CRISPRi Screening in Cancer Spheroids to Investigate Factors in Genome Stability

BME 400

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Section 305

Design Matrix

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Table 1. Design matrix for Cell Line.

Design Criteria (weight)	NCI-H23		A549		OVCAR-5	
	Score X/5	Weighted score	Score X/5	Weighted score	Score X/5	Weighted score
Viability (20)	4	16	5	20	3	12
Adhesion (20)	3	12	4	16	4	16
Reproduction Speed (20)	3	12	5	20	4	16
Drug Sensitivity (15)	4	12	5	15	3	9
Genetic Mutation (15)	5	15	3	9	2	6
Ease of Procurement (10)	5	10	5	10	5	10
Total Score (100)	77		90		69	

Determination of Criteria and Weights & Justification of Assigned Scores: After discussion about the important criteria to evaluate the initial designs against our Product Design Specifications, the following categories were chosen.

Viability (20):

Cell viability is a measure of the proportion of live, healthy cells within a population [1]. For this category, viability is a measure of how robust the cell is and how well it can withstand various stresses. The highest ranked cell line is predicted to be the healthiest after fluid shear stress, transfection, and cisplatin treatment.

NCI-H23 cells were assigned a score of 4/5 for its and >20% cell death after 72 hours when subjected to [3 μM] of cisplatin and its sensitivity to cisplatin treatment [2]. The NCI H23 cell line showed a higher expression level of DNA repair proteins after cisplatin treatment compared to A549 cells. In previous experiments for both NCI-H23 and A549, a negative correlation between cell viability and DNA damage induction upon cisplatin treatment has been noted [2]. A549 cells were assigned a score of 5/5 for its >20% cell death after 72 hours when subjected to [3 μM] of cisplatin and its greater resistance to cisplatin treatment compared to NCI-H23 cells [2]. OVCAR-5 scored a 3/5 for its severe morphological changes—decreased spheroid-forming capacity, reduced cytoskeleton organization, and chromosomal instability—after exposure to <1 dyne/cm^2 fluid shear stress and >50% cell death after 72 hours when subjected to [2.5 μM] cisplatin [3], [4].

The type of transfection done to these three cell types may either help or hinder the cell health depending on the transfection virus used (lentivirus or siRNA)and what it is used in conjunction with (Nrf2, cisplatin, or both). The trend in scientific literature indicates that these three cell types have the same viability, within 7% ±3%, when transfected under the same conditions [2], [3], [4]. Since the vectors encoding the components necessary for CRISPR/Cas are large, they result in low transfection efficiency and cell viability [5]. To overcome those obstacles, the team can add exogenous small plasmids that increase transfection efficiency up to 40-fold and cell viability up to 6-fold [5].

Adhesion (20):

This criteria refers to the ability of cells to attach to the surface of the culture vessel or substrate they are grown in, as well as to attach to other cells. For this criteria, cell lines were therefore judged on whether they were considered an adherent cell line and on their strength of cell-cell interactions. Because adhesion is an important factor in cells being able to effectively form spheroids, this criteria was given a high weight for scoring.

Although NCI-H23 is considered an adherent cell line [6], it is known to have weaker cell-cell interactions due to the lack of E-cadherin at the cell membrane [7]. Additionally, NCI-H23 has presented difficulty in forming spheroids in previous literature, particularly using the Matrigel method [8]. For these reasons, NCI-H23 scored 3/5. Similarly, although A549 and OVCAR-5 are considered adherent cell lines [9], [10], [11], it is also known to have a lack of E-cadherin and thus weaker cell-cell interactions [7], [12]; thus, A549 and OVCAR-5 scored 4/5.

Reproduction Speed (20):

Reproduction Speed is the time it takes the cells to grow and reproduce. In order to look specifically at the cell line, and exclude any factors caused by the spheroid formation protocol, the team used the cell lines' doubling speed. The doubling speed is the amount of time it takes for a cell population to double in number. Essentially, it is how quickly a cell line replicates itself in culture [13].

As each cell line has a range of doubling times depending on the culture medium used, the most typical doubling times were used to determine this category's score. With the longest doubling time of about 38 hours [6], [14], NCI-H23 scored the lowest at a 3/5. A549 is the fastest of the three cell lines with a typical doubling time of about 22 hours [9], [14], scoring a 4/5. Lastly, OVCAR5 has a doubling time of 27 hours [14], [15] scoring a 4/5.

Drug Sensitivity (15):

Gamma-H2AX foci staining is used for this project to assess the extent of CRISPR-Cas9-based DNA damage. Cell lines that are particularly sensitive to drugs that induce DNA damage will have a baseline of DNA damage to refer to, making them appropriate for this project.

Drug Sensitivity is assessed by Z-scores, also called drug-gene correlation scores, are used to describe whether a cell line is sensitive or resistant to a certain drug compared to other cell lines [16]. A positive z-score means the cell line is resistant to the drug, negative means sensitive, and a difference of ± 1 is significant [16].

