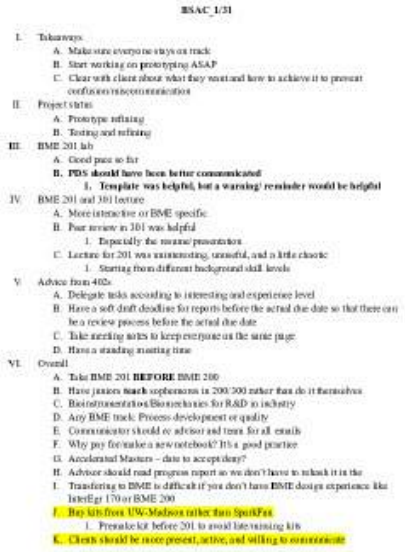
References

[3] “CellTiter-Glo® Luminescent Cell Viability Assay.” Accessed: Feb. 16, 2025. [Online]. Available: https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-luminescent-cell-viability-assay/

[4] “Imager, Reader Control, & Analysis Software | Agilent.” Accessed: Feb. 21, 2025. [Online]. Available: https://www.agilent.com/en/product/microplate-instrumentation/microplate-instrumentation-control-analysis-software/imager-reader-control-analysis-software

BSAC Meeting Notes

Emily Rhine - Feb 14, 2025, 4:27 PM CST



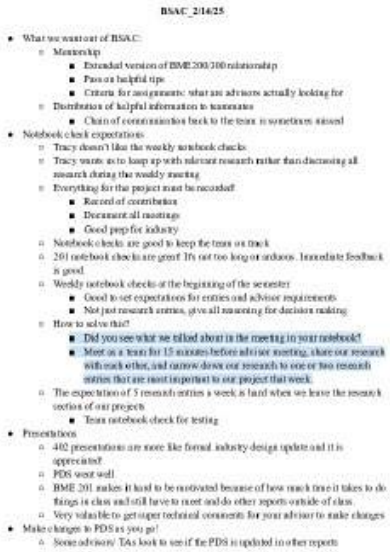
BSAC_1/31

I. Takeaways
  A. Make sure everyone stays on track
  B. Start working on prototyping ASAP
  C. Clear with client about what they want and how to achieve it to prevent confusion/miscommunication
II. Project status
  A. Prototype refining
  B. Testing and refining
III. BME 201 lab
  A. Good pace so far
  B. **PDS should have been better communicated**
    1. **Template was helpful, but a warning/ reminder would be helpful**
IV. BME 201 and 301 lecture
  A. More interactive or BME specific
  B. Peer review in 301 was helpful
    1. Especially the resume/presentation
  C. Lecture for 201 was uninteresting, unuseful, and a little chaotic
    1. Starting from different background skill levels
V. Advice from 402s
  A. Delegate tasks according to interesting and experience level
  B. Have a soft draft deadline for reports before the actual due date so that there can be a review process before the actual due date
  C. Take meeting notes to keep everyone on the same page
  D. Have a standing meeting time
VI. Overall
  A. Take BME 201 **BEFORE** BME 200
  B. Have juniors **teach** sophomores in 200/300 rather than do it themselves
  C. Bio/Instrumentation/Biomechanics for R&D vs industry
  D. Any BME track: Process development or quality
  E. Communicate should ee advisor and team for all emails
  F. Why pay for/make a new notebook? It's a good practice
  G. Accelerated Masters – date to accept/deny?
  H. Advisor should read progress report so we don't have to rehash it in the
  I. Transferring to BME is difficult if you don't have BME design experience like InterEgr 170 or BME 200
  J. Buy kits from UW-Madison rather than SparkFun
    1. Premake kit before 201 to avoid late running kits
  K. Clients should be more present, active, and willing to communicate

Download

BSAC_1_31.pdf (74.1 kB)

Emily Rhine - Feb 14, 2025, 4:25 PM CST



BSAC_2/14/25

- What we want out of BSAC:
  - Mentorship
    - Extended version of BME200/300 relationship
    - Pass on helpful tips
    - Criteria for assignments: what are advisors actually looking for
  - Distribution of helpful information to teammates
    - Chain of communication back to the team is sometimes missed
- Notebook check expectations
  - Tracy doesn't like the weekly notebook checks
  - Tracy wants us to keep up with relevant research rather than discussing all research during the weekly meeting
  - Everything for the project must be recorded!
    - Record of contribution
    - Document all meetings
    - Good prep for industry
  - Notebook checks are good to keep the team on track
  - 201 notebook checks are great! It's not too long or arduous. Immediate feedback is good
  - Weekly notebook checks at the beginning of the semester
    - Good to set expectations for entries and advisor requirements
    - Not just research entries, give all reasoning for decision making
  - How to solve this?
    - Did you see what we talked about in the meeting in your notebook?
    - Meet as a team for 15 minutes before advisor meeting, share our research with each other, and narrow down our research to one or two research entries that are most important to our project that week
  - The expectation of 5 research entries a week is hard when we leave the research section of our projects
    - Team notebook check for testing
- Presentations
  - 402 presentations are more like formal industry design update and it is appreciated!
  - PDS went well
  - BME 201 makes it hard to be motivated because of how much time it takes to do things in class and still have to meet and do other reports outside of class.
  - Very valuable to get super technical comments for your advisor to make changes
- Make changes to PDS as you go!
  - Some advisors/ TAs look to see if the PDS is updated in other reports

Download

BSAC_2_14_25.pdf (73.5 kB)

Emily Rhine - Feb 28, 2025, 12:58 PM CST

BSAC Meeting_2/28/25

Small Group

- Machine training was easy
  - Intro to shop tools
  - Intro to machining
- Report requirements for 201
  - 2,000 words maximum for electronics report
  - Not fun to grade, but can go over without removing too many words
- Machine shop mills and lathes were really busy
- SolidWorks
  - Had to explore different tools and undo the work they had done
  - Harder to do on Mac
- 301 - student with first design experience was great
- 301 - getting feedback from 402s will be very beneficial
- 301s and 402s - show and tell
  - March 21st 12:05 - 2:05 pm
- Exchanged project advice
- Many people didn't fill out feedback fruits because they forgot
- New Journal Article format for 402s
  - An adjustment, but helpful to learn

Big Group

- Show and tell is right before spring break
  - Who will be there?
  - Can it be moved?
- Feedback Fruits
  - Posted late this year
- It's annoying that 430 and 315 are not offered every semester
  - Everyone needs it and it fills up quickly, so it can be hard to schedule
- Helpful Advising day links. Send this out?
  - https://[illegible].engineering.wisc.edu/biomedical-engineering/
  - https://docs.google.com/presentation/d/[illegible]
  - Go to BMES advising day?

[Download](#)

BSAC_Meeting_2_28_25.pdf (87.6 kB)

Graphing Spheroid Formation Results_2/27/25

Althys Cao - May 05, 2025, 8:07 PM CDT

Title: Graphing Spheroid Formation Results

Date: 2/27/25

Content by: Althys

Content: as coded on MATLAB

```
clear


% import result spreadsheet
data = load("6_condition_raw_sizes.mat");



%%
% clean up Nan from result
names = fieldnames(data);

for i = 1:length(names)
    name = names{i};
    vector = data.(name);
    data.(name) = vector(~isnan(vector));
end



% A - 50k, 0.75
% B - 50k, 1
% C - 50k, 1.25
% D - 75k, 0.75
% E - 75k, 1
% F - 75k, 1.25
```

```
% from now, use data from data variable

sizes_50k_075 = {data.A1 data.A2 data.A3 data.A4};
sizes_50k_1 = {data.B1 data.B2 data.B3 data.B4};
sizes_50k_125 = {data.C1 data.C2 data.C3 data.C4};
sizes_75k_075 = {data.D1 data.D2 data.D3};
sizes_75k_1 = {data.E1 data.E2 data.E3 data.E4};
sizes_75k_125 = {data.F1 data.F2 data.F3 data.F4};

counts_50k_075 = [length(data.A1) length(data.A2) length(data.A3) length(data.A4)];
counts_50k_1 = [length(data.B1) length(data.B2) length(data.B3) length(data.B4)];
counts_50k_125 = [length(data.C1) length(data.C2) length(data.C3) length(data.C4)];
counts_75k_075 = [length(data.D1) length(data.D2) length(data.D3)];
counts_75k_1 = [length(data.E1) length(data.E2) length(data.E3) length(data.E4)];
counts_75k_125 = [length(data.F1) length(data.F2) length(data.F3) length(data.F4)];


%% find mean and std of spheroid sizes of each individual well
mean_50k_075 = mean_list(sizes_50k_075);
mean_50k_1 = mean_list(sizes_50k_1);
mean_50k_125 = mean_list(sizes_50k_125);
mean_75k_075 = [mean(data.D1) mean(data.D2) mean(data.D3) 0];
mean_75k_1 = mean_list(sizes_75k_1);
mean_75k_125 = mean_list(sizes_75k_125);

std_50k_075 = std_list(sizes_50k_075);
std_50k_1 = std_list(sizes_50k_1);
std_50k_125 = std_list(sizes_50k_125);
std_75k_075 = [std(data.D1) std(data.D2) std(data.D3) 0];
std_75k_1 = std_list(sizes_75k_1);
std_75k_125 = std_list(sizes_75k_125);
```

```
% plot mean and std of spheroid sizes of each individual well
x = categorical({'50k, 0.75%', '50k, 1.0%', '50k, 1.25%', ...
    '75k, 0.75%', '75k, 1.0%', '75k, 1.25%'})
mean_grouped_individual_well = [mean_50k_075; mean_50k_1; mean_50k_125; mean_75k_075; mean_75k_1; mean_75k_125]
std_grouped_individual_well = [std_50k_075; std_50k_1;
    std_50k_125; std_75k_075; std_75k_1; std_75k_125];
b = bar(x, mean_grouped_individual_well);


ylim([0,10000])


legend('Well 1', 'Well 2', 'Well 3', 'Well 4')


hold on


[ngroups,nbars] = size(mean_grouped_individual_well);
x = nan(nbars, ngroups);
for i = 1:nbars
    x(i,:) = b(i).XEndPoints;
end


errorbar(x',mean_grouped_individual_well,std_grouped_individual_well,'k','linestyle','none')
legend('Well 1', 'Well 2', 'Well 3', 'Well 4');
ylabel('Spheroid Area \mum^2');
title('Average Spheroid Area of Each Well at Different Cell Density and Methylcellulose Concentration');
set(gca, 'Fontsize', 15);



hold off
```

```
%% graph spheroid counts
x = categorical({'0.75% methylcellulose', '1.0% methylcellulose', ...
    '1.25% methylcellulose'});
mean_counts = [mean(counts_50k_075) mean(counts_75k_075);
    mean(counts_50k_1) mean(counts_75k_1);
    mean(counts_50k_125) mean(counts_75k_125)];
std_counts = [std(counts_50k_075) std(counts_75k_075);
    std(counts_50k_1) std(counts_75k_1);
    std(counts_50k_125) std(counts_75k_125)];

b = bar(x,mean_counts);
ylim([0,170])

hold on

[ngroups,nbars] = size(mean_counts);
x = nan(nbars, ngroups);
for i = 1:nbars
    x(i,:) = b(i).XEndPoints;
end

errorbar(x',mean_counts,std_counts,'k','linestyle','none')
legend('50,000 cells/cm^2', '75,000 cells/cm^2');
ylabel('Spheroid Count');
title('Average Spheroid Count at Different Cell Density and Methylcellulose Concentration');
set(gca, 'Fontsize', 15);


hold off
```

```
%% graph spheroid sizes - group 4 wells together

sizes_50k_075_grouped = [data.A1' data.A2' data.A3' data.A4'];

sizes_50k_1_grouped = [data.B1' data.B2' data.B3' data.B4'];

sizes_50k_125_grouped = [data.C1' data.C2' data.C3' data.C4'];

sizes_75k_075_grouped = [data.D1' data.D2' data.D3'];

sizes_75k_1_grouped = [data.E1' data.E2' data.E3' data.E4'];

sizes_75k_125_grouped = [data.F1' data.F2' data.F3' data.F4'];


x = categorical({'0.75% methylcellulose', '1.0% methylcellulose', ...
    '1.25% methylcellulose'});
mean_sizes = [mean(sizes_50k_075_grouped) mean(sizes_75k_075_grouped);
    mean(sizes_50k_1_grouped) mean(sizes_75k_1_grouped);
    mean(sizes_50k_125_grouped) mean(sizes_75k_125_grouped)];
std_sizes = [std(sizes_50k_075_grouped) std(sizes_75k_075_grouped);
    std(sizes_50k_1_grouped) std(sizes_75k_1_grouped);
    std(sizes_50k_125_grouped) std(sizes_75k_125_grouped)];


b = bar(x,mean_sizes);
% ylim([0,2000])


hold on


[ngroups,nbars] = size(mean_sizes);
x = nan(nbars, ngroups);
for i = 1:nbars
    x(i,:) = b(i).XEndPoints;
end


errorbar(x',mean_sizes,std_sizes,'k','linestyle','none')
legend('50,000 cells/cm^2', '75,000 cells/cm^2');
ylabel('Spheroid Area \mum^2');
```

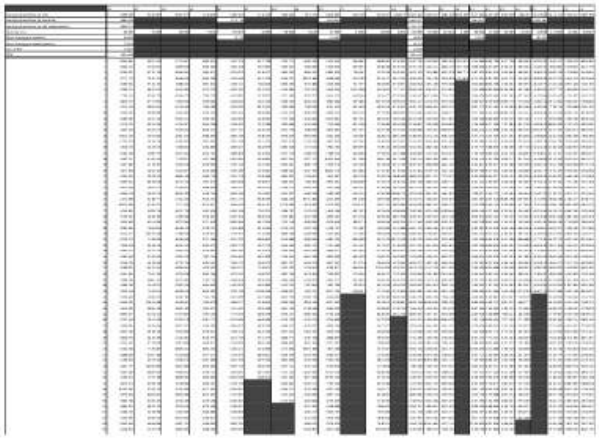
```
title('Average Spheroid Area at Different Cell Density and Methylcellulose Concentration');

set(gca, 'Fontsize', 15);



hold off

%%


function mlist = mean_list(cell_array) % input cell array of spheroid size to recieve mean of spheroid sizes of each well

    mlist = [];

    for i = 1:length(cell_array)

        mlist = [mlist mean(cell_array{i})];

    end

end



function stdlist = std_list(cell_array) % input cell array of spheroid size to recieve mean of spheroid sizes of each well

    stdlist = [];

    for i = 1:length(cell_array)

        stdlist = [stdlist std(cell_array{i})];

    end

end
```

Althys Cao - May 05, 2025, 8:07 PM CDT

Download

6_condition_raw_sizes.mat (15.4 kB)

Althys Cao - May 05, 2025, 8:07 PM CDT



Download

Spheroid_images_2_Results_and_Analysis.xlsx (83.8 kB)

Althys Cao - May 05, 2025, 8:07 PM CDT

```
clear

% import result spreadsheet
data = load("6_condition_raw_sizes.mat");

%%
% clean up keys from result
names = fieldnames(data);

for i = 1:length(names)
    name = names{i};
    vector = data.(name);
    data.(name) = vector(~isnan(vector));
end


% A - 50k, 0.75
% B - 50k, 1
% C - 50k, 1.25
% D - 75k, 0.75
% E - 75k, 1
% F - 75k, 1.25
% from now, use data from data variable

sizes_50k_075 = [data.A1 data.A2 data.A3 data.A4];
sizes_50k_1 = [data.B1 data.B2 data.B3 data.B4];
sizes_50k_125 = [data.C1 data.C2 data.C3 data.C4];
sizes_75k_075 = [data.D1 data.D2 data.D3];
sizes_75k_1 = [data.E1 data.E2 data.E3 data.E4];
sizes_75k_125 = [data.F1 data.F2 data.F3 data.F4];

counts_50k_075 = [length(data.A1) length(data.A2) length(data.A3)
length(data.A4)];
counts_50k_1 = [length(data.B1) length(data.B2) length(data.B3)
length(data.B4)];
counts_50k_125 = [length(data.C1) length(data.C2) length(data.C3)
length(data.C4)];
counts_75k_075 = [length(data.D1) length(data.D2) length(data.D3)];
counts_75k_1 = [length(data.E1) length(data.E2) length(data.E3)
length(data.E4)];
counts_75k_125 = [length(data.F1) length(data.F2) length(data.F3)
length(data.F4)];

%% find mean and std of spheroid sizes of each individual well
mean_50k_075 = mean_list(sizes_50k_075);
mean_50k_1 = mean_list(sizes_50k_1);
mean_50k_125 = mean_list(sizes_50k_125);
mean_75k_075 = [mean(data.D1) mean(data.D2) mean(data.D3) 0];
mean_75k_1 = mean_list(sizes_75k_1);
mean_75k_125 = mean_list(sizes_75k_125);

std_50k_075 = std_list(sizes_50k_075);
std_50k_1 = std_list(sizes_50k_1);
std_50k_125 = std_list(sizes_50k_125);
std_75k_075 = [std(data.D1) std(data.D2) std(data.D3) 0];
std_75k_1 = std_list(sizes_75k_1);
std_75k_125 = std_list(sizes_75k_125);

% plot mean and std of spheroid sizes of each individual well
x = categorical({'50k, 0.75k', '50k, 1.0k', '50k, 1.25k', ...
```

Download

untitled6.m (5.5 kB)

Spheroid Seeding - 24-well_3/5/25

Althys Cao - May 05, 2025, 8:00 PM CDT

Title: Spheroid Seeding Calculations for 24-well plates

Date: 3/5/25

Content by: Althys

Content:

3 / 5 / 25

name: Althys

topic: Cell seeding – 24 well

notes:

Area in 1 well: 1.9 cm^2

1 well, 15k, 0.75% cells/cm^2 mc
- total media: 900 µL
- 142 500 cells
- 338 µL 2% mc

3.8 mil cells enough for 26 wells

serum-free

18 wells

↳ scale up to (17)

- 4845 µL of 500,000 cells/mL media ← take 4.5 mL of left-over media (P2V4) → spin → resuspend in 4845 µL serum-free DMEM
- 5746 → 5750 µL of 2% mc
- 4705 µL serum-free DMEM

take 2.38 mL of left-over media (P2V4) → spin → resuspend in 2565 µL full DMEM

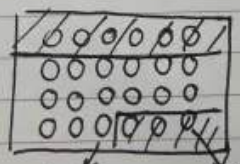
full DMEM

8 wells → scale up to (9)

- 2565 µL of 500,000 cells/mL media
- 3042 → 3045 µL 2% mc
- 2490 µL ~~serum-free~~ full DMEM

SPIN @ 200g, 5min

action items



☒ full DMEM

☐ serum-free

☆ last (smaller volume)

☆ last (same volume)

Spheroid Seeding - 6-well_4/2/25

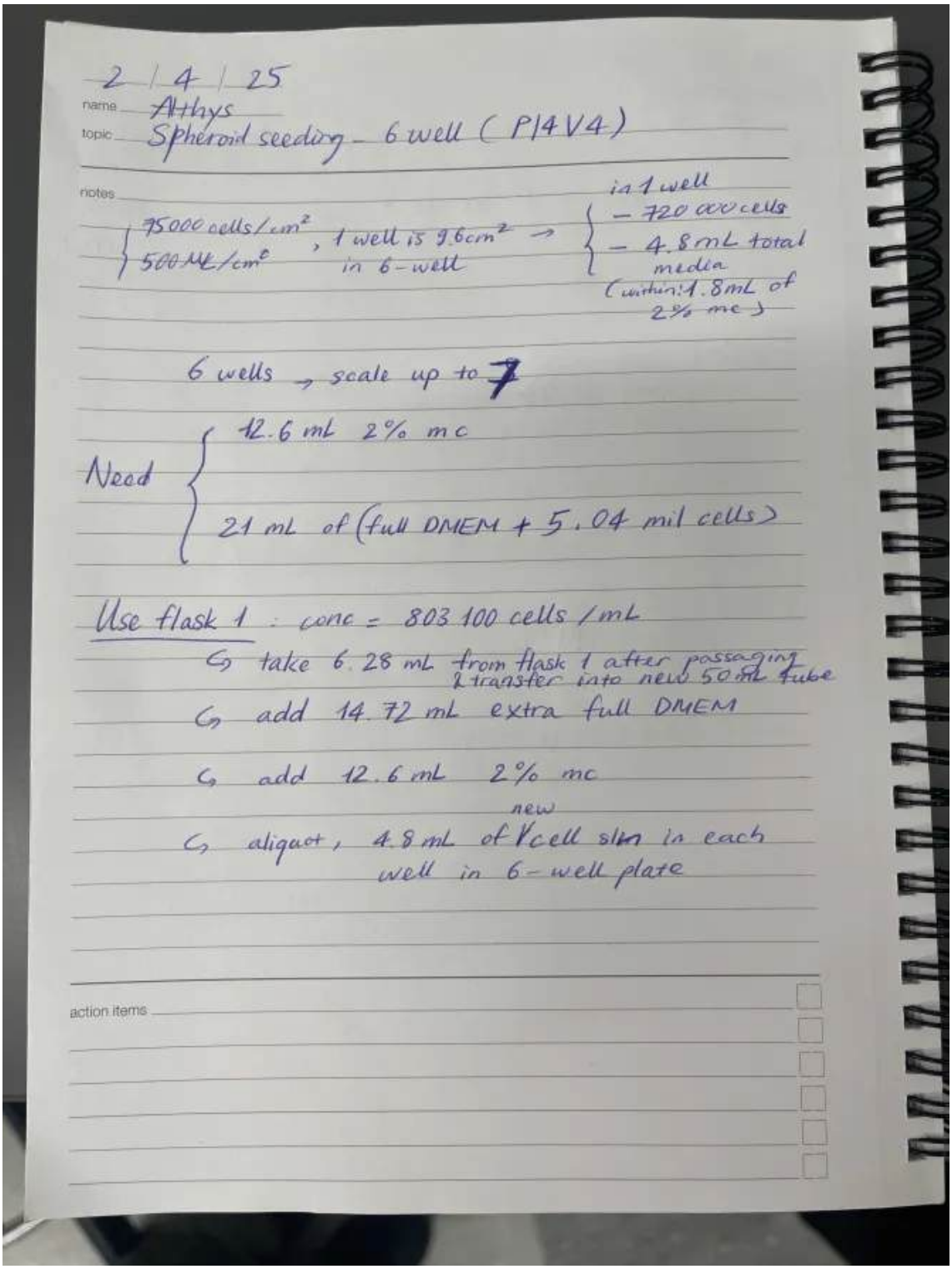
Althys Cao - May 05, 2025, 8:01 PM CDT

Title: Spheroid Seeding Calculations for 6-well Plates

Date: 4/2/25

Content by: Althys

Content:



2/4/25

name Althys

topic Spheroid seeding - 6 well (P14V4)

notes

75000 cells/cm² , 500 µL/cm² , 1 well is 9.6 cm² in 6-well → in 1 well: - 720 000 cells; - 4.8 mL total media (within 1.8 mL of 2% mc)

6 wells → scale up to 7

Need:
- 12.6 mL 2% mc
- 21 mL of (full DMEM + 5.04 mil cells)

Use flask 1 : conc = 803 100 cells / mL

↳ take 6.28 mL from flask 1 after passaging & transfer into new 50 mL tube

↳ add 14.72 mL extra full DMEM

↳ add 12.6 mL 2% mc

↳ aliquot, 4.8 mL of new Vcell slm in each well in 6-well plate

action items

2025/05/05 - Cell Culture Passage Number

ANA MARTINEZ - May 05, 2025, 1:54 PM CDT

Title: "Passage number of cancer cell lines: Importance, intricacies, and way-forward"

Source: [1] “Passage number of cancer cell lines: Importance, intricacies, and way-forward - Prasad - 2023 - Biotechnology and Bioengineering - Wiley Online Library.” Accessed: May 05, 2025. [Online]. Available: https://analyticalsciencejournals-onlinelibrary-wiley-com.ezproxy.library.wisc.edu/doi/full/10.1002/bit.28496

Date: 05/03/2025

Content by: Ana Martinez

Present: Ana Martinez

Goals: To research more about the possibility of effects seen in our yH2AX stain due to our passage number used.

