**Reverse siRNA/sgRNA Transfection Protocol for Users (ThermoFisher Multidrop Combi)**

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Date: 08/14/2021

Revision: 3

This protocol is used at HiTIF for functional genomics screens that use siRNA/sgRNA oligos spotted and dried on the bottom 384-well imaging assay plates. The protocol assumes that the barcoded imaging assay plates were previously spotted with the appropriate amount of siRNA/sgRNA in all columns, dried and stored at -30 0C.

Equipment used:

* Labcyte ECHO525 acoustic dispenser (Room D304)
* ThermoFisher Multidrop Combi dispenser (Room D304, cell culture hood)
* ThermoFisher Standard tube dispensing cassette (Cat# 24072671)
* 384-well LDV plate (Source)
	+ Echo Qualified 384-Well Low Dead Volume Plus Microplate (Beckman, Cat# LPL-0200)
* 384-well imaging plate (Destination)
	+ Perkin Elmer, CellCarrier-384 ultra +LID, Cat# 6057308
* BioRad TC20 Automated Cell Counter

Reagents:

* 40 ml of freshly prepared (Same day) 10% Bleach in a 50 ml conical tube
* 2 X 40 ml of sterile water in 2 separate 50 ml conical tubes
* 40 ml of 0.1% Tween 20% in water in a 50 ml conical tube
* 2 x 40 ml of sterile PBS in a 50 ml conical tube
* Medium with 20% FBS.
* Serum-free medium
* Medium for Transfection (You can use OptiMEM or regular medium for your cells): Opti-MEM™ Reduced Serum Medium, GlutaMAX™ Supplement (ThermoFisher Cat# 51985-034)
* RNAiMAX (ThermoFisher, Cat# 13778150) in one or two wells of LDV plate.

Cells:

* Medium: All cells used in HiTIF have grown well using OptiMEM for the cell optimization and reverse transfection protocols. HiTIF suggests using OptiMEM.
* Count cells using an automated cell counting instrument. Always use the same instrument for downstream applications. The HiTIF is equipped with the BioRad TC20 Automated Cell Counter.
* At least 40 ml of cells resuspended in Medium + 20% FBS at the appropriate concentration in a 50 ml conical tube
	+ For Reverse Transfection
		- Calculate 15 ml for initial priming, then 10 ml volume for an ENTIRE plate
		- Add 20 ul per well

**Tips and Tricks:**

1. Do NOT run the tubing dry between solution changes. It is our experience that bubbles form at the nozzles and interferes with volume dispensing and sterility.
2. Follow the solution sequence as specified with priming volumes.
3. Beware of the BLACK cords. If these wrap around the peristaltic pump wheel, you **cannot** attach the cassette. Ensure that these are INSIDE the outer WHEELS. This is a very common problem.
4. Submerge the end of the dispensing tubes to the bottom of the tube without touching it with your hands and avoiding it touching other non-sterile surfaces, since you want to you keep the tubing as sterile as possible.

**Preliminary Steps:**

1. Set up the ECHO525 to dispense the appropriate volume of RNAiMAX to each well of your assay plate.
2. Prepare the solutions to sterilize the tubing.
3. Use the keypad on the Multidrop to have the correct program displayed and ready to go.

**Prepare the Multidrop by sterilizing the cassette tubing.**

1. Wear gloves
2. Mount the dispensing cassette on the Multidrop.
3. Remember to pull the blue protective shield on the Multidrop all the way forward.
4. Connect the drain tube to the aspirator and turn on the vacuum.
5. Submerge the aspiration end of the cassette tubes in the bleach tube, all the way to the bottom of the tube.
6. Prime the cassette with 20 ml of Bleach
7. Prime with 20 ml of water (Tube 1)
8. **Change the water tube**
9. Prime with 20 ml of water (Tube 2)
10. Prime the cassette with 20 ml of PBS
11. Prime the cassette with 20 ml of Serum-free medium

**Resuspend the oligo siRNA/sgRNA and complex it with the transfection reagent.**

1. **Add your plate to the stage**.
2. **Remove the plate lid**!
3. Dispense 20 ul of Serum-free medium to each well.
4. Place the plate lid back onto the plate.
5. Process the next plates as needed.
6. Use the ECHO to dispense the appropriate volume of RNAiMAX to the wells.
7. Incubate at RT for 30 minutes. (With lid on and in the hood).

**Harvest cells from your flask(s).**

1. Centrifuge to pellet cells. (1500 RPM for 5 minutes)
2. Resuspend cells in 10 ml Serum-free medium.
3. Conduct a cell count using the Auto Cell Counter.
4. Resuspend cells at the appropriate cell density in 40 ml Medium with 20% serum. I generally set up the cells as a dilution:
	1. 8 ml FBS
	2. X ml Cells
	3. Y ml of Medium with 20% serum.
	4. Final volume of **40 ml per Tube**. The number of tubes is dependent on the total number of plates you are transfecting.
	5. The first 40ml is for **priming** and the **first plate**.
	6. The next 40 ml are for 3 plates. I do not try to squeeze a 4th plate.
5. I always try to have an extra tube of cells, just in case. Some key cell numbers:
	1. 600 cells/20 ul per well at 3 x 104 cells/ml
	2. 1000 cells/20 ul per well at 5 x 104 cells/ml
	3. 1500 cells/20 ul per well at 7.5 x 104 cells/ml
	4. 2000 cells/20 ul per well at 1 x 105 cells/ml

**Add Cells to the Transfection mix**

1. **Gently vortex your cells to mix**.
2. Prime the cassette with 20 ml of Cells
3. **ADD YOUR PLATE TO THE STAGE**.
4. **Remove the plate lid!**
5. Dispense 20 ul of cells to each well.
6. Place the plate lid back onto the plate.
7. Process the next plate as needed.
8. Incubate at RT for 30 minutes. (With lid on and in the hood).
9. Incubate at 37oC for 72 hours.

**Clean and Disinfect the Multidrop Cassette**

1. Prime the cassette with 20 ml of PBS
2. Prime the cassette with 20 ml of 0.1 Tween 20%
3. Prime the cassette with 20 ml of Bleach
4. Prime with 20 ml of water
5. **CHANGE WATER TUBE**
6. Prime with 20 ml of water
7. Slide the Blue shield back
8. Put the cassette back in the box
9. Write down the estimated total volume used in the experiment (Including all washes)