**RNAi/CRISPR-KO Screen Assay Development SOP**

The assay development process for imaging-based RNAi or CRISPR/Cas9 knock-out (KO) screens in 384-well format involves the optimization of experimental conditions for cell growth, oligo siRNA/sgRNA transfection, and image acquisition and image analysis. All these parameters need to be tested in advance of the actual siRNA/sgRNA primary screen. In this document, we break down the RNAi/CRISPR-KO screen assay development process in 4 steps, we describe the goals of each step, and we provide a list of all the necessary reagents/consumables that the user will need to purchase in advance, or that HiTIF will provide at a cost to the user. A complete list of consumables, reagents and instrumentation used for the assay development and the primary screen is provided at the end of the document.

Additionally, we identify useful Milestones that need to be reached BEFORE proceeding to the next step.

**MILESTONE 1:** The user needs to identify and show the data of one or two relevant **biological siRNA controls** that will elicit a significant measurable change compared to the negative control in the assay. These controls will be used in Steps 2 and 3 of the assay development. HiTIF will provide the **transfection controls**.

**MILESTONE 1A**: Should the endpoint of the siRNA/sgRNA screen be visualized by immunofluorescence (IF), an optimized IF protocol with primary and secondary antibodies along with supporting data will also be required.

**STEP 1: Optimize cell seeding density in 384-well imaging plates.**

The goal of this step is to identify cell growth conditions that allow optimal high-throughput imaging acquisition and analysis. The initial cell seeding density is the key parameter optimized here. Different numbers of cells per well will be seeded in different columns of the 384-well plate (The actual numbers tested are dependent on the cell line used and the length of the incubation, but a reasonable starting range is between 600 and 2,000 cells/well for 72 hrs of growth).

We suggest testing the following number of cells per well, **40 ul** final volume:

600 cells/well = 1.5 x 104 cells/ml

800 cells/well = 2.0 x 104 cells/ml

1,000 cells/well = 2.5 x 104 cells/ml

1,200 cells/well = 3.0 x 104 cells/ml

1,500 cells/well = 3.75 x 104 cells/ml

2,000 cells/well = 5.0 x 104 cells/ml

Seed 2 full columns for each cell density.

At the end of the incubation period (which must be the same as the one planned for the actual RNAi screen experiment), cells will be fixed in 4% paraformaldehyde (PFA), stained with a DNA fluorescent marker (DAPI, DRAQ5, Hoechst 33342, or similar), and the Columbus image analysis software will be used to quantify the number of nuclei per well in the images generated by the Yokogawa CV7000S or CV8000 high-throughput microscopes. Visual estimation of the images will also be used as a criterion to estimate the best initial number of cells to be seeded. Cells should neither be to too sparse, nor too confluent. As a rule of thumb, ideal cell seeding conditions should lead to ~70 - 80% confluency at the end of the incubation period.

Reagents/Consumables required for this step:

* 1, re-useable Multidrop Combi Standard tube dispensing cassette, 5 to 2,500µL dispensing range (ThermoFisher Scientific, Cat 24072670).
* 1, CellCarrier 384 Ultra imaging plate (PerkinElmer, Cat# 6057300, 50 plates/case).
* 2 vials of 16% paraformaldehyde (PFA, 10x10 ml, EMS, Cat# 15710) per plate to prepare 20 ml of 8% PFA in PBS. 40 ul 8% PFA will be added per well.
* Nuclear stain
	+ DAPI (Sigma, Cat# D9542-5MG) Stock at 2.5 mg/ml. The working concentration is 2.5 ug/ml.
	+ Hoechst 33342 (Sigma, Cat# B2261-25MG) Stock at 20mg/ml. The working concentration is 1 ug/ml.
	+ DRAQ5 (Biostatus Cat# DR50200) Stock at 5 mM. The working concentration is 1: 5,000.
	+ Or any other appropriate nuclear stain.

At this time, users will receive training on the use of the ThermoFisher Multidrop liquid dispenser, the Yokogawa CV7000S/CV8000 high-throughput microscopes, and Columbus for image analysis.

**Milestone 2**: The user analyzes the cell seeding density and schedules a consultation with the Facility Director for review. Once the optimal seeding density has been established, the user proceeds to STEP 2.

**STEP 2: Optimize transfection conditions in 384 well imaging plate.**

The goal of this step is to identify the optimal quantity of transfection to efficiently reverse-transfect siRNA or sgRNA oligos, while at the same time minimizing cellular toxicity due to the transfection itself. The ECHO525 acoustic liquid handler will be used by HiTIF personnel to automate the dispensing of the various test siRNA/sgRNA oligos on the assay imaging plate. A non-targeting siRNA/sgRNA will be used as a negative transfection control. In addition, for CRISPR-KO screens, an sgRNA pool against the *ORF105A* olfactory receptor gene will be used as a non-biologically relevant, but DNA cutting negative control. An siRNA or sgRNA against either *PLK1* or a combination of toxic siRNA’s will be used as a positive transfection control with cell killing or cytostasis as the visual readout. Negative and positive controls will be matched to the actual chemistry/format (Pooled vs. singles) of the actual siRNA/sgRNA oligo library used for the RNAi/CRISPR-KO screen.