Content:

- "Among cancer cells, the most promising models appear to be cancer cell lines that are generated from an early-stage and/or lower-grade disease. The condition of P53 (100%) and ERBB2 (93%) were two parameters that demonstrated good agreement in comparison studies between the primary tumor tissues, and the early-stage cell lines developed from these tumors (Burdall et al., **2003**).These findings indicate that these cells effectively replicate the characteristics of in vivo tumor cells, accurately emulating the original tumor."
- When cancer cells are cultured for extended periods, it might result in a loss of phenotypic characteristics and other molecular changes, including changes to several cellular pathways (Wistuba et al., **1998**). Typically, cancer cell lines exhibit significant gene amplifications, allelic loss, oncogene mutations, chromosomal rearrangements, and site-specific epigenetic abnormalities upon long-term culture."
- "Continuous subculturing over an extended period of time imposes selective pressure on cell line traits, leading to the dominance of faster-growing cells (or specific clones) over slower proliferating cells within the overall cell population. Moreover, long-term cultured cell lines may experience mutations that alter their original functional characteristics, potentially impacting cell lines from earlier passage levels."
- "Collectively, these studies highlight the need for caution when conducting experiments on cancer cell line models, emphasizing the significance of considering the passage number (PN) of the cells as a crucial factor for researchers."

Conclusions/action items: Hess lab has a hard "cut-off" for not using cells passaged past passage 20, but due to it being the end of the year we decided on using passage 19 and 20 cells rather than thawing a whole new flask. In the final report, I addressed this possible confounding variable by saying:

"Of note, A549 cells in passage 19 and 20 were used for staining 2D and 3D cells respectively, which could have impacted results observed due to characterized alterations and further deviation from original tumors seen with cells at high passage numbers."

However, it is important to note that most references to "high passage numbers" are typically higher than 20, so although we were getting close to the Hess Lab cutoff, we were likely not using very high passage number cells.

2025/05/05 - Established "High Amplification" Ct Values for RT-qPCR

ANA MARTINEZ - May 05, 2025, 2:05 PM CDT

Title: “The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments”

Original Source: [11] S. A. Bustin et al., “The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments,” Clinical Chemistry, vol. 55, no. 4, pp. 611–622, Apr. 2009, doi: 10.1373/clinchem.2008.112797.

Article that cited above source: [1] IslandPubDev518, “What is a Ct Value and does it differ from qPCR Cq Values?” Accessed: May 05, 2025. [Online]. Available: https://bitesizebio.com/24581/what-is-a-ct-value/

Date: 04/23/2025

Content by: Ana Martinez

Present: Ana Martinez

Goals: To confirm with established literature values that our Ct value obtained for our GAPDH control indicated high amplification.

Content:

- "Cq values are inverse to the amount of target nucleic acid in your sample and correlate to the number of target copies in your sample."
- "Lower Cq values (typically below 29 cycles) indicate high amounts of the target sequence. Higher Cq values (above 38 cycles) mean lower amounts of your target nucleic acid. High Cq values can also indicate problems with the target or the PCR set-up"
- "Real-time PCR records the amount of fluorescence emitted during the reaction where all PCR components are abundant. In this way, Cq values are usually consistent across replicates in real-time PCR. By the time the PCR reaction endpoint is reached, accumulated inhibitors, inactivated polymerases, and limiting reagents create a lot of variation in endpoint values, and this is why conventional PCR cannot be used quantitatively.

Conclusions/action items: From the given established threshold value of <29 for Ct values that indicate high amounts of target sequence, and given that our GAPDH Ct values for both 2D and 3D cells were below this threshold, we concluded that we had high amplification of GAPDH. I addressed this in the final report as follows:

"RT-qPCR results indicated that SOX2 was not amplified on either 2D or 3D conditions. Given that it is well established that Ct values below 29 indicate a high amount of target sequence, results indicated that GAPDH was sufficiently amplified on both 2D and 3D conditions (Figure 4). Similar GAPDH Ct values in this study indicated that genetic information was efficiently obtained in both conditions for the RT-qPCR assay, and thus the lack of SOX2 amplification was likely not due to errors in RNA extraction or cDNA synthesis."

2025/05/05 - Alternative Chemotherapy Drugs for yH2AX Stain

ANA MARTINEZ - May 05, 2025, 2:20 PM CDT

Title: "p21 promotes gemcitabine tolerance in A549 cells by inhibiting DNA damage and altering the cell cycle"

Source: [1] T. Fu *et al.*, “p21 promotes gemcitabine tolerance in A549 cells by inhibiting DNA damage and altering the cell cycle,” *Oncol Lett*, vol. 26, no. 5, p. 471, Sep. 2023, doi: 10.3892/ol.2023.14059.

Date: 05/04/2025

Content by: Ana Martinez

Present: Ana Martinez

Goals: To explore alternative chemotherapy drugs that can be used to induce DNA damage in A549 cells, in case a higher concentration of etoposide does not work in future yH2AX stains.

Content:

- "The reduction in p21 expression in A549/G+ cells promoted gemcitabine-induced accumulation of DNA damage and apoptosis, which may sensitize cells to gemcitabine."

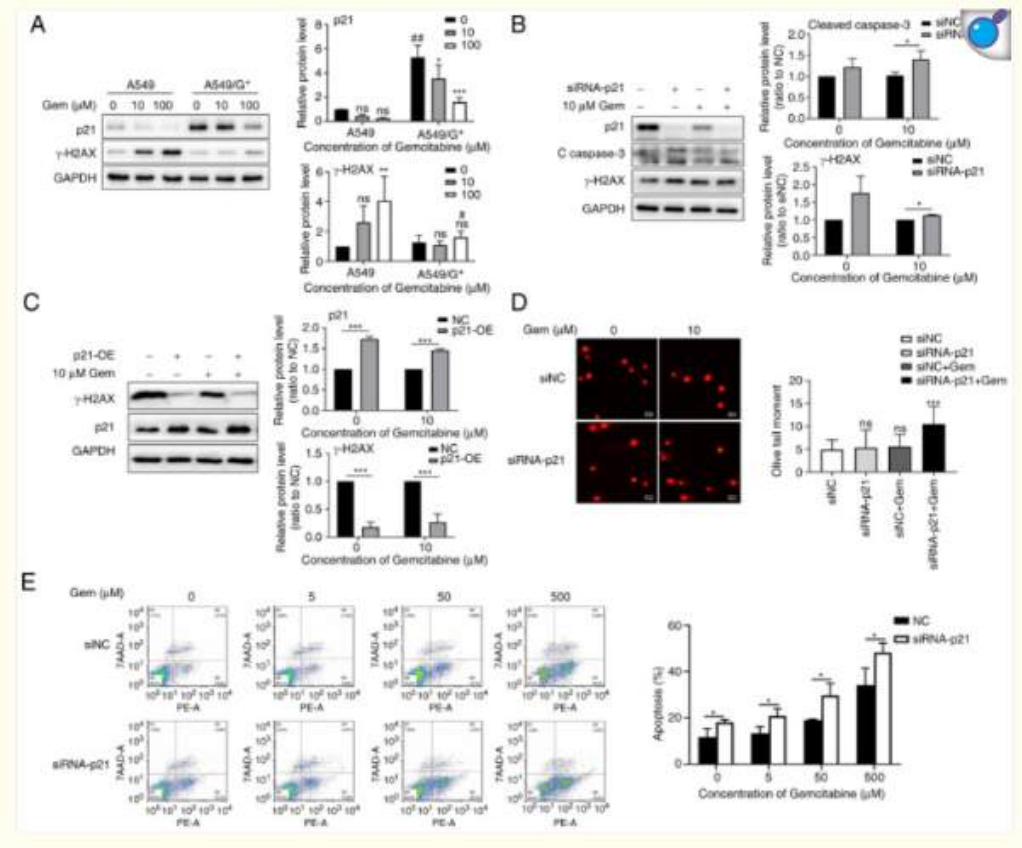


Figure Legend: Knockdown of p21 expression results in accumulation of unrepaired DSBs and induction of apoptosis by gemcitabine. (A) Western blotting detection of DNA double-strand replication-related protein (γ-H2AX) in A549 cells and A549/G+ cells coupled with various concentrations of gemcitabine (0, 10 and 100 μM). (B) Western blotting detection of apoptosis and DNA double-strand replication-related protein (γ-H2AX and cleaved caspase-3) in siNC- or siRNA-p21-transfected A549/G+ cells treated with different concentrations of gemcitabine (0 and 10 μM). (C) Western blotting detection of DNA double-strand damage was observed with p21 overexpression. (D) Representative images of the comet assay in A549/G+ cells treated with siRNA-p21 (or siNC) combined with gemcitabine. (E) Flow cytometric analysis of apoptosis in siNC- or siRNA-p21-transfected A549/G+ cells treated with various concentrations of gemcitabine (0.0, 5.0, 50 and 500 μM). Gem, gemcitabine; A549/G+, gemcitabine-resistant

A549; siRNA, small interfering RNA; NC, negative control; OE, overexpressed. *P<0.05, **P<0.01 and ***P<0.001 vs. respective controls, #P<0.05 and ##P<0.01 vs. the same drug concentration in A549 cells.

Conclusions/action items: This paper basically tells us that knockdown of p21 increases gemcitabine-induced DNA damage in A549 cells, though without this knockdown DNA damage was still induced. Thus, if a higher etoposide concentration does not work in future yH2AX studies, using gemcitabine (perhaps also in a higher concentration) may be an alternative solution.

2025/05/05 - Previous Work with Etoposide for yH2AX stain on A549s

ANA MARTINEZ - May 05, 2025, 9:16 PM CDT

Title: “Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay,”

Source: [1] F. K. Noubissi, A. A. McBride, H. G. Leppert, L. J. Millet, X. Wang, and S. M. Davern, “Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay,” Sci Rep, vol. 11, no. 1, p. 8945, Apr. 2021, doi: 10.1038/s41598-021-88296-3.

Date: 05/04/2025

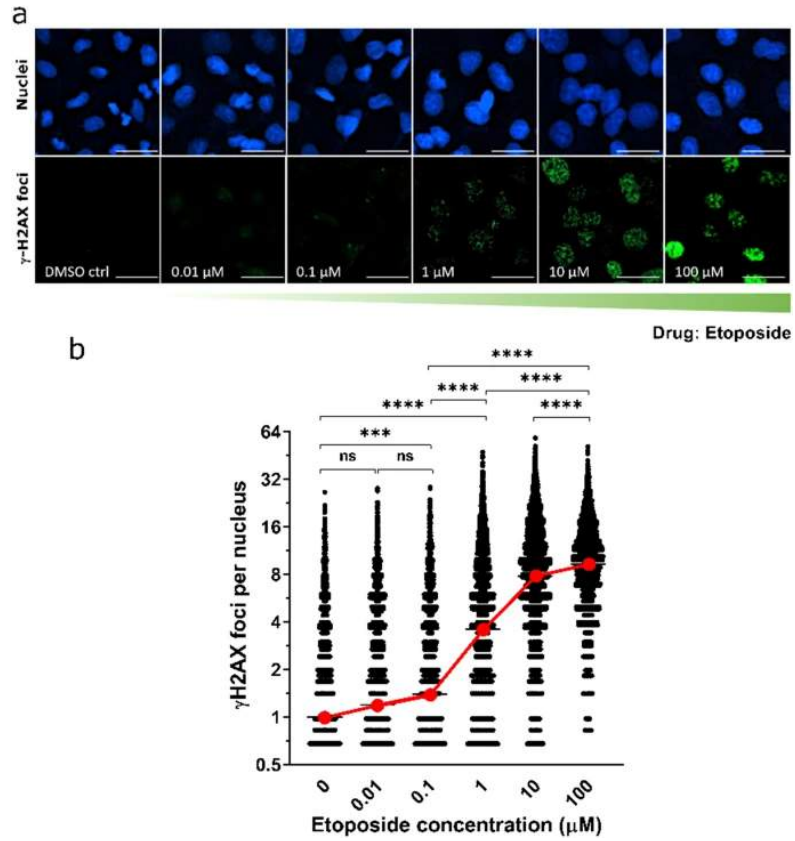
Content by: Ana Martinez, Emily Rhine

Present: Ana Martinez

Goals: To learn more about a previous study that similarly used etoposide to induce DNA damage in A549 cells and performed yH2AX staining.

Content:

- "A549 cells seeded at 10,000 or 20,000 cells per well (320 mm_2, 96 well plate) and grown for 24 h exhibited no significant response to 10 μM etoposide but showed an increased expression of γ-H2AX after exposure to 100 μM"
- "A549 cells of the same seeding density grown for 48 h showed a detectable increase in γ-H2AX expression upon exposure to both 10 and 100 μM etoposide"
- "An increased cell seeding density of 30,000 or 40,000 cells per well followed by 24 h growth, and subsequent etoposide treatment (10–100 μM, 1.5 h) yielded a statistically significant increase of γ-H2AX foci as reported in aggregate fluorescence intensity per well"
- "Cells grown for 48 h prior to etoposide treatment (10 μM and 100 μM) yielded a statistically significant increase in γ-H2AX foci with increasing cell seeding density"
- "There is a threshold of fluorescence intensity required to detect a signal. This fluorescence intensity threshold appears to correlate with cell density and the amount of DNA damage induced."



[1]

Figure legend: γ-H2AX foci of double-strand breaks quantified by automated spot counting algorithm. (a) Representative images of γ-H2AX foci in A549 cells treated with etoposide (1.5 h, 0–100 µM); γ-H2AX (green) and nuclear DNA stained with DAPI (blue). Scale bar is 20 µm. (b) Data summarizing Spot Count analysis and quantitation of individual γ-H2AX foci per nucleus (black) and average γ-H2AX foci per nucleus (red). The data are presented as mean ± standard deviation of 1500 nuclei per sample with four biological replicates, **P < 0.01. ***P < 0.001, ****P < 0.0001, one-way ANOVA. Average γ-H2AX for each concentration reveals a dose–response relationship (red line).

Conclusions & Action Items: For our yH2AX staining trial, we diluted the 16uM Etoposide in our protocol per the client's instructions. However, this study shows that, depending on seeding density, a minimum of 10 uM was required for yH2AX foci to appear. Thus, we should implement this study's protocol in future experiments to get a sufficient amount of etoposide-induced DNA damage in our 2D and 3D cells.

Emily Rhine-May 03, 2025, 7:52 PM CDT

Conclusions/action items:

CRISPR Screening in Cancer Spheroids (article 1)

JULIA SALITA - Sep 11, 2024, 11:54 AM CDT

Title: CRISPR Screening in Cancer Spheroids (article 1)

Date: 9/11/2024

Content by: Julia Salita

Present: Self

Goals: To understand CRISPR use with cancer spheroids

Content:

1. unclear which gene mutations are functional cancer drivers
    1. "have to scalably investigate these genes in relevant cancer models to assign causality and identify cancer specific vulnerabilities"
    2. have to look into the genes in cancer models to see what causes it and point out its weaknesses
2. In vivo and in vitro models are used to look at cancer but both have their limitations
    1. In Vivo (genetically engineered mouse models)
        1. summarizes' tumor growth and microenvironment
        2. limited by scale, time and cost
    2. In Vitro (Xenograft based models)
        1. limited in scale and manipulability
3. 2D cell culture has limitations in many disease features; ex: hypoxia, altered cell-cell contacts and rewired metabolism.
    1. in vitro organoid models lesson some of those missing pieces but can't be scaled like that
4. DepMap: use CRISPR screens to characterize cancer dependencies on a genome scale in hundreds of cell lines to reveal many (previously) undiscovered cancer drivers.
5. in article made scalable way to (re)produce lung cancer spheroids and use CRISR screens genome-wide in both 2D monolayers and 3D spheroids.

Conclusions/action items:

What is CRISPR screening

JULIA SALITA - Sep 11, 2024, 12:40 PM CDT

Title: What is CRISPR screening

Date: 9/11/2024

Content by: Julia Salita

Present: Self

Goals: To understand what CRISPR screening is an its subsequent parts.

Content:

1. What is CRISPR screening
    1. "CRISPR screening is a large-scale genetic loss-of-function Experimental approach designed to find the equivalent of a few needles in a haystack."
    2. helps the discovery of key genes or sequences that cause a certain function or phenotype for a cell type
    3. Hypothesis of CRISPR screening: "that there are a few genetic sequences or genes in the genome that have a certain physiological effect, ant that these few genetic sequences can be identified"
    4. Success in CRISPR screening is a list of genes or genetic sequence that potentially partake in producing the specific physiological effect that is being investigated
    5. starts with a broad hypothesis but ends with creating narrower hypotheses that need to be further investigated individually using other biological methods
    6. CRISPR is a method to cut double-strand DNA at targeted sites.
    7. When used in in genomic DNA in cells, the cells DNA repair system mends the cut, however the repair process is imprecise and results in mutations that knock out the targeted gene.
    8. The knockout event is what most scientists want to happen when they use CRISPR screening
2. CRISPR screening examples:
    1. identify genes or DNA sequences that cause cells to be resistant or sensitive to a drug
    2. identify genes of DNA sequences that affect susceptibility to environmental toxins
    3. identify components of cellular pathway
    4. identify genes of DNA sequences that lead to a particular disease state
3. How does CRISPR screening work?
    1. mostly done in cell culture
    2. basic idea: knock out every gene that could be important, but only one gene per cell
    3. the mixed cell population with different knocked out genes in each cell sit and some will die, some will live, and some may even excel in growth, becoming the predominant cell type.
    4. then Next generation sequencing (parallel sequencing) is performed to see which sequences are there and which are depleted (absent)
        1. high-throughput, rapid, and scalable sequencing alternative to first generation sanger sequencing. allows for sequence of millions of DNA fragments from hundreds of samples on a single sequencing run.
    5. identifies genetic sequence that are necessary for survival in normal conditions
    6. used to identify specific genes or sequenced that survive under special conditions (drug treatment of other physiological conditions of interest)
    7.

Conclusions/action items:

JULIA SALITA - Sep 12, 2024, 12:45 PM CDT

Title: What is CRISPR screening (continued)

Date: 9/12/2024

Content by: Julia Salita

Present: Self

Goals: To understand what CRISPR screening is an its subsequent parts.

