Single cell dilution protocol for transfected (adherent) cells in 96-well plates

Reagents and Equipment Required

	Tick Box
15 mL and 50 mL conical bottom tubes	
20 µL and 200 µL filter pipette tips	
Single channel manual pipettes or similar	
1.5 mL microcentrifuge tubes	
5, 10 and 25 mL stripettes	
10 mL sterile reagent reservoir	
Multichannel pipettes or similar	
96 well cell culture plates (up to 30)	
Cell culture media	
Phosphate buffered saline (PBS)	
TrypLE™ (Life Technologies 12605010) cell dissociation reagent or similar	
250 mL sterile bottles	
Disposable hemocytometer (or equivalent)	
Trypan blue	

Day 1: Single Cell Dilution

1. Warm media for at least 10 minutes at 37ºC in a water bath.
2. Transfer cell culture vessel containing transfected cells from the humidified CO2 incubator to a MSC.
3. Remove existing media from cells and dispose to waste bottle.
4. Wash cells with PBS.
5. Aspirate PBS from flask and place in waste bottle.
6. Add 1x TrypLE™ (or other appropriate cell dissociation reagent) to cells (adjust volumes of TrypLE™ added according to the size of the cell culture vessel).
7. Incubate the cells for 5 minutes at 37ºC and 5% CO2 until the cells detach.
8. Transfer flask back to MSC.
9. Triturate cells 3-4 times with 5ml stripette to break up any cell clumps.
10. Add equal volume of media to that of the TrypLE™ added to the flask to neutralize the TrypLE™ activity.
11. Transfer the cells to a 15 mL conical bottom tube.
12. Add 20 µL 1x Trypan Blue to a microcentrifuge tube.
13. Add 20 µL neutralized cells (from step 11) to the same microcentrifuge tube.
14. Mix cells by gentle pipetting.
15. Transfer 10 µL of cell suspension to a well of disposable hemocytometer.
16. Transfer the hemocytometer to an inverted microscope.
17. Count the number of live cells in 3 fields of the hemocytometer (where each field is a 4x4 grid) and determine the mean of these counts [(count1+ count2 + count3)/3].
18. Calculate the concentration of cells (cells/mL) in the cell suspension: Concentration (cells/ml) = Mean x 2 x 10^4.
19. Dilute cells to 1 x 10^4 cells/mL in 1 mL of fresh media: volume of cells to add (in ml) can be calculated as follows:

    ($1x10^4$) x volume required (mL) / Cell concentration (from step 18).
20. Below is the calculated volume of cells (at 1x104 cells per mL) required to be added to 220 mL media for ten 96-well plates for each of 3 cell densities:

1 cell/well (5 cells/mL)	2 cells/well (10 cells/mL)	5 cells/well (25 cells/mL)
5 (cells/mL) x 220 (mL) / $1x10^4$ (cells/mL)	10 (cells/mL) x 220 (mL) / $1x10^4$ (cells/mL)	25 (cells/mL) x 220 (mL) / $1x10^4$ (cells/mL)
110 µL	220 µL	550 µL

21. Aliquot 220 mL of complete cell culture media into a suitable sterile vessel (e.g. 250 mL sterile bottle). Label each vessel appropriately with the cell concentration.
22. To the media in the bottle add the appropriate volume of cell suspension.
23. Transfer the diluted cells (1 cell/well, 2 cells/well, 5 cells/well) to large reservoir.
24. Dispense 200 µL/well in all 12 columns of ten 96-well plates using a multichannel pipette.
25. Dispose of excess cell suspension to waste bottle.
26. Repeat steps 23 to 25 for each cell dilution.
27. Dispose of waste according to the local regulations.
28. Transfer plates to a humidified incubator at 37ºC, 5% CO2.
29. Visually inspect the plates periodically from 7 to 10 days under a microscope to assess if colonies are establishing.

Day 10-14: Visual Identification

30. Visually screen plates using a microscope for clear single colonies. Mark these by circling the well on the lid and/or record in a spreadsheet. When colonies reach 70% confluence, the cells may be harvested to assess for the engineering event.

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