Integration of RNAi and Small Molecule Screens to Identify Targets for Drug Development

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Abstract

Cellular models for siRNA and small molecule high-throughput screening have been widely used in the last decade to identify targets for drug discovery. As an example, we present a twofold readout approach based on cell viability and multipolar phenotype. To maximize the discovery of potential targets and at the same time reduce the number of false positives in our dataset, we have combined focused and rationally designed custom siRNA libraries with small molecule inhibitor libraries. Here we describe a cellular model for centrosome amplification as an example of how to design and perform a multiple readout/multiple screening strategy.

Key words

High-throughput screening
Centrosome amplification
Centrosome clustering
High-content screening
siRNA screening
Target discovery

1. Introduction

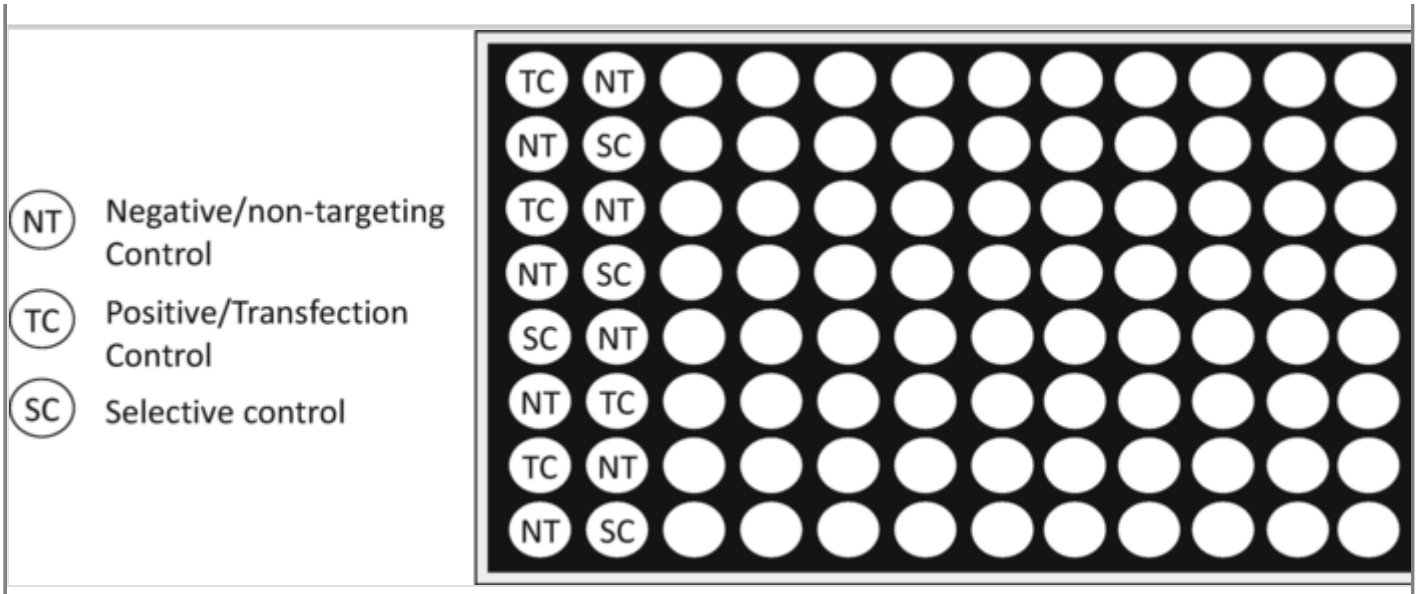
Despite intensified efforts to identify new targets for drug development in cancer, in the last decades, only a very small fraction reaches the preclinical development phase. The most common complications reported are drug selectivity and lack of understanding of the underlying mechanisms and biomarkers. The recent advances in RNA interference (RNAi) and CRISPR-Cas9 technologies have permitted a wide adoption of high-throughput approaches for the discovery of new targets within their natural context. However, these strategies are still beset by selectivity problems due to off-target effects, especially in mammalian cells [1, 2]. Network and protein connectivity analyses have contributed to shedding light on the potential therapeutic mechanism of a particular target, but these are still depended on high-quality input data in order to be successfully applied [3]. Several strategies have been suggested for improving the quality of data from high-throughput screens, including the use of isogenic cell lines (cell lines of genetically identical background), use of multiple RNAi per gene, and multiplexing viability data with high-throughput microscopy data [4]. In order to improve the discovery of verifiable targets and facilitate identification of false positives, as well as the understanding of the underlying mechanisms, we use a twofold readout approach, also limiting our assay to a specific phase of the cell cycle where possible. In addition, in order to further reduce screening artifacts and false positives, we utilize isogenic cell lines with or without centrosome amplification that are screened under identical conditions and siRNA transfection efficiency.