Content:

1. What is CRISPR screening
    1. "CRISPR screening is a large-scale genetic loss-of-function Experimental approach designed to find the equivalent of a few needles in a haystack."
    2. helps the discovery of key genes or sequences that cause a certain function or phenotype for a cell type
    3. Hypothesis of CRISPR screening: "that there are a few genetic sequences or genes in the genome that have a certain physiological effect, ant that these few genetic sequences can be identified"
    4. Success in CRISPR screening is a list of genes or genetic sequence that potentially partake in producing the specific physiological effect that is being investigated
    5. starts with a broad hypothesis but ends with creating narrower hypotheses that need to be further investigated individually using other biological methods
    6. CRISPR is a method to cut double-strand DNA at targeted sites.
    7. When used in in genomic DNA in cells, the cells DNA repair system mends the cut, however the repair process is imprecise and results in mutations that knock out the targeted gene.
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5. identifies genetic sequence that are necessary for survival in normal conditions
6. used to identify specific genes or sequenced that survive under special conditions (drug treatment of other physiological conditions of interest)
7.

Conclusions/action items:

Spheroids as a Type of Three-Dimensional Cell Cultures—Examples of Methods of Preparation and the Most Important Application

JULIA SALITA - Sep 24, 2024, 1:21 PM CDT

Title: Spheroids as a Type of Three-Dimensional Cell Cultures—Examples of Methods of Preparation and the Most Important Application

Date: 9/24/2024

Content by: Julia Salita

Present: Self

Goals: to find a cell line that we can use to make into spheroids

Content:

1. 96-well NanoCulture plate
2. colon cancer cell lines HCT-116wt, HCT-116 wt/GFP and HCT-116 HRP EGFP

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7503223/#:~:text=3%2D%20and%206%2Dday%20spheroids,by%20spheroid%20formation%20%5B42%5D

Conclusions/action items:

1. Look more into these cell lines and their formation

colorectal carcinoma spheroids

JULIA SALITA - Sep 24, 2024, 1:56 PM CDT

Title: colorectal carcinoma spheroids

Date: 9/24/2024

Content by: Julia Salita

Present: self

Goals: to understand more about colorectal carcinoma spheroids, and see if they are a good option to pursue.

Content:

1. first link:
    1. HCT116, DLD-1 and SW620 were used in this article.
    2. spheroid formation capacity in ultra low attachment round bottom 96-well plates
2. Second Link:
    1. "Enrichment of CSC-related features in HT-29 and Caco-2 (for the first time without applying special scaffold/biochemical) spheroids, suggests spheroid culture as robust, reproducible, simple and cost-effective model to imitate the complexity of in vivo tumors including self-renewal, drug resistance and invasion for in vitro research of CRC-CSCs."- second link
        1. I think this would be a good thing to investigate more. It seems Colorectal carcinoma makes good spheroids.

https://www.nature.com/articles/s41598-018-19384-0#:~:text=Formation%20of%20colon%20cancer%20spheroids,round%20bottom%2096%2Dwell%20plates.

https://cancerci.biomedcentral.com/articles/10.1186/s12935-021-01898-9#:~:text=Relapse%20and%20metastasis%20in%20colorectal,detailed%20morphological%20and%20molecular%20characteristics.

https://www.eurekaselect.com/article/33210

https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/abs/10.1002/biot.200700228

https://onlinelibrary.wiley.com/doi/10.1111/cas.13155

https://link.springer.com/article/10.1186/s12943-017-0691-y

Conclusions/action items:

1. once again look into the specific properties of it
2. talk with group about helping find it/ understanding the wording of the articles

Mutation Research

JULIA SALITA - Nov 13, 2024, 10:34 AM CST

Title: Julia Salita

Date: 03- Oct- 2024 (added 13- Nov-2024)

Content by: Julia Salita

Present: Julia Salita

Goals: To understand common genetic mutations that may contribute to causing cancer

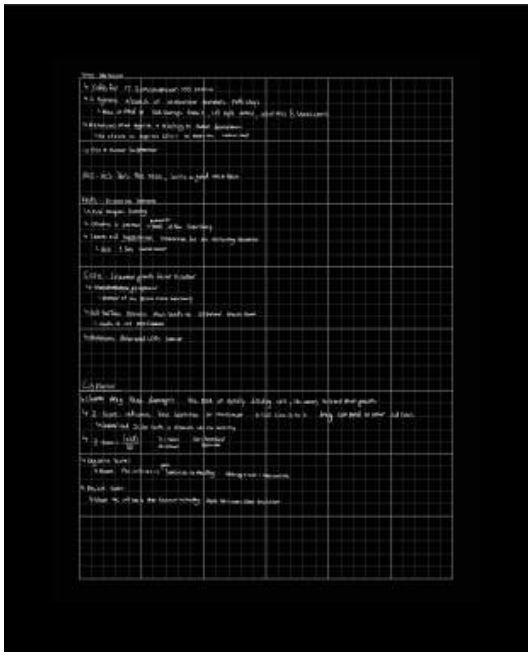
Content:

See notes attached below

Conclusions/action items:

1. add to preliminary presentation

JULIA SALITA - Nov 13, 2024, 10:42 AM CST



Download

Note_Oct_3_2024_1_.pdf (708 kB)

Understanding qPCR

JULIA SALITA - Nov 13, 2024, 10:39 AM CST

Title: Understanding qPCR as explained by our client

Date: 08-Nov-2024

Content by: Julia Salita

Present: Whole team

Goals: to understand how a qPCR works and what results/data would be produced

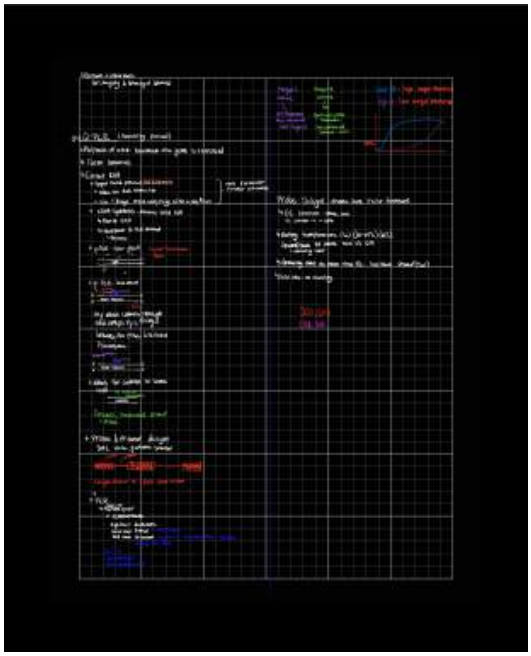
Content:

See notes below

Conclusions/action items:

1. Discuss with team on whether to proceed or not

JULIA SALITA - Nov 13, 2024, 10:43 AM CST



Download

Note_Nov_8_2024.pdf (1.08 MB)

Spheroid Formation Protocols

JULIA SALITA - Nov 16, 2024, 3:32 PM CST

Title: Spheroid formation using Ultra-Low Attachment Surface in MCF-7 Human Breast cancer cell line

Date: 11/16/2024 (inputted - no Idea when initially found)

Content by: Julia Salita

Present: Myself

Goals: To find a way to form spheroids using Ultra-Low Attachment plates

Content:

1. They didn't make the plates they used pre-made Ultra-Low Attachment Surface (Catalog number 3815_ but I couldn't find it) and TCT (430639) made by Corning.
2. Did a few other things with the cells as well but for spheroid:
    1. seeded onto an Ultra-Low Attachment surface with 384 wells, black with clear bottom microplate. with a density of 80,000 cells/cm^2 (0.49 mL/cm^2) and incubated for 96 hours (4 days).
    2. they did immunostaining with spheroids so they did that here, along with a few other things
        1. fixed and permeabilized for a few hours as well as exposed to other chemicals that are not as relevant for our project

Conclusions/action items:

1. could be good start for a seeding density and timing of incubation, but we would most likely need to make our own plates, because of money, and control over variables of concentrations in the coating.

JULIA SALITA - Nov 16, 2024, 3:32 PM CST


Corning® Ultra-Low Attachment Surface Promotes Spheroid Formation in MCF-7 Human Breast Cancer Cell Line

CORNING

SnAPPShots

Download

Corning_ULA_Surface_Promotes_Spheroid_Formatn_in_MCF-7.pdf (244 kB) Original article

JULIA SALITA - Nov 16, 2024, 3:51 PM CST

Title: Spheroid formation from Corning using multiple types of cell line including A549

Date: 16-Nov-2024 (input- originally found Oct 24th)

Content by: Julia Salita

Present: Whole team

Goals: to find a spheroid formation protocol to have a base for forming necessary spheroids

Content:

1. Culture and maintain as normal, using standard methods
    1. they used F-12K (Kaighn's Mod.) medium
2. Seeding densities used: 40, 200, 1,000, 5,000, and 10,000 cells/ 100uL of media per well (96-well spheroid microplates from Corning, Catalog number: 4520)
3. shperoids analyzed at 0, 24, 48, and 72 hours using a CEll Titer-Glo 3D cell viability assay
4. using same seeding method for all cell lines used:
    1. harvest cells, single cell suspension
    2. perform 5mL dilutions for each seeding density to seed eight wells per seeding density for each time point (96 well plate per cell line)
    3. add 100 uL of cell suspension to 8 wells per each concentration (add control by adding media without cells) avoiding scratching the well to not scrape the plate coating off
    4. incubate plate and evaluate ate specified hours

Conclusions/action items:

1. This seems like a good thing to maybe do but with just our cell line to see what is the best seeding density and time.

JULIA SALITA - Nov 16, 2024, 3:51 PM CST

Corning® Spheroid Microplates

Spheroid Formation Protocol

Download

CLS-AN-308.pdf (497 kB) Full article

JULIA SALITA - Nov 16, 2024, 3:52 PM CST

Title:

Date:

Content by:

Present:

Goals:

Content:

Conclusions/action items:

10/14/2024 WIMR Lab Visit- Cell Passage 1 - Copy

JULIA SALITA - Oct 14, 2024, 12:06 PM CDT

Title: Cell Passage 1

Date: 10/14/2024

Content by: Julia Salita

Present: Julia Salita and Jayson O'Halloran

Goals: To correctly passage our cells, get a better understanding of how to passage cells, learn to use the required equipment, and understand why each step of passaging cells is important.

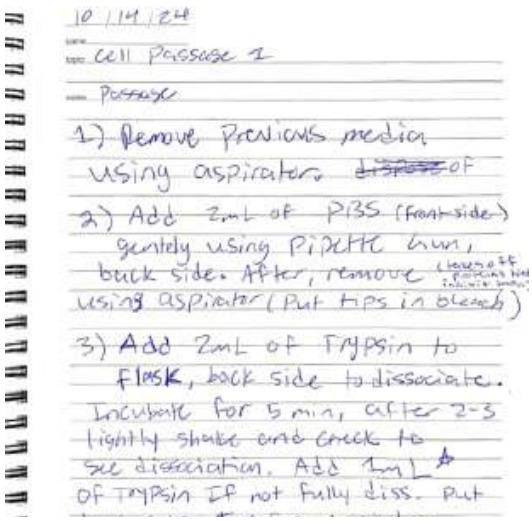
Content:

1. See file below for in lab notes
2. Start as normal: look at your cells get, everything ready, then aspirate the current media, use 2mL of PBS to wash the cells GENTLY, aspirate the PBS out, **you're supposed to add 2mL of trypsin but we needed 3mL**, then let sit in the incubator for about 3 minutes, then add 7 mL of D-MEM, and mix up and down with the serological pipet making sure to wash the back of the flask, take about 0.5 mL and put it in a small centrifuge tube and close it, then go to they cytoflex
3. Fill in table: Run the cytoflex and get the # of alive cells (# of events in the shape you make) and multiply by 100. This will give you cells per mL. We got 5309 which means there is 530,900 cells in 1mL which in 10 mL is 5,309,000 cells total in flask.
    1. We were at about 110% confluency (100% = 5,000,000 cells) with 5,309,000 cells.
4. Then we used a doubling time calculator (https://www.cellseeker.org/cellcalc/growth-rate-and-doubling-time/) to calculate that they had a doubling time of 28.2 hr.
    1. We cut back the cells to 1,000,000 cells, so we left a volume of 1.9 mL of the ~10mL solution of the current flask and added back in 8.1 mL of D-MEM for anew total of 10mL.
5. Then record the passage and put the cells back in the incubator.

Conclusions/action items:

1. Passage cells again on Wednesday October 16th.

JULIA SALITA - Oct 14, 2024, 12:06 PM CDT


10/14/24

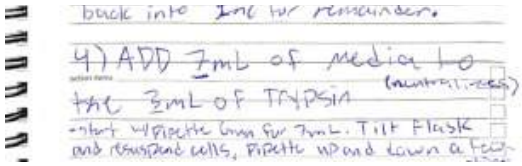
Cell Passage 1

Passage

1) Remove Previous media using aspirator ~~dispose~~ of

2) Add 2mL of PBS (front side) gently using pipette then, back side. After, remove using aspirator (Put tips in bleach)

3) Add 2mL of Trypsin to flask, back side to dissociate. Incubate for 5 min, after 2-3 lightly shake and check to see dissociation. Add 1mL of Trypsin If not fully diss. Put


back into 1mL for remainder.

4) ADD 7mL of Media to (neutralizes)
the 3mL of TRYPSIN
-start w/ pipette tip for 7mL. Tilt flask
and resuspend cells, Pipette up and down a few

Download

CamScanner_10-14-2024_.pdf (1.19 MB)

Passage Records

JULIA SALITA - Nov 13, 2024, 12:11 PM CST

Title: Passage Records

Date: Added 13-Nov-2024

Content by: Julia Salita

Present: Julia for all, Jayson for some

Goals: to passage and take care of our cell line

Content:

Performed 5 passages as of when inserted

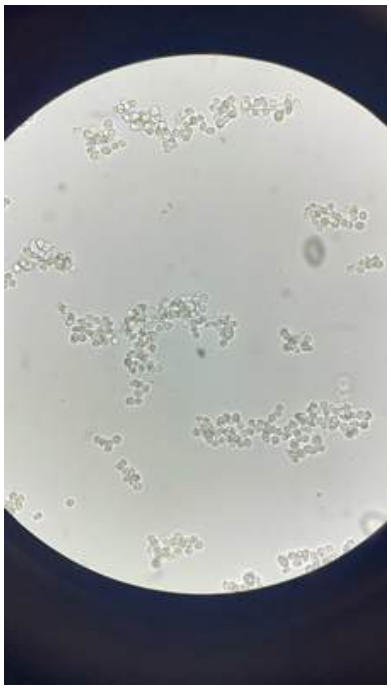
1. Passage 1: 14-Oct-2024 , No image
2. Passage 6: 25-Oct-2024 , No image
3. Passage 7: 28-Oct-2024
4. Passage 10: 04-Nov-2024
5. Passage 13: 11-Nov-2024

See some images below

Conclusions/action items:

Continue to passage.

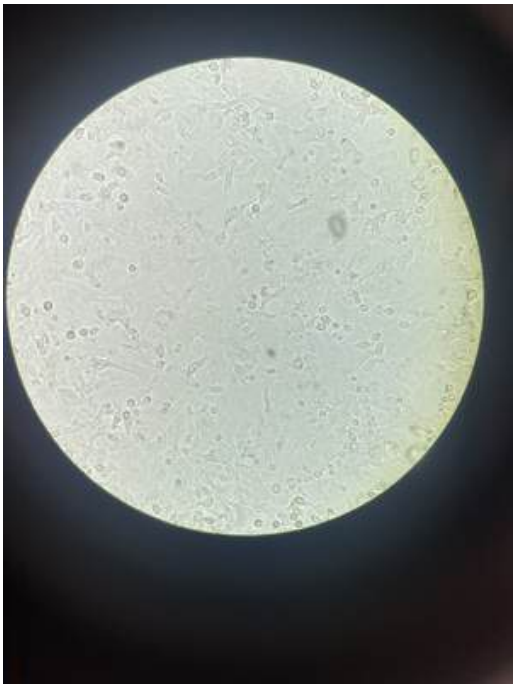
JULIA SALITA - Nov 13, 2024, 12:09 PM CST



Download

IMG_2702_1_.JPG (2.22 MB) Cells detached with trypsin during passage 7

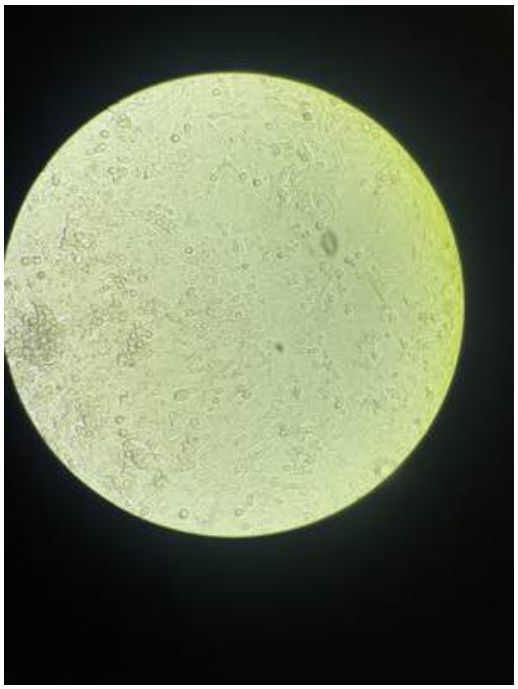
JULIA SALITA - Nov 13, 2024, 12:11 PM CST



Download

IMG_2788_1_.jpg (2.82 MB) Adhered cells before passage 10

JULIA SALITA - Nov 13, 2024, 12:13 PM CST



Download

IMG_2814_1_.jpg (3.01 MB) Adhered cells before passage 13

JULIA SALITA - May 06, 2025, 2:00 PM CDT

Title: Julia Salita

Date: Whole semester but added 5/6/2025

Content by: Julia Salita

Present: Various team members

Goals: to passage the 2D vials of A549

Content:

I performed multiple passages throughout the semester (didn't record every one)

I would usually save excess cells for experiments as well

I most likely did a passage every other week on average (as I would help with other things while someone else passaged.

Conclusions/action items:

Keep passaging and doing data analysis/ gathering data.

Data analysis

JULIA SALITA - May 05, 2025, 4:08 PM CDT

Title: Image data

Date: 5/5/2025

Content by: Julia Salita

Present: me

Goals: to analyze Spheroid images.

Content:

To begin, choose the most in-focus image (figure 8a) for the desired well and upload it to an image analysis tool named ImageJ. Measure the scale bar in the corner of the image using the line tool and measurement feature. Set the scale under ‘Analyze’ → ‘Set Scale’ by setting the ‘distance in pixels’ to the measured amount of pixels from the images scale bar length. Then set the known length to the scale bar length in µm, and changing the ‘Unit of length’ to read um in order to output results in micrometers. Select the ‘Global’ box to retain the scale bar incase of needing to restart the analysis process with an image, then select ‘OK’.

Starting image analysis, change the image type to a 16-bit under ‘Image’ → ‘Type’ → ‘16-bit’ in order to use the ‘Threshold’ feature. Next, select ‘Image’ → ‘Adjust’ → ‘Threshold’. Uncheck the box titled ‘Dark background’ if selected, then move the ranges until the desired section shows the spheroids highlighted in red, as seen in figure 8b, then hit ‘Apply’. Threshold recognizes pixels containing cells, or in this case spheroids. Next, go to ‘Process’ → ‘Binary’ → ‘Watershed’, which separates any spheroids that were close together but were recognized as one by the ‘Threshold’ feature. In order to block out any background noise from the edges of the well use the ellipse tool to encircle only the center of the well being analyzed (Figure 8c).

To analyze the highlighted spheroids click ‘Analyze’ → ‘Analyze Particles’. Set the size range to 4147-infinity, as to ensure only spheroids of 20 or more cells were being analyzed. In the dropdown menu under ‘Show:’ select ‘Outlines’ then underneath select the ‘Display results’, ‘Clear results’, and ‘Exclude on edges’ boxes before hitting ‘OK’.

To save the results one of two windows will pop up. 1. A window asking to save measurements or 2. A results window. Should option 1 appear, select save measurements, but if option 2 appears, select ‘File’ → ‘Save As’ then save results as an excel file to a secure and known location. Combine the excel files into one master document for data analysis.

Conclusions/action items:

run the results through excel to analyze

JULIA SALITA - May 05, 2025, 4:18 PM CDT

Title: Data Analysis

Date: 5/5/2025

Content by: Julia Salita

Present: Myself

Goals: to organize and understand the spheroid data

Content:

The results from the ImageJ analysis were compiled into one master document to further analyze. The average spheroid size and the average number of spheroids per cell seeding density and methylcellulose concentration were compared.

During the imaging process, the image for the 4th well for the 75k cells/cm^2, 0.75% methylcellulose concentration did not have good quality, resulting in the number of spheroids captured by ImageJ to be lower than actual. Thus, data regarding this well was excluded for data analysis as an outlier.