The three drugs used in this assessment will be bleomycin, cisplatin and/or oxaliplatin as they directly "attack" DNA. Bleomycin is a radiomimetic drug that can induce double-strand breaks in DNA [17]. Cisplatin and oxaliplatin both have platinum cores and induce DNA damage by forming adducts at DNA sites [18].

Z-scores for NCI-H23, A549, and OVCAR-5 are obtained from the Genomics of Drug Sensitivity in Cancer Project [19]. NCI-H23 has a Z-score average of -1.22 for bleomycin, -0.73 for cisplatin and +0.41 for oxaliplatin [20]. A549 has a Z-score average of -1.04 for bleomycin, -0.12 for cisplatin, and -1.45 for oxaliplatin [21]. OVCAR-5 has a Z-score average of +0.67 for bleomycin, +0.52 for cisplatin, and -0.18 for oxaliplatin [22]. A549 has 2 significant negative Z-scores, scoring a 5/5. NCI-H23 has a negative Z-score for 1 of the drugs, scoring a 4/5. OVCAR-5 has none, scoring a 3/5.

Genetic Mutation (15):

Analysis of genetic mutations inherent to various cell lines is crucial in determining the ideal cell line for our project. A high score in this category means genetic mutations that are helpful must work with for any step in the adhesion process, Cas9 attachment, CRISPR edit, or screen. Using Han's ten selected lung cancer cell lines, a set of ideal cell line mutations were created including: TP53 (tumor protein p53), a gene that encodes a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains [23]; EGFR, a cell surface protein that binds to epidermal growth factor leading to cell proliferation [24]; and

KRAS, Kirsten rat sarcoma viral oncogene homolog causes malignancies based on an error encoding a protein that is a member of the small GTPase superfamily [25]. These mutations may either cause or enhance the malignancy of existing cancer cells. Since TP53 is the most frequently mutated gene in human cancer, the presence of this mutation is crucial for biological relevance to half of all cancer cases [26].

NCI-H23 has all of the key mutations including TP53, KRAS, EGFR, a high degree of c-myc DNA amplification, and no counterproductive mutations, so it was given the highest score of 5/5 [6]. A549 scored second highest with 3/5 because it contains EGFR and TP53 which are key, but also PIK3CA, ALK, and PTEN which aren't key but won't impede the success of the project [27]. The protein encoded by PIK3CA, phosphatidylinositol 3-kinase, is oncogenic and represents the catalytic subunit, which uses ATP to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P2 [28]. ALK, anaplastic lymphoma receptor tyrosine kinase, a chromosomal rearrangement which results in creation of multiple fusion genes in tumorigenesis. PTEN guards the genome by controlling multiple processes of chromosome inheritance [29]. Although OVCAR-5 has KRAS, a key mutation, it was ranked the lowest at 2/5 due to the lack of essential protein TP53 and the presence of additional mutation CLDN4, which is a mutation of integral membrane proteins [30], [31], [32].

Ease of Procurement (10):

This criteria assesses how easy it is to obtain the cell line. It is expensive to purchase an entirely new cell line from ATCC, Sigma-Aldrich or similar sources. Therefore, the chosen cell line should be either already available in Hess Lab or can be obtained from other labs, preferably within Wisconsin Institutes for Medical Research (WIMR); however, that process can be time-consuming.

All 3 chosen cell lines are readily available in Hess Lab, both wild-type and genetically-modified, and therefore all scored a 5/5.

Table 2. Design matrix for Spheroid formation.

Design Criteria (weight)	Treated Tissue Culture Plates		Hanging Drop		Matrigel	
	Score X/5	Weighted score	Score X/5	Weighted score	Score X/5	Weighted score
Ease of Fabrication (30)	5	30	4	24	3	18
Scalability (20)	4	16	3	12	5	20
Uniform properties (20)	4	16	3	12	2	8
Cost (15)	3	9	4	12	2	6
Safety (15)	5	15	5	15	5	15
Total Score (100)	86		75		67	

Determination of Criteria and Weights & Justification of Assigned Scores: After discussion about the important criteria to evaluate the initial designs against our Product Design Specifications, the following categories were chosen.

Ease of Fabrication (30):

This criteria assesses the complexity of the 3D spheroid formation protocol, which is one of the important factors when choosing an appropriate fit-for-purpose 3D culture model [33]. Specifically, this criteria assesses whether additional specialized training or equipment will be needed for the protocol, on top of those necessary for 2D traditional cell culture methods. Also, the time needed to complete the protocol once will also be considered, as it can directly affect the length of the project due to the large number of cells needed for genome-wide CRISPR screening.