The centrosomes are microtubule-organizing centers which play an essential role in organizing the mitotic spindle and maintaining spindle bipolarity in mammalian cells [5]. Centrosome amplification is one of the most common abnormalities in cancer cells. In order to avoid the deleterious effects of multipolar mitotic spindles, cancer cells cluster supernumerary centrosomes in a bipolar fashion during mitosis, a process which can be exploited to selectively kill cancer cells [6]. The cellular model that we use consists of chromosomally stable human colon cancer DLD1 diploid (2N) and tetraploid (4N) cells, along with tetraploid centrosome-amplified (4NCA) cells. In order to generate 4NCA cells, we use dihydrocytochalasin B (DCB) to transiently block cytokinesis and induce tetraploidization and centrosome amplification in DLD1 cells. Centrosome amplification in 4NCA cells is transient, and therefore they need to be generated from 2N cells for each run of the assay. We then use this model to screen a custom siRNA library representing all known microtubule-binding proteins with the rationale that centrosomes, which are microtubule-associated organelles, depend on microtubules and their associated proteins for their motility. In addition, we use a kinase-related siRNA library and a small molecule kinase inhibitor library in order to identify the regulatory networks that lie upstream of the microtubule-associated effectors.

The siRNA screening is performed in duplicate as shown in Fig. 1. The first replica is used for viability readout and the second for phenotypic readout where we look at spindle structure by using a high-content screening platform. The small molecule library screening is also performed in duplicate with the first replica used for viability readout. In contrast to the siRNA libraries, small molecule libraries give us the opportunity to carefully time the treatment to include mostly one phase of the cell cycle, thus reducing the possibility for "noise" introduced by off-target inhibition of kinases that might be important in other phases of the cell cycle. For this reason, the second replica, which we use for the phenotypic readout, cells are synchronized in mitosis by treatment with the APC/C inhibitor pro-TAME [7] for 3.5 h and then placed at 4 °C for 10 min to induce a reversible disorganization of the mitotic spindle. Finally, the cells are treated with the compound library in the presence of MG132 for 3 h, to limit the effects of the treatment only in mitosis. Treatment with pro-TAME arrests the cells in metaphase without inducing mitotic spindle defects in the short term [7]. Incubation of the plates at 4 °C aims to emulate the cells entering mitosis in the presence of the compounds in a uniform manner. MG132 is a proteasome inhibitor that prevents the degradation of spindle assembly checkpoint proteins and cyclin B; therefore, it helps to maintain the metaphase arrest. Metaphase is the optimal phase of mitosis where the number of spindle poles/cell can be quantified by automated analysis. Using this approach, we identify a number of hit genes that selectively kill cells with centrosome amplification validating at the same time both the underlying mechanism and the regulatory signaling involved in this process.

Fig. 1

Example 96-well plate with controls on columns 1 and 2. The rest of the plate is occupied by the samples (siRNA or small molecule). siRNA and small molecule libraries often come with columns 1 and 2 or 1 and 12 empty for the purpose of leaving space for the desired controls. Positioning the controls on columns 1 and 2 is preferable than the 1 and 12 option. Ideally, the controls should be dispersed throughout the plate if possible