Regarding spheroid area, overall, the average spheroid area per well of each condition is similar among each other (Figure 2). The 50k cells/cm^2 seeding density had an average of 6006.751 µm^2 and the 75k cell seeding density had an average of 6265.328 µm^2 (Figure 3). The 0.75% methylcellulose concentration had an average of 6248.067 µm^2, the 1% methylcellulose concentration had an average of 5893.392 µm^2 , and the 1.25% methylcellulose concentration had an average of 6292.092 µm^2 (Figure 3).

50k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 6085.596 µm$^{2>}$ (Figure 3). 50k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 5721.736 µm^2 (Figure 3). 50k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 6250.038 µm^2 (Figure 3). 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 6410.537 µm^2 (Figure 3). 75k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 6065.047 µm^2 (Figure 3). 75k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 6334.146 µm^2 (Figure 3).

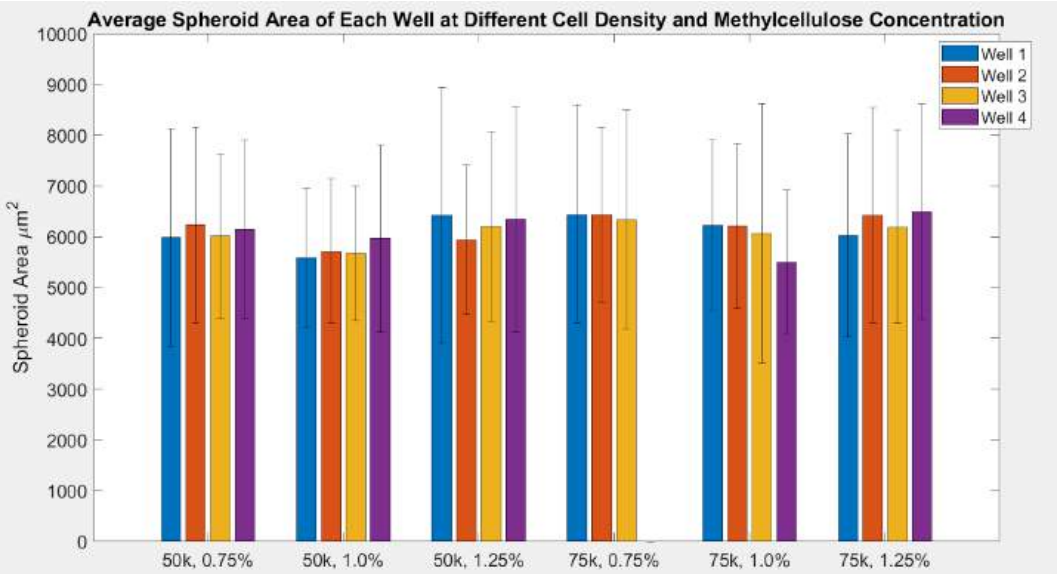


Figure 2. Average spheroid area of each well at different cell density and methylcellulose concentration. Error bars represent standard deviation. Condition notations are shortened: 50k and 75k stand for the corresponding cells/cm^2 seeding density; 0.75%, 1.0%, and 1.25% stand for the corresponding methylcellulose concentration. Each condition has 4 replications or 4 wells. Data for the 4th well of 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration is excluded.

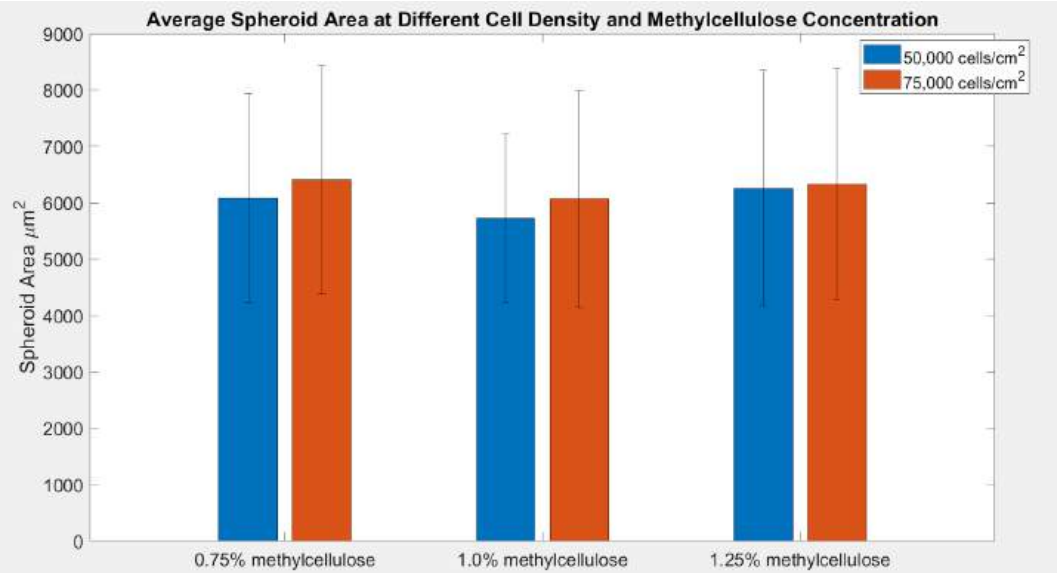


Figure 3. Average spheroid area of each different cell seeding density and methylcellulose concentration. Error bars represent standard deviation. Data for the 4th replicate of 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration is excluded.

Regarding spheroid count, the 50k cells/cm^2 seeding density had an average of 70.3 spheroids and the 75k cells/cm^2 seeding density had an average of 94.3 spheroids (Figure 4). The 0.75% methylcellulose concentration had an average of 89.3 spheroids, the 1% methylcellulose concentration had an average of 85.1 spheroids , and the 1.25% methylcellulose concentration had an average of 72.4 spheroids (Figure 4).

50k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 84.5 spheroids (Figure 4). 50k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 70.75 spheroids (Figure 4). 50k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 55.5 spheroids (Figure 4). 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had an average of 124 spheroids excluding the replicate with poor image quality (Figure 4). 75k cells/cm^2 seeding density and 1% methylcellulose concentration condition had an average of 99.5 spheroids (Figure 4). 75k cells/cm^2 seeding density and 1.25% methylcellulose concentration condition had an average of 89.3 spheroids (Figure 4).

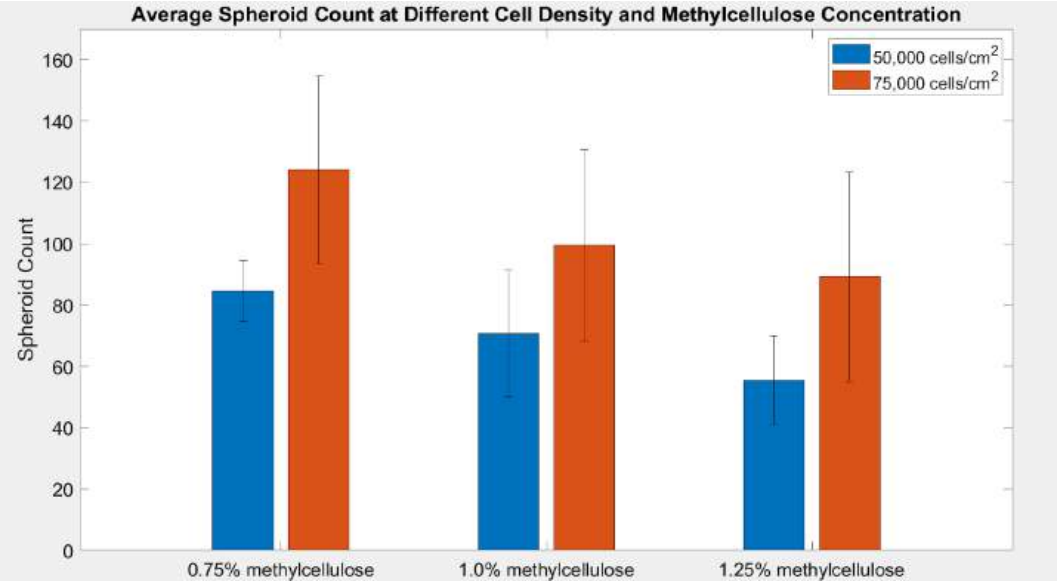


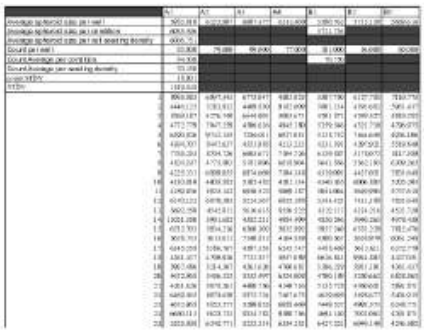
Figure 4. Average spheroid count of each different cell seeding density and methylcellulose concentration. Error bars represent standard deviation. Data for the 4th replicate of 75k cells/cm^2 seeding density and 0.75% methylcellulose concentration is excluded.

Conclusions/action items:

Continue on to qPCR and Gamma H2AX staining

Sorry for imputing this so late I just kept getting sidetracked by classes and lab work and would forget to add stuff to lab archives.

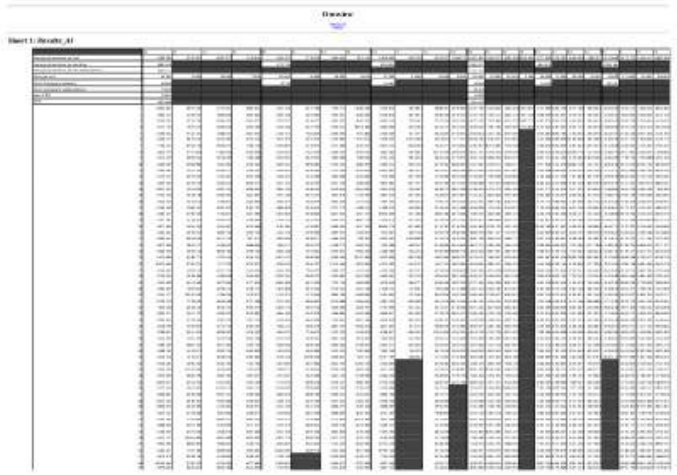
JULIA SALITA - May 06, 2025, 2:10 PM CDT



Download

Spheroid_images_2_Results_and_Analysis.xlsx_-_Results_A1.pdf (540 kB)

JULIA SALITA - May 06, 2025, 2:10 PM CDT



Download

Spheroid_images_2_Results_and_Analysis.xlsx (129 kB)

Poly HEMA Stock

JULIA SALITA - May 06, 2025, 1:57 PM CDT

Title: Making PolyHEMA stock solution

Date: entered 5/6/2025, made/completed 3 times throughout the semester

Content by: Julia Salita

Present: Myself

Goals: to make more Poly HEMA stock solution.

Content:

1. Grabbed 250 mL Erlenmeyer flask, poly(2-hydroxyethyl methacrylate) (poly-HEMA), 99% ethanol, a graduated cylinder, Aluminum foil, stir bar, sharpie, tape, weigh boat, a scale, and a hot plate with a magnetic stir bar.
2. next I placed the Erlenmeyer flask on the hot plate and placed the stir bar in the flask.
3. I then added 33mL of the ethanol to the flask.
4. I then heated the ethanol at around 40 degrees Celsius for a few minutes until sufficiently warm (not all the way to 40 but enough where it is no longer cold as it helps the polyHEMA dissolve better), also ensuring the stir bar is running and set to 100 RPM.
5. after turning the heat down to 37 degrees Celsius I then added 1.3 g of poly(2-hydroxyethyl methacrylate) (poly-HEMA) to the flask and centered it to ensure even stirring.
6. I then would cover the top of the flask with aluminum foil.
7. then I would label the flask with what it is, who it belonged to and how to get in contact should something go wrong. (with the tape and sharpie)
8. Finally I wrote to leave the solution stirring and heating overnight at 37 degrees, who it belonged to, and contact information.
9. Then I would clean up

Conclusions/action items:

1. Next was to place it in a bottle to store it and use it for the well plates then sterilize under UV for 30 minutes when using.

Luminescence

JULIA SALITA - May 06, 2025, 2:08 PM CDT

Title: Cell Titer Glo assay

Date: entered on 5/6 but completed earlier in the semester (February)

Content by: Julia Salita

Present: (for different days) Emily, Althys, and Ana

Goals: to perform the Cell Titer Glo assay and get the results

Content:

Performed cell titer glo assay as specified by the instructions then collected data in a spread sheet

from there I set out (for many hours) to normalize and graph the data to look at cell viability. In the end Althys ended up needed to help me analyze and graph the data (on fancy software).

The average luminescence results from he assay was normalized by subtracting the average luminescence of each condition from the average luminescence of just the media (gets rid of background noise)

this resulted in the 75K cells/cm^2 seeding density and 0.75% methylcellulose concentration condition had the highest luminescence, meaning it had the highest number of alive cells, or the highest cell viability.

Conclusions/action items:

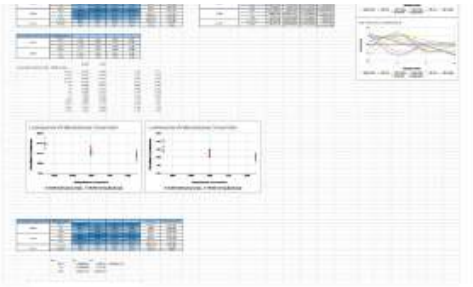
compare with spheroid data to choose optimal conditions for next experiments.

JULIA SALITA - May 06, 2025, 2:10 PM CDT

Download

Cell_Titer_Glo_results.xlsx (17.7 kB)

JULIA SALITA - May 06, 2025, 2:10 PM CDT



Download

Cell_Titer_Glo_results_-_Sheet1.pdf (285 kB)

Introductory Research I

JAYSON O'HALLORAN - May 04, 2025, 11:21 PM CDT

Title: Introductory Research I

Content by: Jayson O'Halloran

Goals: To gain an understanding of the field of CRISPR engineering

Resource: [1] D. Shriner , “What are genome editing and CRISPR-Cas9?: Medlineplus Genetics,” MedlinePlus, https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/ (accessed Sep. 9, 2024).

Content:

Genome Editing:

- Technologies that enable scientists to modify an organism's DNA.

- Allows addition, removal, or alteration of genetic material at specific genome locations.

- Includes various methods, with CRISPR-Cas9 being a prominent example.

CRISPR-Cas9:

- Stands for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9.

- Adapted from a bacterial immune defense mechanism.

- Bacteria use CRISPR arrays to remember and cut viral DNA using Cas9 enzyme.

- Researchers use guide RNA and Cas9 to target and cut specific DNA sequences in cells.

- Cas9 is commonly used, but other enzymes like Cpf1 can also be employed.

Applications and Potential:

- Used in research to understand diseases and for potential treatment.

- Explored for treating single-gene disorders (e.g., cystic fibrosis, hemophilia, sickle cell disease) and complex diseases (e.g., cancer, heart disease, HIV).

- Promises significant advancements in medicine.

Ethical Considerations:

- Most editing is done on somatic cells, which are not inherited by future generations.

- Editing germline cells or embryos can affect future generations and raises ethical issues.

- Concerns include potential use for enhancing traits and long-term impacts.

- Germline and embryo editing are illegal in the U.S. and many other countries due to ethical and safety concerns.

Conclusions

- In conclusion, CRISPR-Cas9 has emerged as a new tool in genetic engineering, providing scientists/researchers with precision and efficiency in editing DNA. Its ability to target specific genetic sequences and make precise modifications holds immense promise for advancing medical research, developing new treatments, and improving agricultural practices. However, the technology also brings significant ethical and safety considerations, particularly concerning off-target effects and germline editing. As more progress is gained in CRISPR, it is crucial to navigate potential ethical concerns carefully, ensuring that its applications are regulated and used responsibly to maximize benefits while minimizing risks.

Spheroid Research II

JAYSON O'HALLORAN - May 04, 2025, 11:26 PM CDT

Title: Spheroid Research II

Content by: Jayson O'Halloran

Goals: To gain an understanding of spheroids and their uses

Resource: [2] K. Białkowska, P. Komorowski, M. Bryszewska, and K. Miłowska, “Spheroids as a type of three-dimensional cell cultures-examples of methods of preparation and the most important application,” International journal of molecular sciences, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7503223/ (accessed Sep. 10, 2024).

Content:

Abstract

- Importance of Cell Cultures: Cell cultures are essential for testing materials, drugs, and understanding cell biology and mechanisms.

- Limitations of 2D Cultures: Traditional two-dimensional (2D) cultures, typically monolayers, do not accurately replicate the natural cell environment, lacking essential cell–cell and cell–extracellular matrix (ECM) interactions.

- Advantage of 3D Cultures: Three-dimensional (3D) cultures, particularly spheroids, provide a more realistic in vitro model that better mimics the complexity of living tissues.

- Spheroid Applications: Spheroids are utilized in drug testing, nanoparticle evaluation, and disease modeling, highlighting their versatility and relevance in R&D.

Introduction

- Cell Sourcing: Cells can be obtained directly from tissues or from established cell lines, which are adapted for laboratory use, allowing for controlled experimental conditions.

Spheroids as a Type of 3D Cell Culture

- Spheroids: Spheroids are defined as aggregates of cells that self-assemble in environments that prevent attachment to flat surfaces, allowing for more natural cell behavior.

Formation Process:

  - Initial Aggregation: Dispersed cells aggregate due to long-chain ECM fibers that contain RGD (arginine-glycine-aspartic acid) motifs, facilitating binding to cell-surface integrins.

  - Cadherin Expression: This binding leads to the upregulation of cadherin, a protein crucial for cell adhesion.

  - Tightening Connections: Hemophilic cadherin–cadherin interactions between neighboring cells strengthen the connections, resulting in the formation of stable spheroids.

- Role of Integrins: Integrins are involved in activating focal adhesion kinase (FAK), which is linked to tumor invasiveness and poor patient prognosis when overexpressed.

Methods of Spheroid Preparation

Hydrogels:

- Provide a versatile platform for controlled microtissue production, allowing for the creation of spheroids with specific shapes and sizes.

- Some cell lines require ECM proteins in the culture medium to form spheroids effectively.

Rotary Cell Cultures:

- Cells are cultured in bottles with agitators, preventing attachment and promoting aggregation.

- This method is simple and allows for large-scale spheroid production but has drawbacks, such as variability in spheroid size and potential mechanical damage to cells.

Microgravity Simulation

- A variation involves rotating flasks around a horizontal axis, simulating microgravity and minimizing hydrodynamic forces, leading to larger and more uniform spheroids.

- Hanging Drop Method: Cells are cultured in droplets suspended by surface tension, allowing them to aggregate without a solid substrate.

- Microfluidic Systems: These systems provide precise control over the microenvironment, enhancing spheroid viability and homogeneity.

Applications of Spheroids

- Phenotypic Stability: Spheroids maintain their phenotypic characteristics over time, making them reliable for long-term studies.

- Co-culture Capabilities: Primary human hepatocytes (PHH) can be co-cultured with non-parenchymal cells (e.g., Kupffer cells), enhancing their viability and mimicking liver tissue interactions.

- Proteome Analysis: Studies show that spheroids exhibit fewer changes in protein expression compared to 2D cultures, indicating a more stable cellular environment. After 24 hours, 457 proteins showed altered expression in 2D cultures, while spheroids demonstrated significantly fewer changes.

- Inter-individual Differences: Spheroids retain the unique characteristics of the donor liver tissue, which is a driver for personalized medicine and understanding individual responses to drugs.

- Functional Studies: Spheroids maintain hepatocyte-specific functions, such as stable albumin secretion, over prolonged culture periods.

- They are effective models for studying liver pathologies, such as cholestasis (indicated by bile acid accumulation) and steatosis (indicated by increased neutral lipids), facilitating drug screening and understanding disease mechanisms.

Conclusions/action items:

- In conclusion, I found this paper incredibly helpful for developing a background in spheroid creation. It clearly outlines various 3D cell culture techniques and explains why they are better than traditional 2D methods, which often don't mimic the natural environment of cells. The insights on different spheroid formation methods, like using hydrogels and

microfluidic systems, are particularly useful for our project. I also appreciate the focus on extracellular matrix components and cell interactions, as these factors are necessary for achieving consistent and viable spheroids.

γH2AX Research III

JAYSON O'HALLORAN - May 04, 2025, 11:25 PM CDT

Title: γH2AX Research III

Content by: Jayson O'Halloran

Goals: To gain an understanding of γH2AX and its properties as a biomarker

Resource: [3] A. Ivashkevich, C. E. Redon, A. J. Nakamura, R. F. Martin, and O. A. Martin, “Use of the γ-H2AX assay to monitor DNA damage and Repair in translational cancer research,” Cancer letters, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3329565/ (accessed Sep. 11, 2024).

Content: Information regarding γH2AX from the article above

Biomarker for DNA Damage: The formation of γH2AX in response to DNA double-strand breaks (DSBs) provides the basis for a sensitive assay of DNA damage in human biopsies. This assay is particularly useful for monitoring the clinical response to DNA-targeted therapies such as chemotherapy and radiation therapy [T4].

Clinical Trials and Monitoring: Since its discovery, γH2AX has been extensively used in basic research, but its application as a pharmacodynamic marker in clinical studies is a relatively recent development. In the last four years, there has been a sharp increase in its use to monitor chemotherapy-induced DNA damage in cancer patients, with more than 35 clinical trials utilizing γH2AX for evaluation of drug response [T1].