HiTIF uses the reverse transfection method for introducing RNAi/sgRNA oligos into the cells. First, the oligos are spotted and dried onto the image plate. The plate is then sealed with AluFoil and stored at minus 30oC for up to 6 months. The user will be introduced to the subsequent steps of adding the relevant reagents using the Multidrop Combi (for dispensing serum-free media and cells) and the ECHO525 (for dispensing the transfection reagent).

Reagents/Consumables required for this step:

* All items from STEP 1.
* Lipofectamine RNAiMAX (ThermoFisher Scientific, Cat# 13778150), or other transfection reagent.
* Negative and Positive siRNA controls:
	+ Non-Targeting #2 siRNA (Fisher Scientific, Cat#D0012061405).
	+ Human PLK1 siRNA (Fisher Scientific, Cat#M00329001).
	+ AllStars Hs Cell Death (Qiagen, Cat# 1027299).
	+ Silencer® Select Negative Control No. 2 siRNA (Life Technologies: 40 nmol size #4390847).

Users should begin to analyze their data on their own to determine the optimal RNAiMax volume and they should begin to familiarize themselves with the image analysis scripts in Columbus. Based on these data, HiTIF will provide guidance on the optimal experimental conditions.

**Milestone 3**: The user analyzes the transfection results and schedules a consultation with the HiTIF Director for review. Once the optimal transfection condition has been established (RNAiMax volume and siRNA/sgRNA concentration), the user proceeds to STEP 3.

**STEP 3: Test siRNA/sgRNA oligos transfection on an entire 384-well imaging plate to identify possible spatial artifacts. Additional optimization of automated IF staining procedures with the Biotek EL406 plate washer/dispenser (If required).**

HiTIF will utilize the ECHO525 acoustic liquid dispenser to set up a test of a full 384-well imaging assay plate containing an array of both positive and negative siRNA or sgRNA controls. The user will test the optimized transfection protocol on this test plate before conducting the actual transfection on the siRNA or sgRNA library. **Positive biological siRNA or sgRNA oligo controls provided by the user will also be tested at this stage.**

**Reagents/Consumables required for this step:**

* All items from STEP 1.
* 1, 384-well LDV plate (Beckman Coulter, Cat# XYZ).
* One or two biological positive controls siRNA or sgRNA oligos provided by the user. These siRNA or sgRNA olgios must match the format (pooled vs. singles) and the chemistry of the oligos used in the actual RNAi/CRISPR-KO screening.
* Negative and Positive siRNA controls (HiTIF):
	+ All the technical control oligos used for STEP 2.
* Lipofectamine RNAiMAX (ThermoFisher Scientific, Cat# 13778150) and/or other transfection reagent.

**MILESTONE 4A**: The user analyzes the transfection results with their biological controls and HiTIF transfection controls, and schedules a consultation with the HiTIF Director for review. Once the optimal transfection condition has been established (RNAiMax vol and siRNA concentration), the user proceeds to STEP 3.

**MILESTONE 4B**: Optimization of automated IF staining procedures will be conducted and finalized during STEP 3, if the user requires this for their endpoint readout.

**STEP 4: Primary screening of the siRNA/sgRNA library.**

Once STEP 3 has been verified, HiTIF will then spot the siRNA/sgRNA library onto 2 sets of 384-well assay imaging plates to allow for screening of the siRNA/sgRNA library in biological duplicates. At this time, one or two biological controls provided by the users will also be added to the library source plates prior to siRNA/sgRNA spotting. Every 384-well assay imaging plate will include 8 wells each for the negative, transfection-positive, biological control-positive 1, and biological control-positive 2 (if needed) siRNA controls.

Reagents/Consumables required for this step:

* siRNA or sgRNA library daughter plate set.
* All items from STEP 1.
* 1, 384-well LDV plate (See STEP 3).
* One or two biological positive controls siRNA or sgRNA oligos provided by the user and tested in STEP 3.
* Negative and Positive siRNA controls (HiTIF), as tested in STEP 2 and STEP 3.
* Lipofectamine RNAiMAX (ThermoFisher Scientific, Cat# 13778150) and/or other transfection reagent.