2. Materials

The nature of this approach requires cell line-specific optimization and setting up of the assay. There is a variety of commercial lipids and siRNA controls that need to be tested for suitability for a particular cell line/cell model. It is desirable to achieve less than 10% toxicity of the lipid alone relative to growth media alone, less than 10% toxicity of the nontargeting (sequence does not match any known human mRNA) siRNA negative control relative to lipid alone, and more than 90% toxicity of the transfection efficiency control siTOX (highly toxic siRNA pool that will kill every transfected cell) relative to nontargeting siRNA negative control. In addition, cell plating should be optimized so that at the end of the assay, cells are no more than 90–95% confluent for the viability assay and no more than 70–80% confluent for the phenotypic assay. It is important that every screen is set up individually by the person who is going to perform the screening since clonal variation of cell lines, available equipment, normal variation of different batches of transfection lipid, and personal technique can have significant effects in a high-throughput screen even if an exact protocol is followed.

2.1. Generation of Isogenic Populations

1. DLD1 colon adenocarcinoma cells grown in DMEM (41966-029, Thermo Fisher) supplemented with 10% FBS.
2. Dihydrocytochalasin B (DCB, 250225, Merck Millipore), an inhibitor of actin polymerization, required to promote cytokinesis failure.
3. Hoechst 33342 (H3570, Thermo Fisher) for live cell sorting and isolation of diploid/tetraploid populations.

2.2. siRNA Screening

1. Transfection reagent: DharmaFECT 2 (T-2002, Dharmacon).
2. Opti-MEM~~mem~~ I (31985, Thermo Fisher).
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3. siRNA controls (Dharmacon): nontargeting siRNA Pool (D-001206-13), siTOX transfection control (D-001500-01), and siKIFC1 (M-004958-02).

4. Dharmacon kinase-related siRNA library or any custom/catalogue siRNA library.

2.3. Small Molecule Library Screening

1. DMSO for resuspension and dilution of small molecule library and for vehicle control.
2. Griseofulvin (G4753, Sigma): Used as a positive control (5 μM) that selectively kills cells with centrosome amplification.
3. pro-TAME (I-440, Boston Biochem): Used at 8 μM for synchronizing the cells in metaphase. Pro-TAME is a prodrug activated by intracellular esterases; optimal concentration should be determined for each cell line.
4. MG132 (M7449, Sigma): Used at 8 μM to maintain metaphase arrest during treatment. Most cell lines will maintain metaphase arrest for the duration of the assay at this concentration.

2.4. Viability and Phenotypic Readout

1. Opaque white-wall, clear-bottom 96-well tissue culture plates to be used with a luminescent cell viability reagent suitable for high-throughput screening assays (CLS3610-48EA, Corning).
2. CellTiter-Glo Luminescent Cell Viability Assay (G7572, Promega).
3. Black-wall, clear-bottom 96-well tissue culture assay plates to be read by a suitable high-content screening platform (6005182, PerkinElmer).
4. Ice-cold methanol.
5. Anti-Aurora-A (*see* **Note 1**) monoclonal antibody (610939, BD) and anti-histone H3 pS10 (pHH3) polyclonal antibody (ab47297, Abcam).
6. Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit (both from Life Technologies).
7. Antibody solution: 1.5% FBS in PBS.
8. DAPI solution: 1:5000 DAPI (10 mg/mL stock, 40043, Biotium) in PBS.
9. Black adhesive film to seal the plates before reading.
10. Suitable HCS platform such as the *IN Cell Analyzer* (GE Life Sciences), *Opera*/*Operetta* (PerkinElmer), or ImageXpress (Molecular Devices).

3. Methods

3.1. Generation of Isogenic Cell Lines

1. Treat DLD1 diploid cells with normal centrosome number for 24 h with 2 μM DCB, and release for 96 h.
2. Prepare cell suspension of 500,000 cells/mL, and incubate with Hoechst 33342 (10 mg/mL stock) in PBS 1:5000 at 37 °C for 20 min.

3. Cell sort populations according to cell cycle profile. Isolate diploid populations from diploid G1 and tetraploid populations from tetraploid G2 (*see* **Note 2**).

4. Grow isolated populations for 1 week, and verify DNA content by comparing isolated tetraploid populations to the original DLD1 cells. Repeat cell sorting if necessary.