Sensitivity and Methodology: The assay can be performed using various techniques, including immunoblotting and enzyme-linked immunosorbent assay (ELISA), but microscopy is preferred for clinical samples due to its ability to detect large numbers of γH2AX molecules at DNA break sites, creating bright foci that allow for sensitive detection of individual DSBs [T2]. Microscopy also provides valuable information about the extent of DNA damage and the differential response in tumor tissues [T2].

Assessment of Treatment Efficacy: The γH2AX assay has been applied to monitor the formation and persistence of DNA damage in human cancer cells. For instance, studies have shown that γH2AX levels increase in cultured tumor cells, confirming the genotoxic effect of therapies such as radioimmunotherapy [T3]. This monitoring can help assess the anti-tumor efficacy of treatments and guide clinical decisions.

Radiosensitivity and Individualized Treatment: Another important application of the γH2AX assay is the assessment of radiosensitivity in prospective patients. Monitoring normal tissue response in parallel with clinical tumor outcomes could form a basis for routine monitoring of individual radiosensitivity to therapy [T3].

High Throughput and Automation: Recent developments in automation have led to the creation of high throughput analysis platforms, such as the Rapid Automated Bio dosimetry Tool (RABiT), which is designed for high throughput bio dosimetry and can handle large numbers of samples for dosimetry screening in response to radiation incidents. Such systems are adaptable to routine clinical investigations [T1].

Conclusions/action items:

- In conclusion, the γH2AX assay is a powerful and sensitive tool for detecting DNA damage in cells. When it comes to screening for gamma-H2AX (γH2AX), cells or tissue samples are first fixed to preserve their structure. They are then permeabilized to allow antibodies to enter. After blocking non-specific binding, the samples are incubated with an anti-γH2AX primary antibody, followed by a fluorescently labeled secondary antibody. The samples are then mounted and

examined under a fluorescence or confocal microscope to detect and quantify γH2AX foci, which indicate DNA double-strand breaks. (Screening for our project)

Cell Line Research IV

JAYSON O'HALLORAN - May 04, 2025, 11:26 PM CDT

Title: Cell Line Research IV

Content by: Jayson O'Halloran

Goals: To gain an understanding of cell lines used in modeling lung cancer.

Resource: [4] A. F. Gazdar, L. Girard, W. W. Lockwood, W. L. Lam, and J. D. Minna, “Lung cancer cell lines as tools for Biomedical Discovery and Research,” Journal of the National Cancer Institute, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2935474/ (accessed Sep. 15, 2024).

Content:

- The role of tumor cell lines in understanding the molecular biology of lung cancer and the ability to translate these findings to clinical applications would have been severely hampered and delayed without the availability of cell lines.

- Ongoing debate exists regarding the relevance of cell lines and in vitro models for cancer research.

- Cell lines typically maintain the expression of most "hallmarks of cancer," except for angiogenesis, which requires stromal tissues.

- Genomic instability in cancer leads to various genetic and epigenetic changes, including driver and passenger mutations.

- Lung cancer cell lines help distinguish driver mutations from passenger mutations through functionality tests, which are challenging in tumor tissues.

- Key contributions include the identification of TP53 mutations and insights into copy number variations and allele-specific imbalances.

- Initial cytogenetic observations in small cell lung cancer (SCLC) lines revealed frequent deletions on chromosome 3, leading to the discovery of tumor suppressor genes like RASSF1A and FUS1.

- MYC amplification was observed in SCLC lines, influencing the understanding of NMYC and the role of MYC family members in lung cancer.

- High-density genomic techniques have identified recurrent gains and losses in the lung cancer genome, with significant overlap between cell lines and tumors.

- Lung cancer cell lines revealed the role of RB in SCLC pathogenesis and established a model for tumor suppressor pathways.

- The LKB1 gene is frequently mutated in non-small cell lung cancer (NSCLC) and located at a common loss site in cell lines.

- The TITF1 gene, a master transcription factor, was identified as an oncogene frequently amplified in lung cancer.

- Activating mutations in the EGFR kinase domain were discovered through cell line studies, establishing a link to sensitivity to tyrosine kinase inhibitors.

- Cell lines have contributed to understanding both intrinsic and acquired resistance to EGFR-targeted therapies.

yH2AX

- DNA Damage Response: γH2AX acts as an early marker for double-strand breaks (DSBs), indicating activation of DNA repair mechanisms in lung cancer cells.

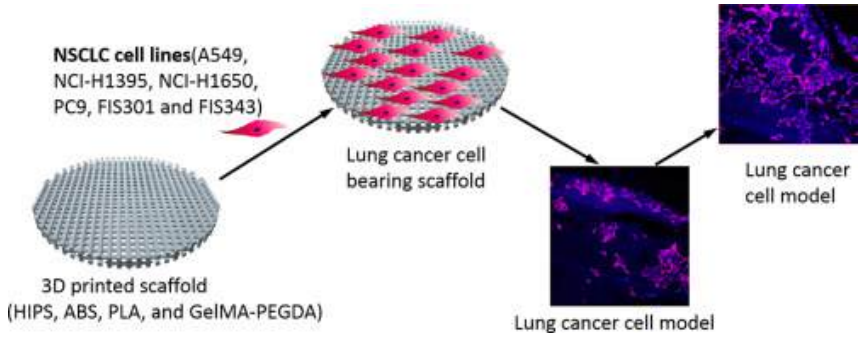
- Genomic Instability: Elevated γH2AX levels in lung cancer cell lines suggest genomic instability, a key hallmark of cancer.

- Evaluating Treatment Efficacy: Measuring γH2AX can help assess the effectiveness of radiotherapy and chemotherapy in inducing DNA damage in lung cancer cells.

- Understanding Resistance Mechanisms: Analyzing γH2AX dynamics provides insights into how lung cancer cells may develop resistance to therapies through unresolved DNA damage.

- Pathway Insights: Correlating γH2AX accumulation with mutations in key genes (e.g., TP53, LKB1) offers insights into the molecular pathways involved in lung cancer progression and treatment response.

Image of possible route for future model of spheroids being held together on a scaffold.



Conclusions/action items:

- Client meeting

- Advisor Meeting

- More research

Cancer Cell Lines Research V

JAYSON O'HALLORAN - May 04, 2025, 11:27 PM CDT

Title: Cancer Cell Lines Research V

Content by: Jayson O'Halloran

Goals: To gain an understanding of the different cell lines in cancer research

Resource: [5] R. Sinha, A. Luna, N. Schultz, and C. Sander, “A pan-cancer survey of cell line tumor similarity by feature-weighted molecular profiles,” Cell reports methods, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9017219/ (accessed Sep. 18, 2024).

Content:

1. Immortalized Cancer Cell Lines

Description: These are cell lines derived from patient tumors that have been modified to proliferate indefinitely in vitro.

Pros:

Availability: "Immortalized cancer cell lines, derived from patient tumors and grown and maintained in vitro, are the most commonly used experimental model in cancer research" .

Consistency: They provide a stable environment for experiments, allowing for reproducibility.

Historical Data: Extensive background data available for many lines facilitates comparative studies.

Cons:

Genetic Drift: "Cell lines generally have more genomic alterations than primary tumors" .

Limited Heterogeneity: "Cell lines typically do not represent all subtypes of cancers nor do they reflect tumor heterogeneity" .

2. Patient-Derived Xenografts (PDXs)

Description: Tumor tissues from patients are implanted into immunocompromised mice, allowing for the growth of human tumors in a living organism.

Pros:

Tumor Microenvironment: "Retain the original tumor microenvironment, providing a more accurate model of human cancer."

Genetic Fidelity: "Better preserve the genetic and phenotypic characteristics of the original tumor."

Cons:

Time-Consuming: Establishing PDX models can take a long time and requires significant resources.

Variability: Differences in host responses can lead to variability in tumor behavior.

3. Organotypic Cultures

Description: These cultures maintain the architecture and cellular composition of the original tissue.

Pros:

Physiological Relevance: "More closely mimic the in vivo environment compared to traditional cell lines."

Cell-Cell Interactions: "Preserve important interactions between different cell types within the tumor."

Cons:

Complexity: More complex to establish and maintain than standard cell lines.

Limited Lifespan: Often have a shorter lifespan and may not be as amenable to long-term studies.

4. Stem Cell-Derived Lines

Description: These lines are derived from cancer stem cells, which are thought to drive tumor growth and metastasis.

Pros:

Heterogeneity: "Capture the diversity of cancer cell populations, including those that are resistant to treatment."

Targeting Cancer Stem Cells: Useful for studying therapies aimed at eradicating cancer stem cells.

Cons:

Isolation Challenges: Difficult to isolate and maintain in culture.

Variability: May exhibit significant variability in behavior and response to treatments.

5. Established Cell Lines (e.g., HeLa, A375)

Description: Well-characterized cell lines that have been used extensively in research.

Pros:

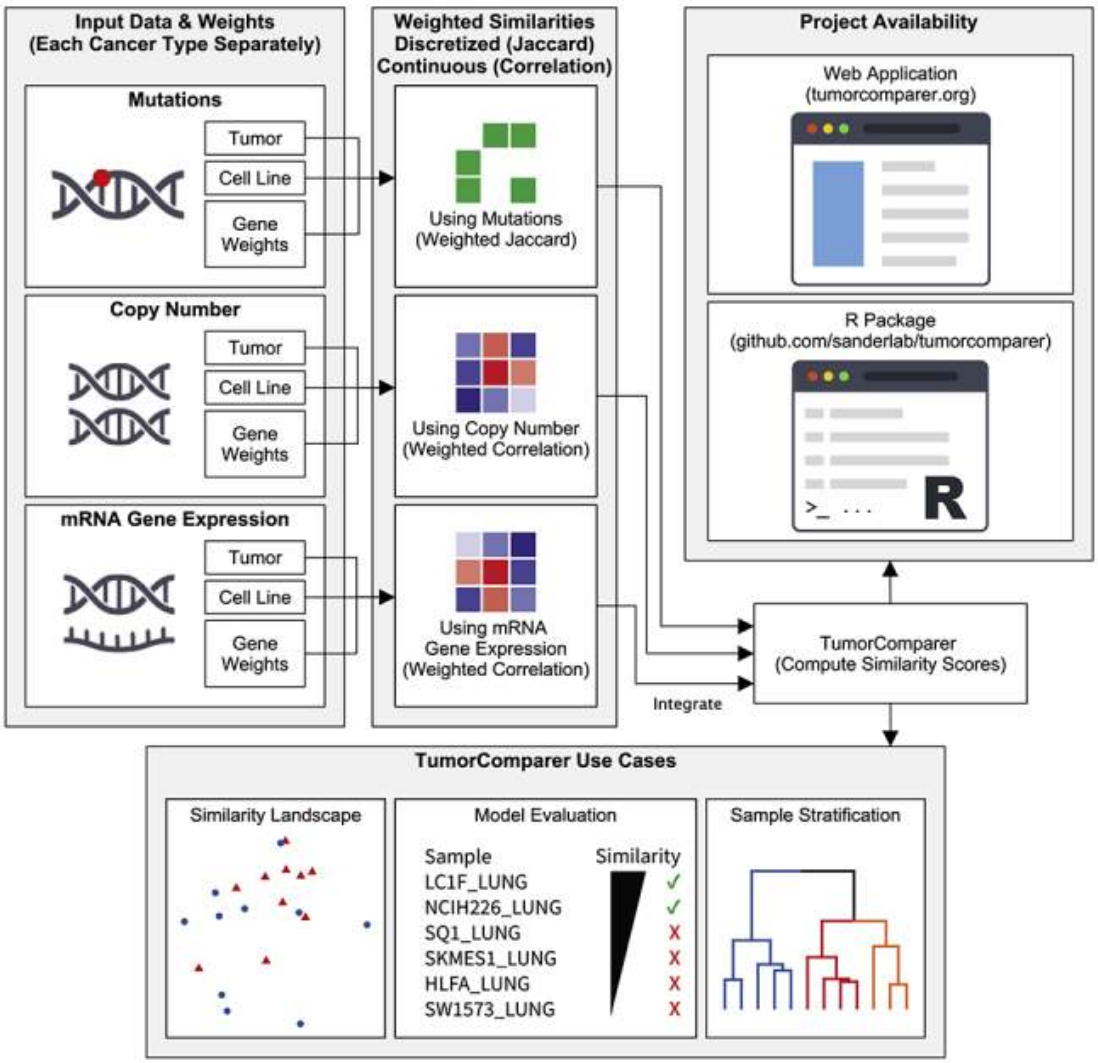
Extensive Literature: "A wealth of information and protocols available for these lines" .

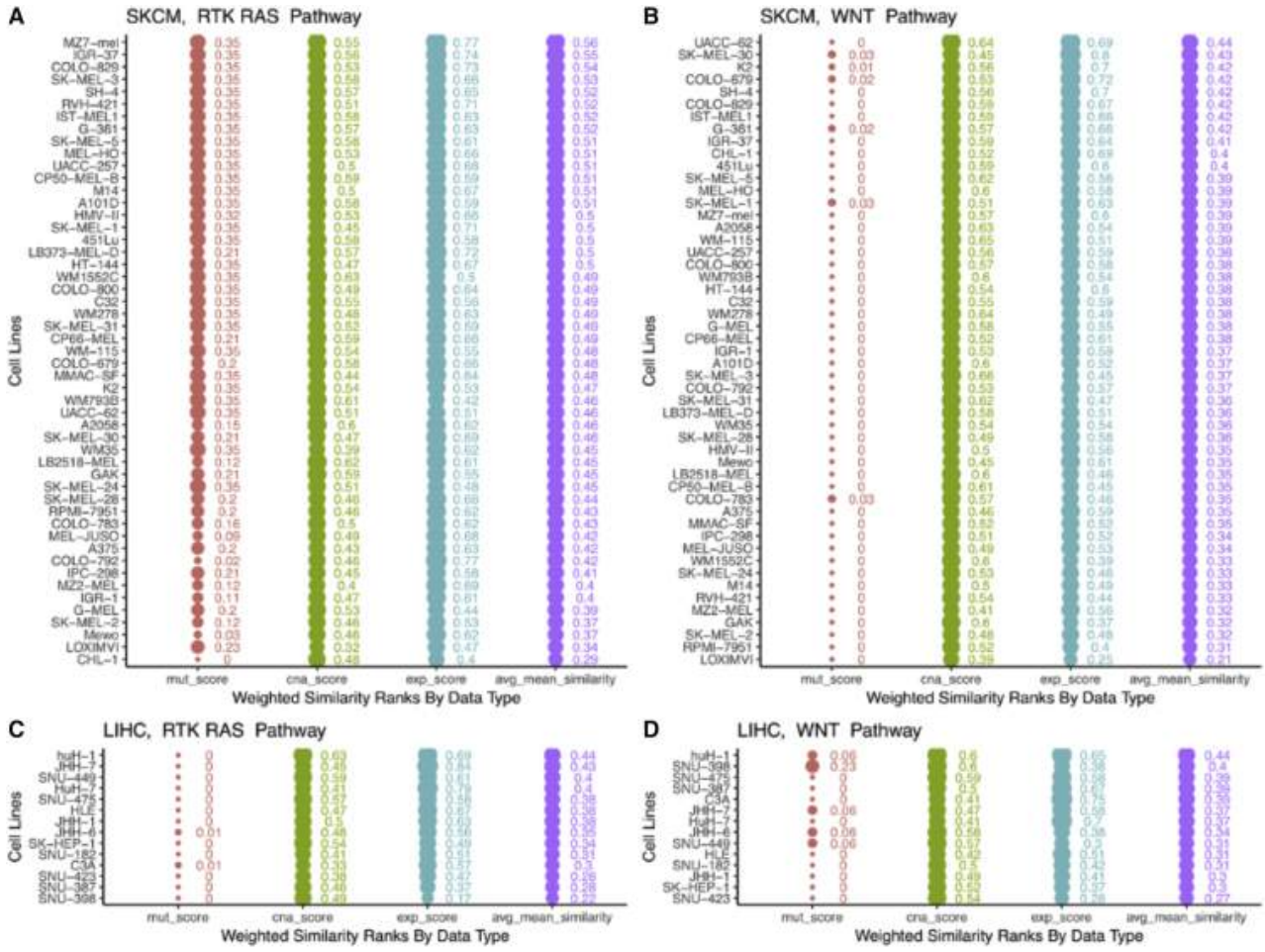
Ease of Use: Generally easy to culture and manipulate genetically.

Cons:

Misidentification: "The issue of misidentification of cell lines is an important and well-known challenge in the field" .

Limited Relevance: "The widespread use of cell lines with a low genomic resemblance to tumors is concerning"


Input Data & Weights
(Each Cancer Type Separately)
Mutations
Tumor
Cell Line
Gene Weights
Copy Number
Tumor
Cell Line
Gene Weights
mRNA Gene Expression
Tumor
Cell Line
Gene Weights
Weighted Similarities
Discretized (Jaccard)
Continuous (Correlation)
Using Mutations
(Weighted Jaccard)
Using Copy Number
(Weighted Correlation)
Using mRNA
Gene Expression
(Weighted Correlation)
Integrate
Project Availability
Web Application
(tumorcomparer.org)
R Package
(github.com/sanderlab/tumorcomparer)
TumorComparer
(Compute Similarity Scores)
TumorComparer Use Cases
Similarity Landscape
Model Evaluation
Sample
Similarity
LC1F_LUNG
NCIH226_LUNG
SQ1_LUNG
SKMES1_LUNG
HLFA_LUNG
SW1573_LUNG
Sample Stratification



The similarity of cell lines and tumors varies by gene set—the best matches might be quite different for different gene sets/pathways

The top two panels show the similarity scores of SKCM tumors and melanoma cell lines when using uniform weights on all features, and genes from (A) RTK-RAS pathway and (B) WNT pathway. Similarly, the bottom two panels (C and D) show corresponding scores for liver cancer cell lines, compared with TCGA LIHC tumors. SKCM cell lines show similar/better similarity scores when using the RTK-RAS pathway than the WNT pathway, whereas LIHC cell lines show lower scores with the RTK-RAS pathway than with the WNT pathway—this is consistent with the frequency of alterations in the member genes of the RTK-RAS and WNT pathways in these cancer types.

Conclusions/action items:

- N/A

A549 Cell Line Research VI

JAYSON O'HALLORAN - May 04, 2025, 11:27 PM CDT

Title: A549 Cell Line Research VI

Content by: Jayson O'Halloran

Goals: To gain an understanding of the A549 cell line and how it can be used in our project.

Resource: [6] “Everything You Need to Know About A549 Cells ,” Synthego A549 Cells , https://www.synthego.com/a549-cells (accessed Sep. 21, 2024).

Content:

A549 Cells: Origin And General Information

- A549 is the most commonly used human non-small cell lung cancer cell line for both basic research and drug discovery.

- A549 Cell Line Origin: The A549 cell line consists of hypotriploid alveolar basal epithelial cells. This cell line was first developed by D. J. Giard et al. in 1972 by removing and culturing pulmonary carcinoma tissue from the explanted tumor of a 58-year-old Caucasian male.

A549 Cells: Morphology and Characteristics

- A549 cells are squamous in nature and are responsible for the diffusion of water and electrolytes across the alveoli. When grown in vitro, these cells grow as a monolayer, adhering to the culture flask.

- These cells have the ability to synthesize lecithin and contain a high percentage of unsaturated fatty acids that are responsible for the maintenance of membrane phospholipids.

A549 Cell Line Information

Information on the cell line such as karyotype, cell size, doubling time, etc.

- Karyotype: A549 cell line is a hypotriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells. It is common to have modal numbers of 64 and 67 with higher ploidies occurring at an infrequent rate (0.4%).

- Cell Size: In a morphological analysis of A549 cells, the mean cell diameter of the cells from inverted microscopy and TEM images was estimated to be 14.93 µm and 10.59 µm respectively.

- Doubling time: These cells are easy to grow. A549 cells doubling time is typically 22 hours, although it can sometimes take up to 40 hours.

- Storage: To ensure that the A549 cells remain viable, the culture should be stored in liquid nitrogen vapor phase pre thaw. Post thaw they can be stored in an incubator with the right media and growth factors.

- Growth Medium: The A549 cells are typically cultured using a base medium F12/K (Gibco/Invitrogen). 10% fetal bovine serum (FBS) is added to the base medium to make the complete growth medium. The cells can also be cultured in complete media consisting of Dulbecco’s MEM (DMEM) modified with 10% FBS.

Research and Applications of A549 Cells

- The A549 cell line has been tested and approved by the FDA and finds use in a variety of applications, including in manufacturing constructs for use in clinical trials.

Adenovirus Production

- The A549 cell line has been used for adenovirus production. Most significantly, it has been utilized for replicating adenovirus constructs that do not need complementation by the viral oncogene, early region 1A (EA1). It has also been used as a negative control in assays to measure the replication of adenoviruses that lack E1A as well as a target cell line for the detection of replication competent adenoviruses.

Disease Modeling

- The A549 cells have been used to model the alveolar Type II pulmonary epithelium. Studies have shown that this can be particularly useful in research for studying the metabolic processing of lung tissue and for identifying mechanisms of drug delivery to the tissue. This cell line has been utilized not only for studying lung cancer but also for other infections related to the lungs like allergies, asthma, and respiratory infections.