In the treated tissue cultures method, cells are seeded in plates covered with an ultra-low hydrophilic attachment and incubated for 24 hours, after which cell media will be replaced. Spheroids can be harvested after up to 4 days [34]. In hanging drop method, cells are seeded in hanging drop plates with added PBS. Extra care is needed to make sure that the hanging drops are formed and confined to the bottom of the plates. Media should be replaced daily, and spheroids can be observed after up to 4 days [35]. In the Matrigel method, cells were first seeded in ultra-low attachment plates and allowed to grow for around 4 days to form aggregate before addition of Matrigel. Spheroids can be observed 2 days after [36].

Treated tissue culture plates method scores a 5/5 as the cell seeding method is not particularly labor-intensive, only requires 1 media change and takes the least time (up to 4 days). Hanging drop method also takes the same amount of time but the cell seeding method requires more careful technique to ensure hanging drop viability and daily media change, thus scoring a 4/5. The Matrigel method does not require extensive cell seeding technique and daily media change but requires longer time (6 days), scoring a 3/5.

Scalability (20):

This criteria assesses the plausibility to use these methods to generate a large amount of viable spheroids. A high-throughput method is preferred because, per the client, each CRISPR screen would require 100-150 million cells per replicate to generate a sufficient amount of spheroids [37]. Because a high-throughput method is very important to successfully completing a spheroid CRISPR screen, this criteria was given a high weight for scoring.

The treated tissue culture plates method is known for its high-throughput capabilities because treated plates have a non-adherent coating that promotes rapid cell aggregation into spheroids [38]. This method also allows for a greater amount of spheroids to be made in a single plate because it can accommodate large volumes of media [39]. For these reasons, this method was given a score of 4/5 for scalability. The Hanging Drop method, in contrast, is considered to be low-throughput in nature. One reason for this is because the method requires a lot of manual handling and is prone to error when setting up and transferring the drops [40]. Additionally, scalability is limited with the Hanging Drop method because each tissue culture plate can only hold a limited amount of drops, and each drop only tends to form one spheroid [41]. For these reasons, this method was given a score of 3/5 for scalability. Lastly, the Matrigel method is considered high-throughput because, like the treated tissue culture plates method, it can allow for a comparatively larger volume of media to generate spheroids compared to the Hanging Drop method. Additionally, this method involves polymerizing a layer of Matrigel matrix onto tissue culture plates to promote spheroid formation [8], and unlike the other two methods, has been shown to improve the growing environment of spheroids due to its mimicry of in-vivo tumor conditions [42]. For these reasons, this method was given a score of 5/5 for scalability.

Uniform Properties (20):

This criteria outlines the reliability of the spheroid formation to produce uniform properties. The ideal formation protocol has no batch-to-batch variability. Minimal variation in size, shape, and porosity is expected so the experiment will have a higher chance of reproducibility and fewer outliers. It is important to note that research has shown that differences in spheroid formation arise from the differences in cell-biomaterial interactions rather than due to differences in cell viability or proliferation [43].

The treated tissue culture plates scored the highest in this category, 4/5, due to their minimal variation between batches. Additionally, treated tissue culture plates have a larger

amount of spheroids per batch— see *Scalability* section— making it easier to harvest a uniform size or shape [33]. Due to the low-throughput nature of Hanging Drop, making it harder to harvest uniform spheroids, and due to its 10% to 15% size variation between drops, this method scored a 3/5 [44]. Matrigel, animal-based basement membrane extract, has a batch-to-batch variation that can hinder reproducibility, so a score of 2/5 was assigned to it [43]. Cells seeded in matrigel also tend to form large cell aggregates with looser morphology rather than spheroids with round, regular shape [43].

Cost (15):

This category is scored based on the expenses of the materials needed to perform the spheroid formation protocol. While the client stressed the importance of a working protocol they also expressed their desire to keep costs to a minimum, as to be able to repeat the protocol and perform it on a larger scale.

Explain scoring:

Treated tissue culture plates scored a 3/5 in this category. Generally, the culture plates range from $0.22-3.78 per well [45], with a need for an abundance of wells. The cost of treated tissue culture plates can vary depending on the manufacturer, the number of wells needed, and the volume of cell culture media required. The Hanging Drop method is rather inexpensive as it does not require specialized equipment or reagents. At around $4 [46], [47] per 100 mm plate the Hanging Drop method is the most cost effective method out of all the options and scored a 4/5 [48]. Lastly, Matrigel scored a 2/5 in this category as the Matrigel itself is rather expensive, at $380 for 10 mL of Matrigel [43], [49].

Safety (15):

This criteria assesses the ability of the method to be used in a laboratory environment without posing significant health risks to the user or the experimental results. This includes evaluating the potential hazards associated with the materials/reagents and processes used, the ease of handling, and the effectiveness of protective measures (e.g., PPE, ventilation). A method should be dependable, minimizing the risk of contamination or adverse effects that could compromise the safety of the personnel and the integrity of the experiments.

All of the methods proposed were able to obtain a 5. This is due to none of the methods using harmful reagents in the process of fabrication. All of the cell lines pose similar contamination risk but can be lowered considerably with proper PPE use.

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