**RNAi Screen Required Reagents and Instrumentation**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Items provided by user** | **Step 1** | **Step 2** | **Step 3** | **Step 4** |
| Cells | Yes | Yes | Yes | Yes |
| Medium, FBS, supplements | Yes | Yes | Yes | Yes |
| OptiMem | - | Yes | Yes | Yes |
| 16% PFA | Yes | Yes | Yes | Yes |
| Nuclear Stain (DAPI, Hoechst, or other) | Yes | Yes | Yes | Yes |
| Lipofectamine RNAiMAX | - | Yes | Yes | Yes |
| siRNA controls: Biological (1 or 2) | - | Yes | Yes | Yes |
| siRNA control: Negative | - | Yes | Yes | Yes |
| siRNA control: Positive | - | Yes | Yes | Yes |
|   |   |   |   |   |
| **Items provided by HiTIF (Later charged to user)** |   |   |   |   |
| 384-well LDV plate (Beckman) | - | Yes | Yes | Yes |
| Multidrop Combo Standard reusable dispensing cassette (\*one charge) | Yes | Yes | Yes | Yes |
| 384-well Ultra CellCarrier imaging plate | Yes | Yes | Yes | Yes |
|   |   |   |   |   |
| **HiTIF equipment Used** |   |   |   |   |
| ThermoFisher Multidrop (automated cell seeding and tranfection) | Yes | Yes | Yes | Yes |
| Yokogawa CV7000S/CV8000 Imager | Yes | Yes | Yes | Yes |
| EL406 plate washer/dispenser | - | - | Yes | Yes |
| ECHO525 acoustic Liquid Handler | - | Yes | Yes | Yes |
| Cell Culture Hood/Incubator | Yes | Yes | Yes | Yes |
|  |  |  |  |  |

**Vendor/Cat#/Pricing for consumables**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Item** | **Vendor** | **Cat #** | **Quantity****used** | **HiTIF charge per item** | **User charge** |
| Multidrop Combi Standard tube dispensing cassette, 5 to 2500µL dispensing range | ThermoFisher Scientific |  24072670 |  **1** | **$483.49** | **$483.49** |
| DAPI | Sigma | D9542-5MG | \*\* |   |   |
| Hoechst 33342 | Sigma | B2261-25MG | \*\* |   |   |
| DRAQ5, 5mM | Biostatus | DR50200 | \*\* |   |   |
| OptiMem | multiple vendors |   | \*\* |   |   |
| Lipofectamine RNAiMAX, 1.5 ml | ThermoFisher Scientific | 13778-150 | **1** | **$373.86** | **$373.86** |
| 16% paraformaldehyde (10x10 ml) | EMS | 15710 | **20 ml/plate** | **$2.70** | **$5.40** |
| NonTargeting #2 siRNA | Fisher Scientific | D0012061405 |  |  |  |
| Human PLK1 siRNA | Fisher Scientific | M00329001 |  |  |  |
| Allstars Hs Cell Death  | Qiagen | 1027299 |  |  |  |
| Silencer® Select Negative Control No. 2 siRNA (40 nmol) | Life Technologies | 4390847 |  |  |  |
| 96-well Greiner 350 ul Microplate | VWR | 82050-678 | **3** | **$2.46** | **$7.38** |
| 384 Small Volume Microplate, 90 ul, storage plate | Greiner BioOne | 784201 | **1** | **$4.20** | **$4.20** |
| p30 MDT 384 tips | Perkin Elmer | 6900028 | **2\*** | **$42.61** | **$85.22** |
| 20 ul RoboRack tips | Perkin Elmer | 6000677 | **2** | **$9.60** | **$19.20** |
| 200 ul RoboRack tips | Perkin Elmer | 6000681 | **1** | **$9.60** | **$9.60** |
| 384 well, CellCarrier, Ultra, Imaging plate | Perkin Elmer | 6057300 | **3\*** | **$17.93** | **$53.79** |

\*: Does not include units required for final primary screen.

\*\*: User provided

RNAi Screen Assay Development Required Reagents Vendor, Cat. Number and Cost

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Item** | **Vendor** | **Cat #** | **Quantity****used** | **HiTIF charge per item** | **User charge** |
| Multidrop Combi Standard tube dispensing cassette, 5 to 2500µL dispensing range | ThermoFisher Scientific |  24072670 |  **1** | **$483.49** | **$483.49** |
| DAPI | Sigma | D9542-5MG | \*\* |   |   |
| Hoechst 33342 | Sigma | B2261-25MG | \*\* |   |   |
| DRAQ5, 5mM | Biostatus | DR50200 | \*\* |   |   |
| OptiMem | multiple vendors |   | \*\* |   |   |
| Lipofectamine RNAiMAX, 1.5 ml | ThermoFisher Scientific | 13778-150 | **1** | **$373.86** | **$373.86** |
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| Human PLK1 siRNA | Fisher Scientific | M00329001 |  |  |  |
| Allstars Hs Cell Death  | Qiagen | 1027299 |  |  |  |
| Silencer® Select Negative Control No. 2 siRNA (40 nmol) | Life Technologies | 4390847 |  |  |  |
| 96-well Greiner 350 ul Microplate | VWR | 82050-678 | **3** | **$2.46** | **$7.38** |
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