A few examples of research studies where A549 cells were used for disease modeling.

- P53, also known as tumor protein is a gene that codes for a protein that regulates the cell cycle and acts as a tumor suppressant. M3814 is a selective pharmacologic inhibitor of the serine/threonine kinase DNA-dependent protein kinase (DNA-PK), and plays a vital role in non homologous end-joining. In a recent study (the one linked), the researchers used isogenic p53-null/wild-type A549 and HT-1080 cell lines to show that M3814 blocks the repair of radiation-induced double-stranded breaks and enhances p53 phosphorylation and activation.

- In another study, the A549 cell line was used to investigate the role of chemokines during the initial local response to infection with Mycobacterium tuberculosis in the human lung. The researchers found that the human alveolar epithelial cell line infected with M. tuberculosis produced the chemokines MCP-1 and IL-8 through upregulation of their respective mRNAs. The production of these cytokines only depended on the intracellular mycobacterial growth and was not related to virulence.

Drug Development

- These cells have proved to be great for in vitro as well as in vivo testing of novel drugs like docetaxel, paclitaxel and, bevacizumab. The in vivo tests are conducted through xenografting, while in vitro tests are conducted in cell culture. The A549 CDX mouse model (cell line derived xenograft) is the most commonly used xenograft lung cancer model.

Enzymatic Studies

- The human A549 cell line has been used to test the ability of 2-(2,4-dihydroxyphenyl)thieno-1,3-thiazin-4-one (BChTT) in the inhibition of cancer cell proliferation and to understand its mechanism of action on a molecular level. It has also been used to study the effects of insulin and insulin-like growth factor 1 on the apoptosis and proliferation of the A549 cells.

CRISPR Delivery

- CRISPR Cas9, as we already know, is a great tool for precisely editing the DNA. However, delivery of the Cas protein into the cells is still an under researched area with a lot of potential for innovation (hence the need for our design project). In a recent study, researchers constructed Cas9 ribonucleoprotein complexes conjugated to the 7D12 nanobody and demonstrated the transfection of Cas9 mediated by receptors into A549 cells via binding to the epithelial growth factor receptor. They also exhibited that transfection with a Cas9 ribonucleoprotein targeting the BRCA2 gene

resulted in an increased sensitivity to Cisplatin, a chemotherapeutic drug and led to a synthetic dose lethality in the cells.

Conclusions/action items:

- The A549 cell line possesses all of the characteristics of a cell line that our team would chose, currently it is the top choice for this semester.

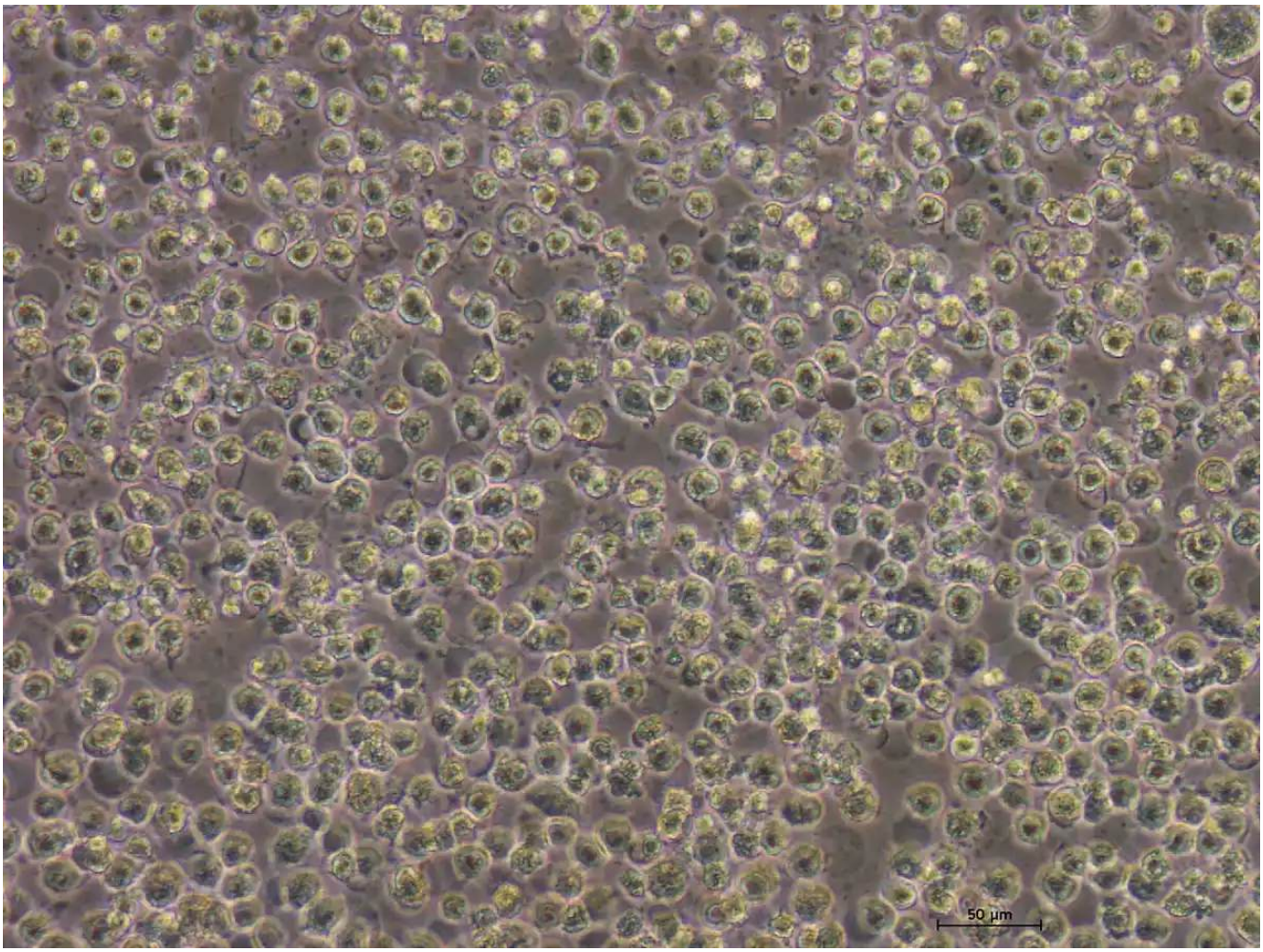


Image of the A549 cell line

Treated Tissue Culture Plates Research VII

JAYSON O'HALLORAN - May 04, 2025, 11:28 PM CDT

Title: Treated Tissue Cultured Plates Research VII

Content by: Jayson O'Halloran

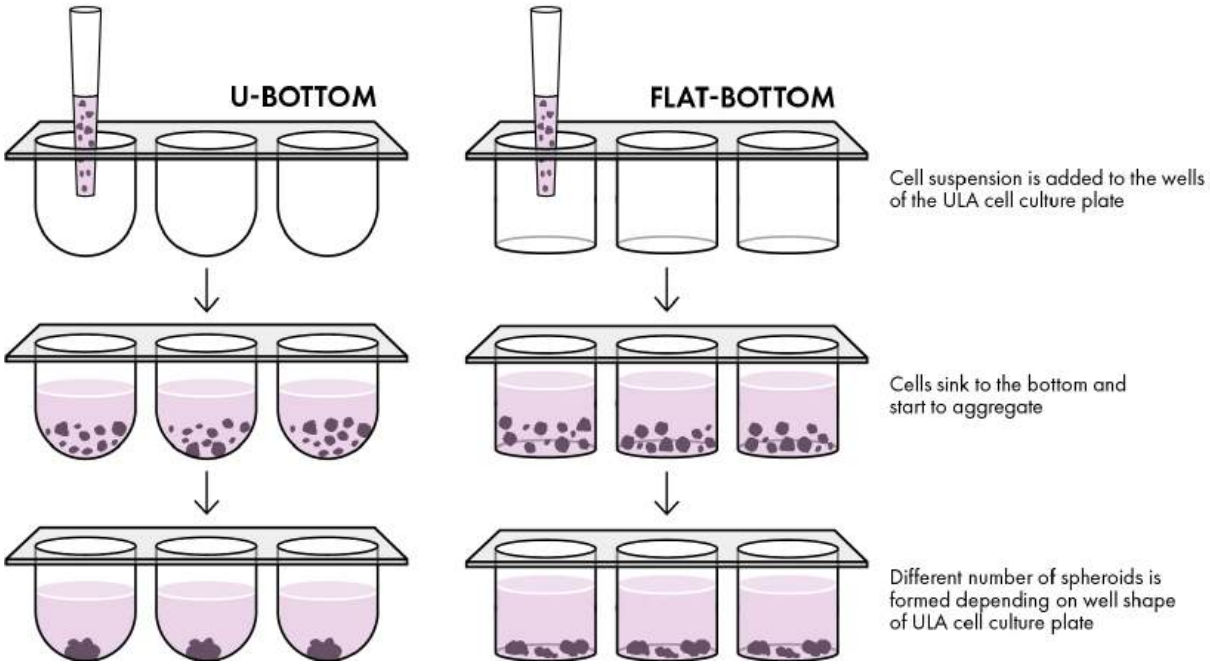
Goals: To gain an understanding of how Treated Tissue Cultured Plates can form spheroids.

Resource: [7] "3D cell culture models: Learning center," 3D Cell Culture Models | Learning Center | UPM Biomedicals, https://www.upmbiomedicals.com/resource-center/learning-center/what-is-3d-cell-culture/3d-cell-culture-models/?gad_source=1&gclid=CjwKCAjwvKi4BhABEiwAH2gcw50g29l2ky8QDWPZUOwSUjHtM5q3kLRI9cX_0t_MsCUBc5R_Y73ksBoCZz4QAvD_BwE (accessed Oct. 7, 2024).

Content:

Ultra-low attachment (ULA) plates were created to produce large scale scaffold-free 3D-cell cultures. These plates are made using liquid overlay techniques where the bottom of a cell culture dish is coated with a non-adhesive material, which prevents cell adhesion and protein absorption. Commonly, ULA-plates are produced by covalently binding a hydrophilic and biologically inert material on the surface of a plate.

When the cell suspension is added to a well of an ULA cell culture plate, cells sink to the bottom of the well, but do not attach to the culture surface which facilitates their aggregation and spheroid formation. There are various well bottom shapes of ULA cell culture plates available, such as U bottom, Flatbottom, Spindle bottom and V-bottom.



U-bottom ULA plates have been created to form and analyse homogenous spheroid populations. Like the hanging drop technique, the U-shape uses gravity to pull down the cells to the bottom of the well. The round geometry will force the cells to aggregate and form a 3D cell complex. The size and shape of cell complex can be easily adjusted by calculating the wanted cell density in the well.

Compared to U-bottom plates, Flat-bottom ULA plates form heterogenous spheroid populations. In each well, cells can freely move on the flat surface and randomly form a cell aggregate. Between plate wells, there may occur varying number of spheroids with different sizes.

ULA Plates are compatible with liquid robotic systems which increases their usage in HTS applications. Downstream analyses and visualization can be done in the same plate as cell culturing. However, in ULA-plate the spheroids float in suspension and are not physically in a fixed position. In live cell imaging, this can cause difficulties to obtain and maintain optical focus when small movements can get spheroids on the

move. Together with this, biological relevance of ULA cell culture plates should be considered because this method lacks tissue-like stiffness and moreover cell-matrix interaction.

Conclusions:

Ultra-Low Attachment (ULA) plates are particularly beneficial for spheroid formation of lung cancer cell lines like A549 and NCI-H23 due to their unique design that facilitates 3D aggregation. The non-adhesive surface prevents cells from attaching, allowing them to sink and aggregate at the bottom of the wells, which promotes the formation of homogenous spheroid populations. The U-bottom configuration effectively uses gravity to encourage cell clustering, creating a consistent environment for spheroid development, which is crucial for studying tumor behavior and drug responses.

Moreover, ULA plates offer compatibility with high-throughput screening (HTS) applications and enable downstream analyses directly in the same culture environment. However, it is essential to recognize potential challenges, such as maintaining optical focus during live cell imaging, as spheroids float and may shift positions. Out of all the options considered for spheroid formations, the ULA plates method is slightly better than the hanging drop method, and much better than matrigel for our specific cell lines.

Etoposide Research VIII

JAYSON O'HALLORAN - May 04, 2025, 11:51 PM CDT

Title: Etoposide Research VIII

Content by: Jayson O'Halloran

Goals: To gain an understanding of etoposides mechanism of action.

Resource: [8] P. L. Olive, J. P. Banáth, and H. H. Evans, “Cell killing and DNA damage by etoposide in Chinese hamster V79 monolayers and spheroids: influence of growth kinetics, growth environment and DNA packaging,” British journal of cancer, vol. 67, no. 3, pp. 522–30, Mar. 1993, doi: https://doi.org/10.1038/bjc.1993.97.

Content:

- Etoposide targets topoisomerase II, leading to double-stranded DNA breaks by stabilizing the cleavage complex during DNA replication, which ultimately induces apoptosis in cancer cells.

- 3D spheroid models better mimic tumor architecture, including hypoxic gradients and cell–cell interactions, which influence the extent and repair of etoposide-induced DNA damage compared to 2D monolayers.

- DNA damage markers like γH2AX are commonly used to quantify etoposide-induced double-strand breaks in spheroids, often revealing reduced drug penetration or delayed DNA damage response in the spheroid core.

- Hypoxia in spheroids can confer resistance to etoposide, as low oxygen levels downregulate topoisomerase II expression and activate DNA repair pathways, limiting drug efficacy.

- Etoposide sensitivity varies with spheroid size, with larger spheroids often showing a more resistant core due to limited drug diffusion and reduced proliferation rates in inner cells.

- Cell cycle arrest at G2/M phase is a hallmark of etoposide treatment; however, cells in the spheroid core may be quiescent, diminishing the drug’s cytotoxic impact.

- Combination therapies using etoposide with PARP inhibitors or hypoxia-targeting agents are being explored to enhance DNA damage and overcome resistance in 3D tumor models.

- Time and dose-dependent effects of etoposide in spheroids reveal non-linear responses, underscoring the need to optimize treatment regimens for effective DNA damage induction.

- High-throughput 3D screening platforms using etoposide-treated spheroids allow for rapid evaluation of genotoxicity, apoptosis, and resistance mechanisms in various cancer types.

- Gene expression analysis post-etoposide treatment in spheroids often shows upregulation of p53, ATM, and DNA repair genes, providing insight into the cellular response to DNA damage.

Conclusions:

- Etoposide-induced DNA damage in cancer spheroids reveals critical insights into drug resistance, penetration, and the spatial complexity of tumor microenvironments. These findings highlight the value of 3D models for evaluating therapeutic efficacy and optimizing combination strategies targeting DNA repair and hypoxia.

CRISPRi Research IX

JAYSON O'HALLORAN - May 04, 2025, 11:57 PM CDT

Title: CRISPRi Research IX

Content by: Jayson O'Halloran

Goals: To explain CRISPR(i)

Resource: [9] K. Han et al., “CRISPR screens in cancer spheroids identify 3D growth-specific vulnerabilities,” Nature, vol. 580, no. 7801, pp. 136–141, Mar. 2020, doi: https://doi.org/10.1038/s41586-020-2099-x.

Content:

- CRISPR screening in 3D spheroids of NSCLC more accurately recapitulates tumor growth phenotypes observed in vivo compared to 2D monolayers, enhancing the identification of cancer-specific vulnerabilities.

- Genes with strong differential effects in 3D vs. 2D cultures were significantly enriched for known mutations in lung cancer, highlighting the translational relevance of 3D CRISPR screens.

- CRISPRi was used to knock down target genes in pre-formed NSCLC spheroids, enabling assessment of gene function in established tumor-like environments with hypoxia and dense cell-cell contacts.

- CPD (carboxypeptidase D) emerged as a novel 3D-specific vulnerability, required for maturation of IGF1R and MET by removing a C-terminal RKRR motif, with direct consequences for signaling and spheroid growth.

- Loss of CPD impaired IGF1R signaling and reduced spheroid growth, especially in KRAS-mutant NSCLC lines, identifying CPD as a potential synthetic lethal partner for KRAS G12C inhibitors.

- 3D CRISPR screens identified gene modules (e.g., CPD-FURIN-IGF1R) that were co-essential in 3D but not in 2D, reflecting a shift in pathway dependencies between culture models.

- Functional validation with competitive growth assays and inducible CRISPRi confirmed that CPD knockdown reduces viability of NSCLC spheroids but has less effect in 2D cultures.

- Gene knockdown in 3D spheroids predicted patient survival more accurately than 2D models, with high CPD expression correlating with poor prognosis in lung adenocarcinoma patients.

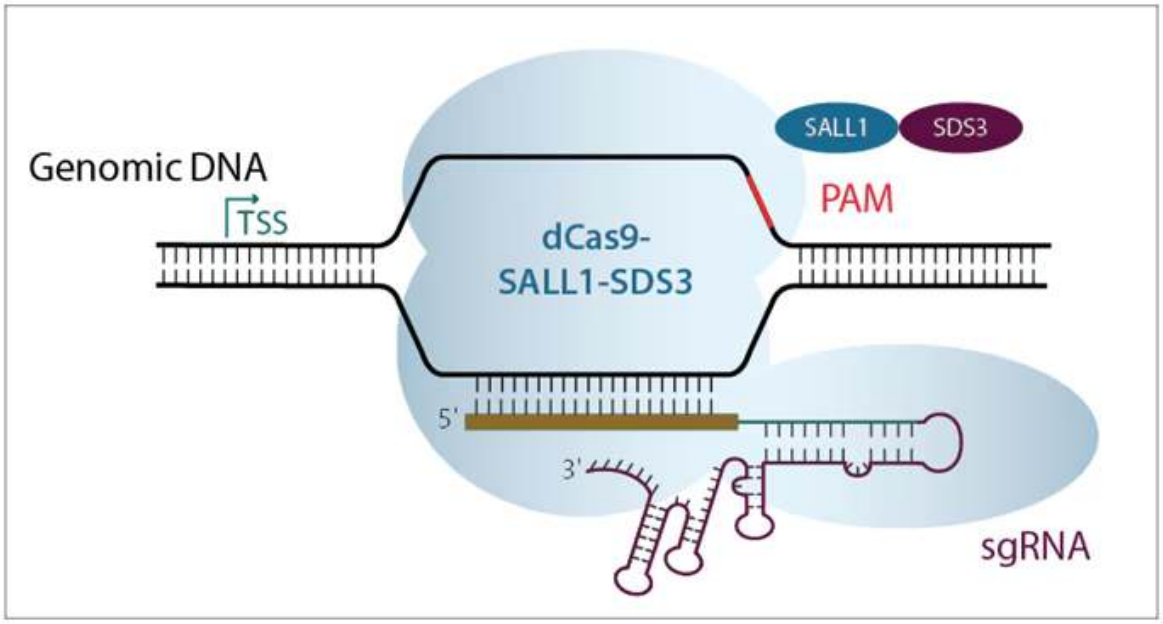
- CRISPR screens across multiple NSCLC lines consistently showed that 3D cultures expose oncogene and tumor suppressor gene dependencies masked in 2D conditions.

- Combining CPD inhibition with KRAS G12C blockade demonstrated synergistic effects in 3D spheroids, underscoring the value of CRISPRi in optimizing targeted therapy combinations in lung cancer models.

Conclusions:

- CRISPRi in NSCLC spheroids enables precise functional interrogation of gene dependencies in a more physiologically relevant 3D environment, revealing vulnerabilities that are often overlooked in 2D cultures. These findings support the use of spheroid-based CRISPR screens to improve translational relevance and identify effective therapeutic targets for lung cancer.

CRISPRi Image



Conclusion Research X

JAYSON O'HALLORAN - May 05, 2025, 12:01 AM CDT

Title: Conclusion Research X

Content by: Jayson O'Halloran

Goals: To write conclusions about the semester/project.

Resource: [10] Madison, “University of Wisconsin-Madison Login,” Wisc.edu, 2025. https://bmedesign.engr.wisc.edu/selection/projects/9c548911-8fa6-41ae-95ab-adc0cd32aec0 CRISPRi screening in cancer spheroids.

Conclusions:

- 3D spheroid culture using A549 cells offers a more physiologically relevant model of NSCLC compared to 2D monolayers, enhancing the investigation of tumor-specific genomic stability.

- Optimal spheroid conditions were determined to be 75,000 cells/cm² with 0.75% methylcellulose, based on diameter, count, and cell viability assays.

- Despite established literature on SOX2 upregulation in NSCLC spheroids, our RT-qPCR did not detect amplification in either 2D or 3D conditions, meaning we needed to troubleshoot the assay and could have done immunostaining as well.

- γH2AX staining successfully labeled DNA damage, but no significant difference in DNA double-strand breaks (DSBs) was observed between 2D and 3D conditions or drug versus control, likely due to insufficient etoposide concentration (should have seen it)

- The study validated that γH2AX staining can be applied in 3D cultures, as comparable signal penetration and detection were achieved in both 2D and 3D conditions.