3.2. siRNA Screening

All reagents must be at room temperature (RT).

1. Split, pellet, and resuspend 2 N, 4 N, and 4NCA cells at appropriate density (2000 cells/well for viability and 9000 cells/well for phenotypic) in growth media (DMEM) without antibiotics.

2. Add 80 μL PBS in the space between the wells of the 96-well assay plates (*see* **Note 3**).

3. Distribute library and control siRNAs (Fig. 1) in V-bottom 96-well plates (one siRNA pool/well). Add appropriate amount of DharmaFECT 2 in Opti-MEM~~mem~~ (final concentration 0.1 μL per sample) using a 12-channel pipette and precision tips.

4. Gently pipette up and down 3–4 times, and immediately distribute to flat-bottom 96-well plates.

5. Place the lid on the plate, and incubate for 30 min at RT to allow the formation of siRNA-lipid complexes.

6. Add cell suspension on complexes by a single swift pipetting step, and shake on an orbital shaker for 2–3 s at 400 rpm to equally distribute the cells throughout the well.

7. Place the plates in the incubator, avoiding stacking if possible (*see* **Note 3**).

8. After 5–7 h, carefully add 200 μL of growth media, taking caution not to disturb the cells. This is done to minimize lipid toxicity.

9. (Optional) Next morning, remove half of the media in the plates using a multichannel pipette, and carefully add an equal amount of fresh media to further dilute the lipid. This will help to further reduce lipid toxicity effects and at the same time minimize disturbance of cells that will likely happen if the full amount of growth media is replaced.

10. For the phenotypic readout, 48 h after the transfection, remove media, and fix cells adding 150 μL of ice-cold methanol.

11. Incubate for 20 min at −20 °C.

12. Leave for 2 min at RT before removing methanol, and immediately add 150 μL PBS to rehydrate the cells.

13. Remove PBS, and add 85 μL of antibody solution with Aurora-A and pHH3 antibodies at 1:1000.

14. Incubate for 16–24 h at 4 °C.

15. Wash 2 × 2 min with PBS taking care not to leave the cells dry for more than 30 s maximum.

16. Incubate with fluorescent secondary antibodies at 1:1000 and DAPI 1:5000 for 1 h at RT.

17. Wash 2 × 2 min with PBS.

18. Seal the plate with black adhesive film, and acquire images using a high-content platform.

19. For the viability assay, 96 h after the transfection, remove growth media by flicking the plate sideways above a sink, and add 100 µL of DMEM/Cell Titer Glo 1:1 solution.

20. Mix for 2–3 min on an orbital shaker and incubate for 20 min at RT.

21. Read using a luminescence plate reader.

3.3. Small Molecule Library Screening

1. Plate~~s~~ 12,500 4 N cells and 16,000 4NCA cells in black 96-well assay plates, one replica per concentration of the library (*see* **Note 4**). It is important that the cells are evenly dispersed within the wells.

2. Next day, aspirate media with a multichannel aspirator, and add 75 µL of 8 uM pro-TAME in each well with a 12-channel pipette. Aspirate and treat cells row by row to avoid drying out of cells. Incubate assay plates at 37 °C, 5% CO_2 for 3.5 h.

3. During the incubation, prepare 12 µM MG132 in DMEM for the serial dilutions of compounds at 1.5× the final concentration and appropriate DMSO controls. Distribute 350 µL/well in a standard 96-well plate in the same well positions as in the assay plates. Place the plate at 37 °C.

4. Take the assay plates from the incubator, and place them at 4 °C for 10 min.

5. In the hood at RT, using a 12-channel pipette, slowly (dropwise to avoid detachment of mitotic cells) add 150 µL from the serial dilution plates to the corresponding positions on the assay plates. Incubate at 37 °C, 5% CO_2 for 3 h.

6. Continue from Subheading 3.2, **step 10** with the difference that the phenotypic replicas are treated for 3 h instead of 48 h.

3.4. Analysis and Interpretation of the Results

1. For the analysis of viability data, we use the MAD method selecting over one standard deviation [8].

2. For the analysis of phenotypic results, we consider hits the siRNAs or small molecules that induce a multipolar spindle phenotype at a frequency which is at least two times over the one observed in the control-treated centrosome-amplified cells and at the same time do not induce the multipolar spindle phenotype in control cells (Fig. 2).

3. The common hits between the two different readouts represent our high confidence hits, which we validate further for their suitability to be used as targets for drug development for which both the target population and the underlying mechanisms involved are known.

4. Confirmed hits from the small molecule~~The results from the chemical~~ library serve three purposes: to identify tools that can be used at later stages of drug development, for determining the target population, and for providing potential models for structure–activity relationship (SAR). In addition, small molecules have the advantage of being suitable for dose-response experiments that are helpful in validating borderline hits from the kinome siRNA libraries.
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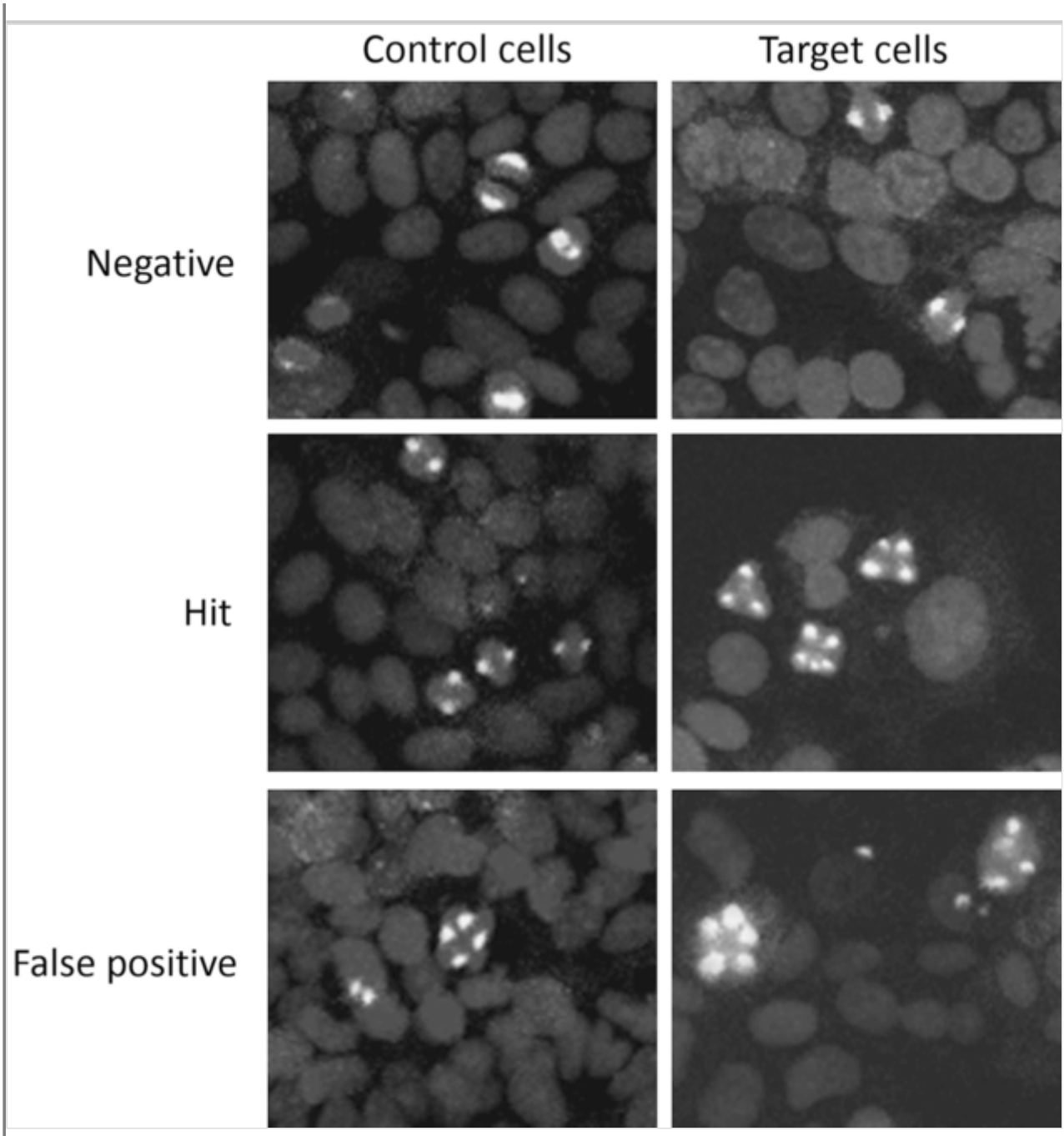
5. Image acquisition and analysis can be performed with most major HCS systems. For imaging, ×20 long working distance objective provides sufficient resolution for downstream analysis either with