- High passage number (passage 19–20) of A549 cells may have impacted gene expression and treatment responsiveness, indicating the importance of using lower passage cells in future work.

- Spheroid dissociation using Accutase was optimized to ensure efficient recovery of viable single cells for downstream assays such as flow cytometry and qPCR.

- CellTiter-Glo assays confirmed viability trends, with the optimal spheroid condition showing the highest ATP activity and suggesting robust metabolic function.

- The project established a robust workflow for pre-screen validation, including spheroid formation, drug treatment, staining, and gene expression analysis, to set the stage for genome-wide CRISPRi screens.

- Future work will involve refining SOX2 detection, increasing etoposide dosage, and employing an A549 CRISPRi cell line to identify 3D-specific regulators of DNA repair and tumor progression.

yH2AX staining protocol as of 4/4/25

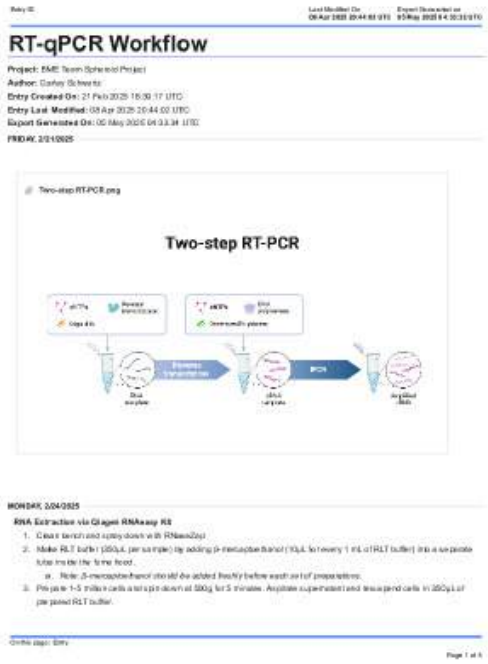
JAYSON O'HALLORAN - Apr 06, 2025, 6:40 PM CDT

Download

γH2AX_staining_protocol.pdf (165 kB)

RT-qPCR Workflow

JAYSON O'HALLORAN - May 04, 2025, 11:38 PM CDT



RT-qPCR Workflow

Project: [illegible]
Author: [illegible]
Entry Created On: 21 Feb 2025 16:39:17 UTC
Entry Last Modified: 08 Apr 2025 20:44:02 UTC
Export Generated On: 05 May 2025 04:33:34 UTC
FRIDAY, 2/21/2025

Two-step RT-PCR.png

MONDAY, 2/24/2025

RNA Extraction via Qiagen RNAeasy Kit

1. Clean bench and spray down with RNaseZap!
2. Make RLT buffer (350µL per sample) by adding β-mercaptoethanol (10µL for every 1 mL of RLT buffer) into a separate tube inside the fume hood.
   a. Note: β-mercaptoethanol should be added freshly before each set of preparations.
3. Prepare 1-5 million cells and spin down at 500g for 5 minutes. Aspirate supernatant and resuspend cells in 350µL of prepared RLT buffer.

[illegible]

[illegible]

Download

RT-qPCR_Workflow_2025-02-21_-_2025-02-24_etr_sP5dxuwh_.pdf (354 kB)

RNA Extraction

JAYSON O'HALLORAN - May 04, 2025, 11:39 PM CDT

Step 1 - RNA extraction - 4/7/2025

Project: EME Team Spheroid Project
Author: Alisyn Cao
Entry Created On: 06 Apr 2025 16:57:34 UTC
Entry Last Modified: 07 Apr 2025 15:59:44 UTC
Export Generated On: 06 May 2025 04:33:55 UTC

SUN547, 4/6/2025

Note: will do 6 sets of experiments: 3 x (2 mil 2D cells) & 3 x (2 mil cells in 3D spheroids)

Reagents & stuff needed:

- RNaseZap
- RLT buffer
- β-mercaptoethanol
- 6 x QiaShredder columns
- 70% ethanol (for experiments)
- 6 x RNAeasy spin columns
- DNase solution: DNase I stock solution & RDD buffer
- RW1 buffer
- RPE buffer
- RNase-free water
- 6 x 1.5 ml Lobes

RNA Extraction via Qiagen RNAeasy Kit (adapted for protocol)

1. Clean bench and spray down with RNaseZap
2. Prepare 6 tubes of cells:
   a. 3 x (2 mil cells in 2D)
   b. 3 x (2 mil cells in 3D)
3. Spin the tubes down at 500 g, 5 mins
   a. While the tubes are spinning down, prepare a new 15 mL conical tube, add 2.3 mL of RLT buffer and 23 µL of β-mercaptoethanol
   b. While the tubes are spinning down, prepare 6 x QiaShredder columns, label accordingly (2D/3D)
4. After tubes are done spinning, aspirate supernatant and resuspend cells in 350 µL of prepared RLT buffer in each tube by pipetting up and down
5. Pipet cell solution onto a QiaShredder column and spin for 2 minutes at 14000 rpm. **Discard column, not flow-through!**
   a. While the tubes are spinning down, prepare 6 x RNAeasy spin columns, label accordingly (2D/3D)
6. Add 350 µL of 70% ethanol to flow through. Transfer mixture to RNAeasy spin column and spin for 30s at 10000 rpm. **Discard flow-through.**
   a. While the tubes are spinning down, prepare a new 1.5 mL tube, add 70 µL of DNase I stock solution in 490 µL of RDD buffer and mix well
      i. DNase in the resuspended form is stored in the -30 in the box with reagents for cDNA synthesis
7. Make sure the flow through is discarded (see step 6).
8. Add 80 µL of DNase I + RDD mix onto the column and let sit at room temperature for 15 min.

On this page: Entry

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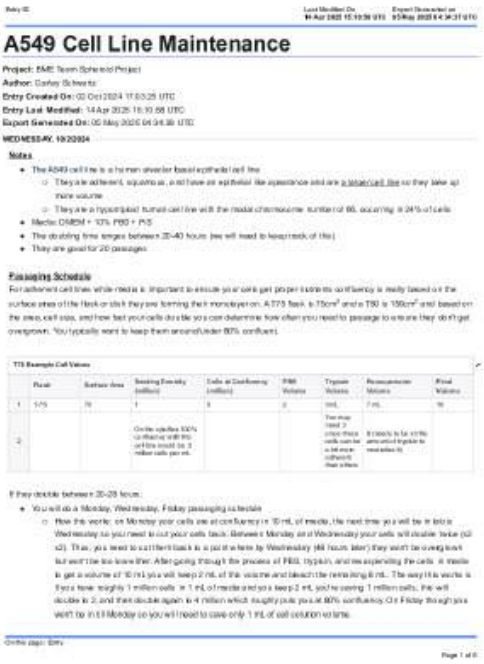
Download

Step_1_-_RNA_extraction_-_4-7-2025_2025-04-06_etr_tGf6maMx_.pdf (115 kB)

A549 Cell line

JAYSON O'HALLORAN - May 04, 2025, 11:41 PM CDT



A549 Cell Line Maintenance

Project: EME Team Spheroid Project
Author: Darley Schwartz
Entry Created On: 03 Oct 2024 17:03:25 UTC
Entry Last Modified: 14 Apr 2025 15:10:58 UTC
Export Generated On: 05 May 2025 04:04:36 UTC

WEDNESDAY, 10/2/2024

Notes

- The A549 cell line is a human alveolar basal epithelial cell line
  - They are adherent, squamous, and have an epithelial like appearance and are a tetraploid line so they take up more volume
  - They are a hypertriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells
- Media: DMEM + 10% FBS + P/S
- The doubling time ranges between 20-40 hours (we will need to keep track of this)
- They are good for 20 passages

Passaging Schedule

For adherent cell lines while media is important to ensure you're cells get proper nutrients confluency is really based on the surface area of the flask or dish they are forming their monolayer on. A T75 flask is 75cm^2 and a T80 is 150cm^2 and based on the area, cell size, and how fast your cells double so you can determine how often you need to passage to ensure they don't get overgrown. You typically want to keep them around/under 80% confluent.

T75 Example Cell Values

	Flask	Surface Area	Seeding Density (million)	Cells at Confluency (million)	PBS Volume	Trypsin Volume	Resuspension Volume	Final Volume
1	T-75	75	1	5	5	1mL	7 mL	10
2			On the splits 100% confluence with this cell line would be 3 million cells per mL			Two may need 2 since these cells can be a bit more adherent than others	8 mL back in for on the second split to resuspend	

If they double between 20-28 hours:

- You will do a Monday, Wednesday, Friday passaging schedule
  - How this works: on Monday your cells are at confluency in 10 mL of media, the next time you will be in lab is Wednesday so you need to cut your cells back. Between Monday and Wednesday your cells will double twice (x2 x2). Thus, you need to cut them back in a split where by Wednesday (48 hours later) they won't be overgrown but won't be too low either. After going through the process of PBS, trypsin, and resuspending the cells in media to get a volume of 10 mL you will keep 2 mL of this volume and bleach the remaining 8 mL. The way this works is if you have roughly 1 million cells in 1 mL of media and you keep 2 mL you're saving 1 million cells, this will double to 2, and then double again to 4 million which roughly puts you at 80% confluency. On Friday though you won't be in till Monday so you will need to save only 1 mL of cell solution volume.

Page 1 of 6

Download

A549_Cell_Line_Maintenance_2024-10-02_etr_5Skd74TL_.pdf (121 kB)

PolyHEMA Stock

JAYSON O'HALLORAN - May 04, 2025, 11:41 PM CDT

PolyHema Stock Protocol

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PolyHema_Stock_Protocol_2025-02-09_etr_u7y3P36m_.pdf (88.1 kB)

CRISPRi Genes of Interest

JAYSON O'HALLORAN - May 04, 2025, 11:42 PM CDT

Preliminary Project Research: Genes of Interest

Project: Defining the DNA Damage Hits

Author: Ellie Webster

Entry Created On: 12 Jun 2023 22:34:47 UTC

Entry Last Modified: 06 Oct 2023 15:36:49 UTC

Export Generated On: 02 May 2025 04:37:07 UTC

MONDAY, 6/12/2023

"OMIM used to generate most gene information."

RAD51 (Contax: 147)

- RAD51 recombinase located mainly in nuclear DNA; gene itself is on chromosome 15
- Interacts with BRCA1 and BRCA2 to fix damaged DNA (is related to cancer because the mutation of BRCA1/2 predisposes one to cancer)
- Important in the binding of damaged DNA in double-stranded break repair and homologous recombination
- Mutation is implicated in congenital mirror movement disorder (in which body movements on one side are accompanied by unintended movements on the other side) and breast cancer
- Protects regions of the genome that are prone to consensus amplifications during replication stress via "replication fork protection" (note: RS can be induced in cells treated with hydroxyurea or aphidicolin)
- Since underreplicated regions are marked with pH2AX foci in mitosis, this was quantified in RAD51-depleted cells exposed to low dose APH in interphase, though pH2AX frequency was not observed to change
- RAD51 localized to be most prevalent in the S phase of the cell cycle in U2OS cells
- Atypical MIDAS sites are associated with cancer genome amplification, which makes sense because MIDAS is typically a marker of the failure of a locus to complete replication in interphase (Bhowmick et. al, 2022)
- High expression in testis, moderate expression in placenta, thymus, pancreas, and colon; weak expression detected in lung, liver, skeletal muscle, kidney, and ovary
- Important in cisplatin resistance
- Sources: https://medlineplus.gov/genetics/gene/rad51/#resources, https://www.sciencedirect.com/science/article/pii/S1097276522006737?via%3Dihub

"OTHER (Contax: 176)

- General transcription factor IIH, polypeptide 4, 52 kDa, in the TFIIH complex and nucleoplasm
- Suggests a lot of inter- and intra-strand crosslinks
- Aids in nucleotide excision, DNA repair, and ATP catabolic processes
- Possesses sequence-specific DNA binding transcription factor activity, as well as kinase and helicase activity
- Functional genetic variants, such as rs 114560632 in chromosome 8, may confer lung cancer susceptibility, as suggested by the results of six GWASs (Wang et al., 2016)
- Not a crazy amount of research done on this gene...PubMed only shows 16 results for the gene name...and many of them aren't particularly specific/relevant

Page 1 of 5

Download

Preliminary_Project_Research-_Genes_of_Interest_2023-06-12_etr_CFUbQew5_.pdf (109 kB)

Cell Seeding Protocol

JAYSON O'HALLORAN - May 04, 2025, 11:42 PM CDT

Copy of Cell Seeding Protocol (3/14/2025) -- 96 well plate (full)

Project: EMS Team Spheroid Project
Author: ISABLY RHINE
Entry Created On: 04 Apr 2025 19:35:31 UTC
Entry Last Modified: 04 Apr 2025 19:58:21 UTC
Export Generated On: 02 May 2025 04:43:30 UTC

WEDNESDAY, 3/26/2025

NOTE: THIS PROTOCOL IS USED FOR 96 WELLS. EACH WELL HAS CELL DENSITY OF 75,000 CELLS/CM^2 AND METHYLCELLULOSE DENSITY OF 0.75%

1. Collect cells from the day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL).**
2. Make a **5 mL cell solution of 500,000 cells/mL (4000000 cells in total)**
   a. **Volume needed to obtain 4000000 cells from the day's cell passage: 4,000,000 (cells) / cell concentration from CytoFLEX (Step 1, cell/mL)**
      1. Can also put in **cell conration (cell/mL) from Step 1** into **Cell #2** from the table below, the volume needed for step 2a will be in **Cell #3**.
   b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**.
   c. Spin down the the tube at **200g for 5 minutes**, then **remove supernatant** to collect cells using vacuum filter.
   d. Resuspend cells in **5 mL of full-serum DMEM**.
3. **Optimized Condition: 75,000 cells/cm², 0.75% methylcellulose**
   a. Into a new **50 mL tube**, add
   b. 6.24 mL of serum-free DMEM
   c. 7.2 mL of 2% methylcellulose stock
   d. 3.76 mL of 500,000 cells/mL cell solution (Step 2)
      1. Mix gently by **pipetting up and down** and make sure the solution is well-mixed
4. **Mix gently by pipetting up and down then Aliquot** the optimized condition mix into each well, add **100 µL of cell mix** into each well (**24 wells total**).
5. Store spheroids in **37C, 5% CO2 incubator.** Spheroids are split every 3-4 days.

Page 1 of 1

Download

Copy_of_Cell_Seeding_Protocol_3-14-2025_--_96_well_plate_full_2025-02-26_etr_LFJsNuJQ_.pdf (88.1 kB)

More Cell Seeding

JAYSON O'HALLORAN - May 04, 2025, 11:43 PM CDT

Cell Seeding Protocol (2/26/2025)

Project: EME Team Spheroid Project
Author: EMILY RHINE
Entry Created On: 26 Feb 2025 05:12:20 UTC
Entry Last Modified: 14 Mar 2025 10:19:00 UTC
Export Generated On: 05 May 2025 04:40:40 UTC

WEDNESDAY, 2/26/2025

NOTE: THIS PROTOCOL IS USED FOR 24 WELLS. EACH WELL HAS CELL DENSITY OF 75,000 CELLS/CM^2 AND METHYLCELLULOSE DENSITY OF 0.7 5%

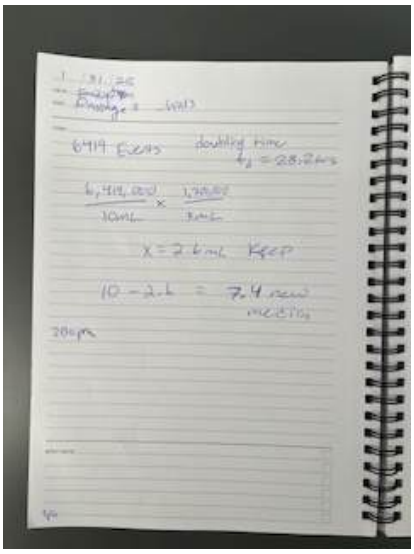
1. Collect cells from that day's passage and **use the CytoFLEX to obtain cell concentration (cells/mL).**
2. **Make a 2 mL cell solution of 500,000 cells/mL (750,000 cells in total)**
   a. **Volume needed to obtain 750,000 cells from the day's cell passage: 1,000,000 (cells) / cell concentration from CytoFLEX (Step 1, cells/mL)=**
      i. Can also put in **cell contration (cells/mL) from Step 1** into **Cell #2** from the table below, the volume needed for step 2a will be in **Cell #3.**
   b. Obtain the volume calculated (Step 2a) of cells and transfer into a **15 mL conical tube**
   c. Spin down the the tube at **200g for 5 minutes.** Then **remove supernatant** to collect cells as by vacuum filter
   d. Resuspend cells in **2 mL of serum-free DMEM**
3. **Optimized Condition: 75,000 cells/cm², 0.75% methylcellulose**
   a. In a new **15 mL tube**, add:
   b. 1.95 mL of serum-free DMEM
   c. 1.8 mL of 2% methylcellulose stock
   d. 1.44 mL of 500,000 cells/mL cell solution (Step 2)
      i. Mix gently by pipetting up and down, make sure the solution is well mixed
4. **Mix gently by pipetting up and down then Aliquot** the optimized condition mix into each well, add **100 µL of cell mix** into each well **(24 wells total).**
5. Grow spheroids in **37C, 5% CO2 incubator.** Spheroids are split every 3-4 days.

Download

Cell_Seeding_Protocol_2-26-2025_2025-02-26_etr_XKpgunXw_.pdf (87.6 kB)

Passaging and work 1/31/25

JAYSON O'HALLORAN - Apr 06, 2025, 3:30 PM CDT



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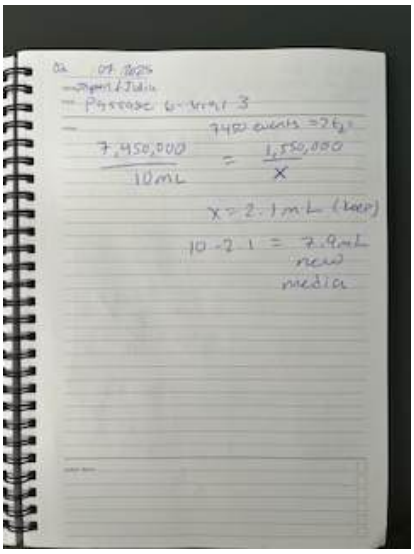
Passaging and work 2/7/25

JAYSON O'HALLORAN - Apr 06, 2025, 3:31 PM CDT



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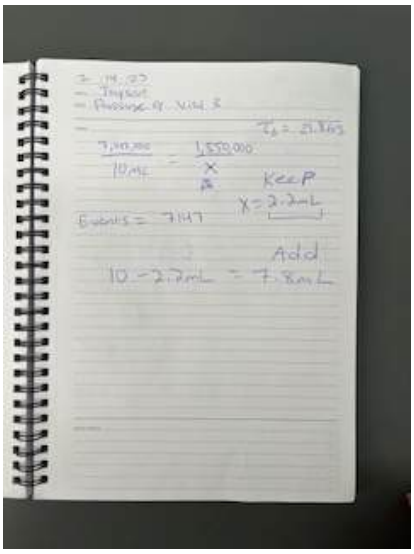
Passaging and spheroids 2/14/25

JAYSON O'HALLORAN - Apr 06, 2025, 3:32 PM CDT



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Celtiterglo and passaging 2/19/25

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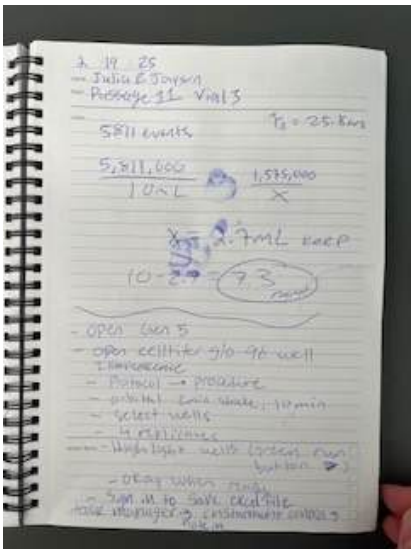

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Spheroids 2/28/25

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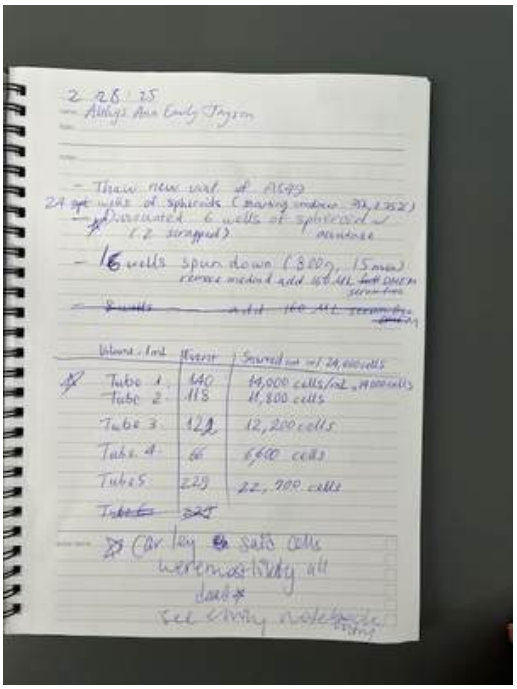