the respective proprietary software for each platform or with third-party software (e.g., CellProfiler). In principle:

- Use the DAPI signal for the initial segmentation (“find nuclei”); this is optional, but it helps to quantify toxicity.
- Select pHH3-positive nuclei (“find mitotic”) for further analysis.
- Expand the borders of the pHH3 signal by 9–10 pixels to make sure the mitotic spindle (Aurora-A signal) is included in the region of interest (“find cytoplasm”). Cytoplasm border can also be determined from the Aurora-A signal. However, in general, outlining the cytoplasm border with the pHH3 signal is more robust, especially in confluent samples.
- Count the number of spindle poles/pHH3-positive nucleus.
- Export single-cell data in CSV format, and calculate in Excel the percentage of multipolar mitoses relative to all mitoses (bipolar and multipolar).

Fig. 2

Example images acquired with IN Cell Analyzer 3000 (GE Healthcare). Control cells are 4 N DLD1 cells with no centrosome amplification; target cells are 4 N DLD1 cells with centrosome amplification. First set shows cells transfected with siControl nontargeting siRNA (negative), second set shows cells transfected with siKIFC1 (hit), and the third set shows cells transfected with siESPL1 (false positive) which is known to cause cytokinesis failure when knocked down. Note that in the first set, no multipolar spindles are observed; in the second set, multipolar spindles are observed only in the target cells; and in the third set, multipolar spindles are observed on both control and target cells



4. Notes

1. Aurora-A staining has the advantage of localizing at the spindle poles (in contrast to α-tubulin staining that decorates the whole spindle) and at the same time producing a large enough area of signal that can be easily separated from background (in contrast to γ-tubulin or pericentrin staining). This protocol can be applied to any cell line as long as they express Aurora-A. Also, it allows visualization of both spindle structure and centrosomes with a single antibody.

2. Do not use more than 250,000 cell/mL as it will make it harder to separate the different phases of the cell cycle. Also, avoid keeping the cells with Hoechst for more than 3 h to avoid toxicity. The procedure will give stable diploid and tetraploid populations due the tendency of the cells that do not originally present centrosome amplification to reduce the centrosome number back to normal over time but maintaining 4 N DNA content. Cells with 4 N content and no centrosome amplification are used as a control cell line for the purpose of screening. 4 N cells with centrosome amplification are generated by 24 h incubation with DCB followed by a 24 h release. These cells will maintain abnormal centrosome numbers for a period of 6–10 days during which they can be used for screening.

3. When the plates are placed in the incubator, temperature and evaporation gradients start to form inside the plates which result in positional biases (edge effects) that introduce noise in the final data. The most affected samples are the ones in the periphery of the plate. Filling the spaces between the wells with PBS reduces the edge effect. Stacking the plates inside the incubator will also influence the evaporation gradient inside the plate, especially when the top plate of a stack is compared to the bottom plate of a stack. If the plates must be stacked, then the plates that are to be compared directly should be in the same position on different stacks.

4. Different small molecules have different efficacies. A very potent inhibitor can inhibit its target at nanomolar concentrations and start being toxic at micromolar concentrations. Similarly, a less potent inhibitor might only work at micromolar concentrations. When screening small molecule libraries, it is best to do it at three different concentrations minimum (e.g., 100 nM, 1 μM, 10 μM) in order to maximize the production of useful data.
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