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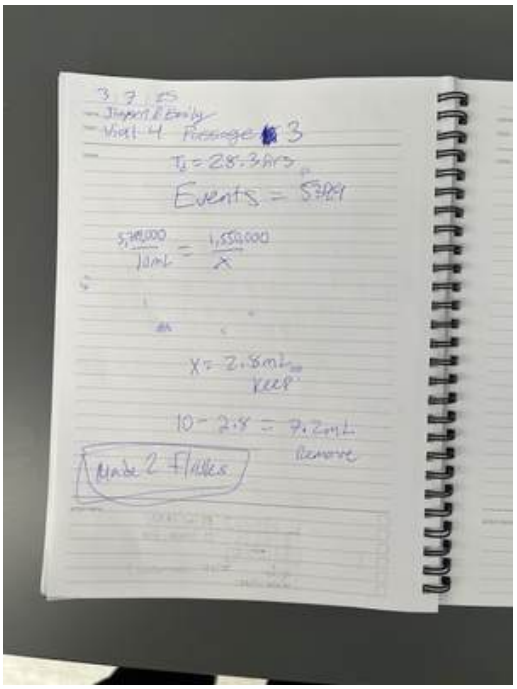
Passaging both flasks 3/7/25

JAYSON O'HALLORAN - Apr 06, 2025, 3:35 PM CDT



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Spheroids and passaging 3/14/25

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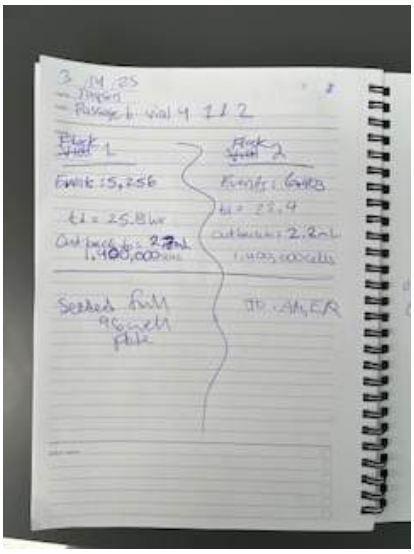

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Spheroids and passaging 3/21/25

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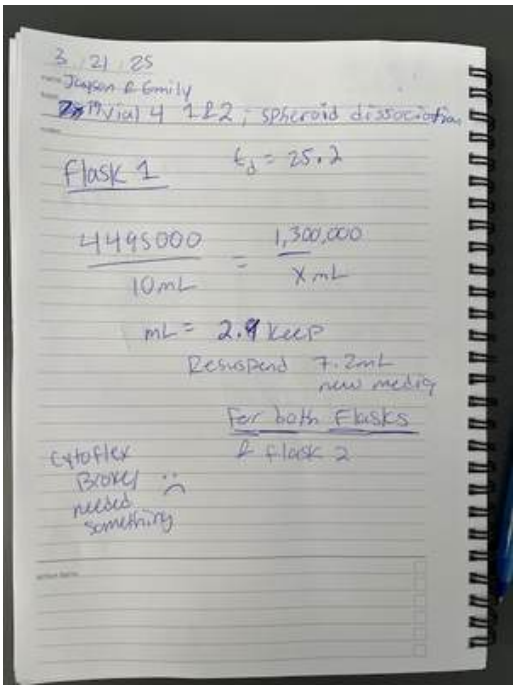

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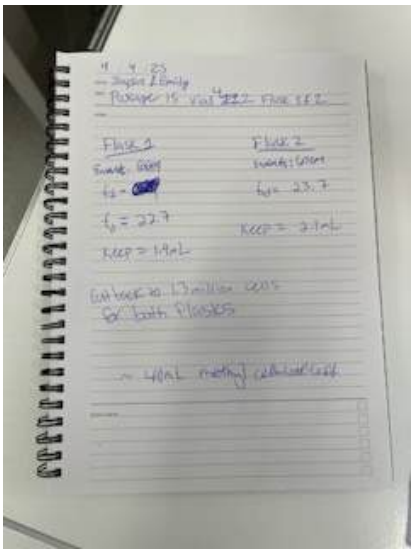
Passaging and work for 4/4/25

JAYSON O'HALLORAN - Apr 06, 2025, 3:21 PM CDT



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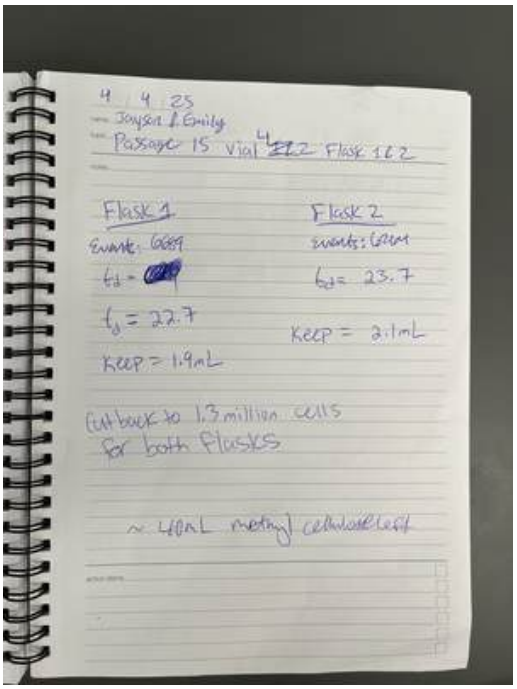
Extra Notes 4/4/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:48 PM CDT



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Extra Notes II 4/4/25

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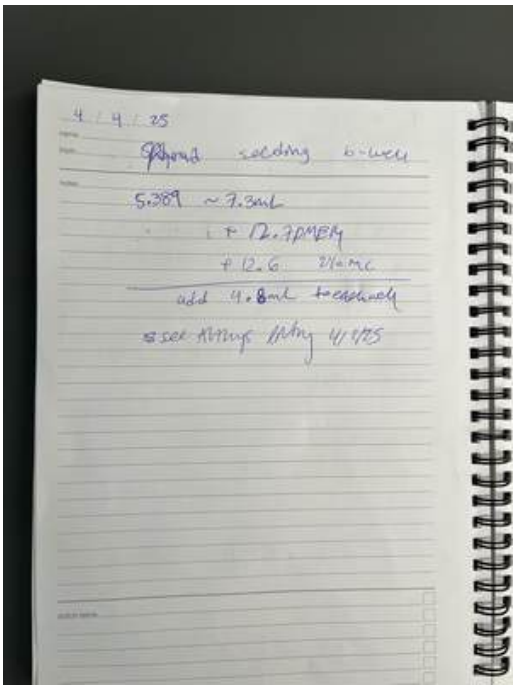

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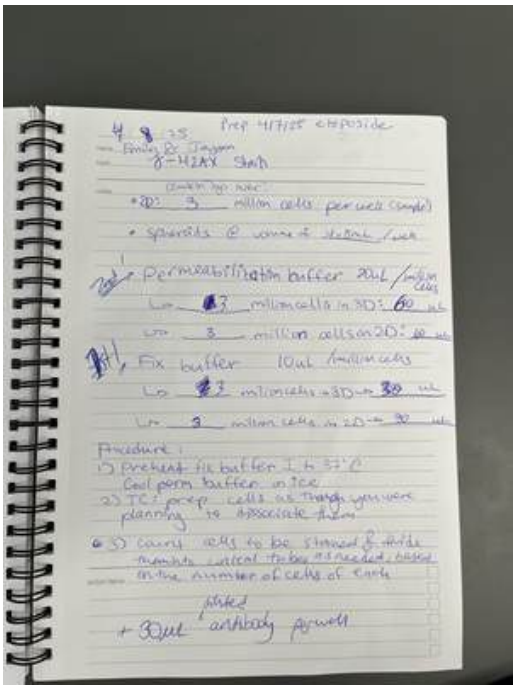
yH2AX stain 4/8/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:50 PM CDT



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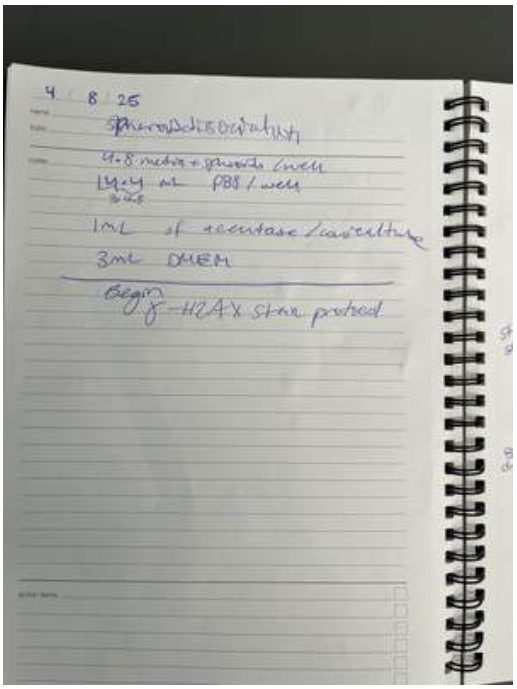
Spheroid Dissociation 4/8/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:50 PM CDT



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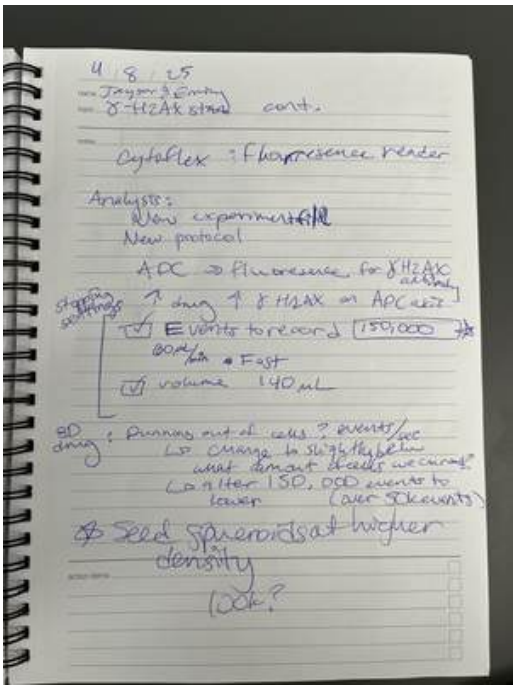
yH2AX stain cont. 4/8/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:51 PM CDT



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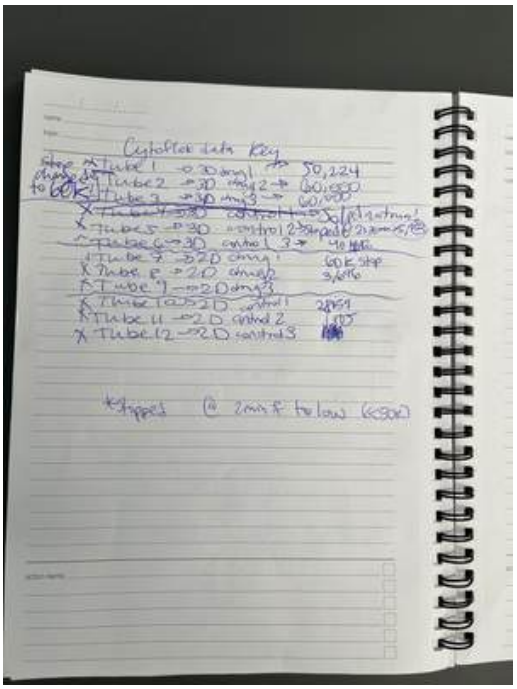
CytoFlex data 4/8/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:51 PM CDT



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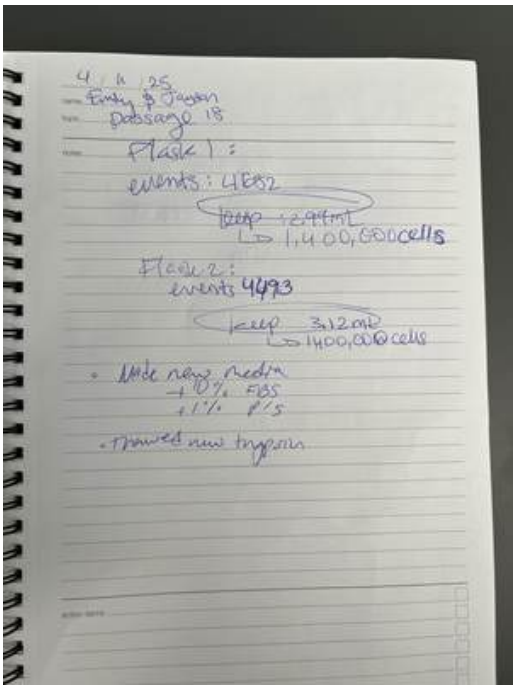
Passaging 4/11/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:54 PM CDT



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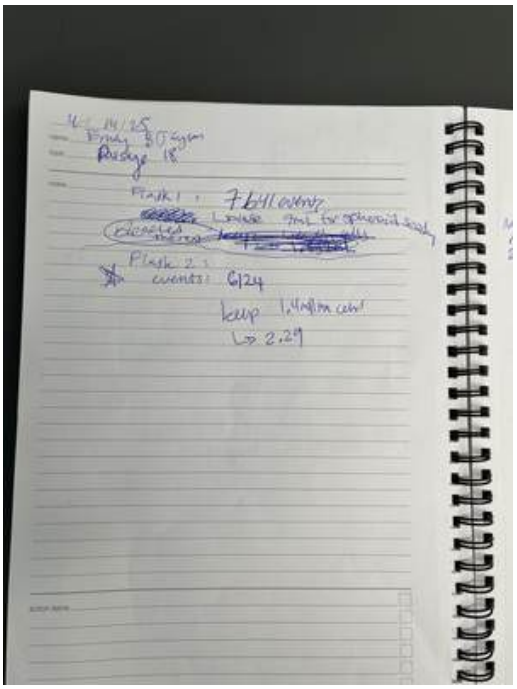
Passaging 4/14/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:55 PM CDT



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IMG_6644.jpg (2.68 MB)

Seeding spheroids 4/14/25

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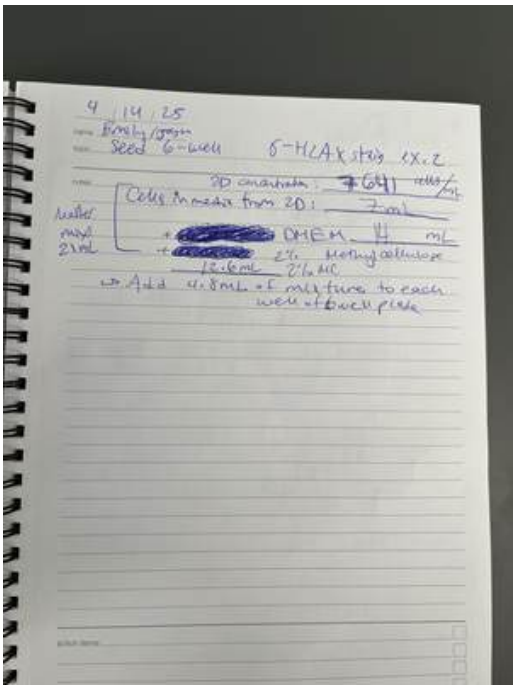

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Etoposide to spheroids 4/18/25

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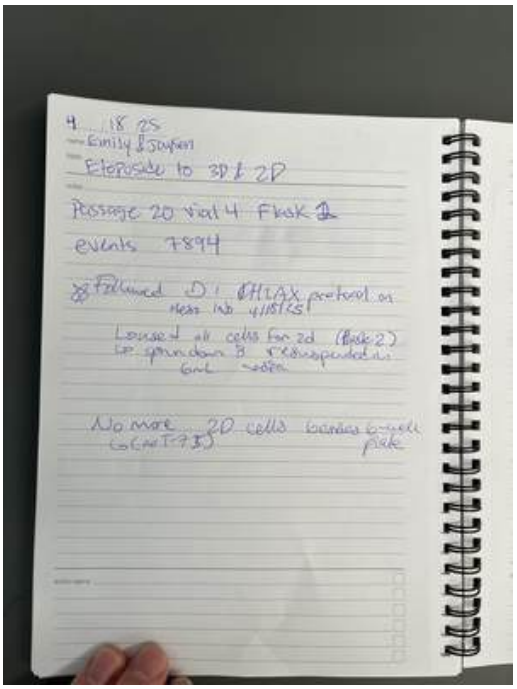

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yH2AX stain 4/19/25

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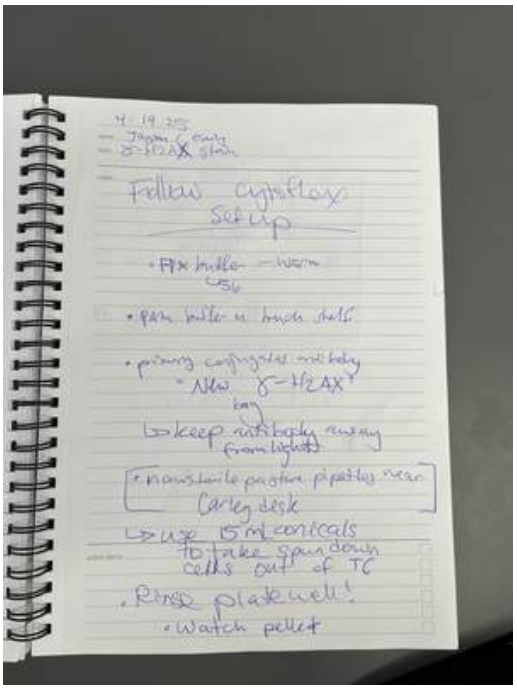

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yH2AX stain cont. 4/19/25

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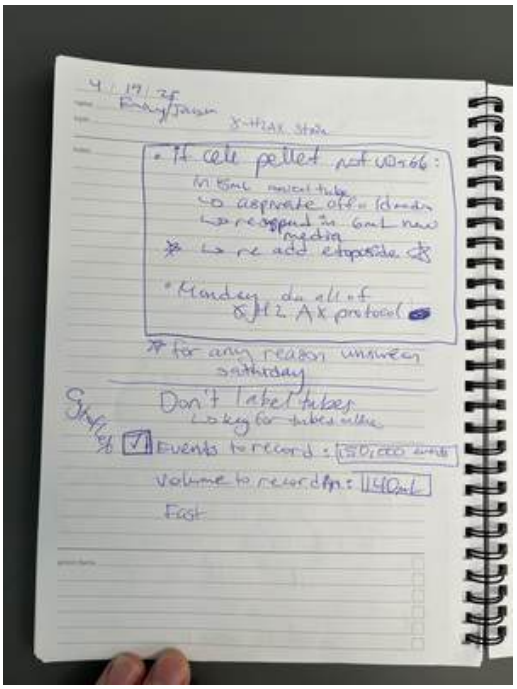

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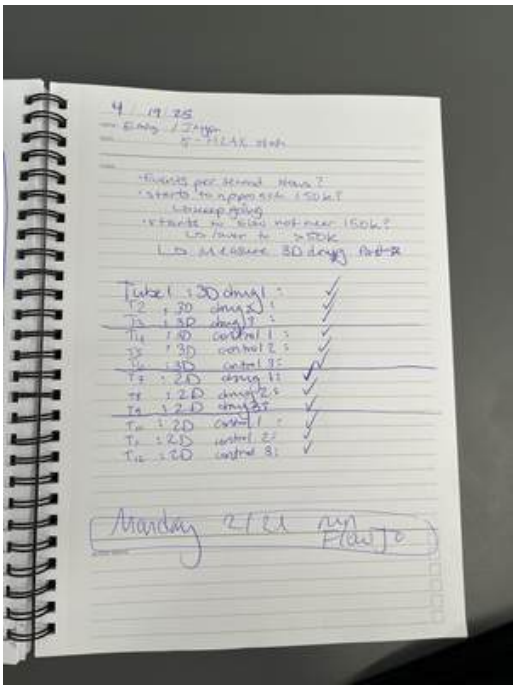
CytoFlex data 4/19/25

JAYSON O'HALLORAN - Apr 23, 2025, 7:58 PM CDT



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2014/11/03-Entry guidelines

John Puccinelli - Sep 05, 2016, 1:18 PM CDT

Use this as a guide for every entry

- Every text entry of your notebook should have the **bold titles** below.
- Every page/entry should be **named starting with the date** of the entry's first creation/activity. subsequent material from future dates can be added later.

You can create a copy of the blank template by first opening the desired folder, clicking on "New", selecting "Copy Existing Page...", and then select "2014/11/03-Template")

Title: Descriptive title (i.e. Client Meeting)

Date: 9/5/2016

Content by: The one person who wrote the content

Present: Names of those present if more than just you (not necessary for individual work)

Goals: Establish clear goals for all text entries (meetings, individual work, etc.).

Content:

Contains clear and organized notes (also includes any references used)

Conclusions/action items:

Recap only the most significant findings and/or action items resulting from the entry.

2014/11/03-Template

John Puccinelli - Nov 03, 2014, 3:20 PM CST

Title:

Date:

Content by:

Present:

Goals:

Content:

Conclusions/action items: