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Library transfection

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Abstract

This protocol details the transfection in 6-well plates.

Materials

Materials

Growing culture of HEK293T-LLP-iCasp9-Blast (Matreyek et al. 2020)

DPBS no calcium no magnesium **Gibco - Thermo Fischer Catalog #**14190250

Trypsin-EDTA 0.05% phenol red **Gibco - Thermo Fischer Catalog #**25300054)

D10 media

- DMEM, high glucose **Thermo Fisher Catalog #**11965118)
- Fetal Bovine Serum, Tet system approved, USDA-approved regions **Thermo Fisher Scientific Catalog #**A4736401
- Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #**15140122

TC-treated Cell Culture Flask 75cm2 **Thomas Scientific Catalog #**1194Z07

TC-Treated Cell Culture Flasks 175cm2 Cell Culture Flask, Vent Cap, TC, Sterile **Thomas Scientific Catalog #**709003

GenClone 25-105, 6-Well Cell Culture Plates Flat Bottom Wells, TC Treated, 100 Plates/Unit **Genesee Scientific Catalog #**25-105

Doxycycline (2mg/mL)

10mg/ml Blasticidin **InvivoGen Catalog #**ant-bl-1

1.7 mL microcentrifuge tubes (Thomas Scientific, Catalog # 1159M35)

Trypan Blue Solution 0.4% **Thermo Fisher Scientific Catalog #**15250061

Hemocytometer

Rimiducid (AP1903) 10nM (MedChem Express, Catalog # HY-16046)

15-mL conical tubes, sterile **VWR International Catalog #**89039-664

Opti-MEM™ I Reduced Serum Medium **Thermo Fisher Scientific Catalog #**31985070

FuGENE(R) 6 Transfection Reagent, 5 X 1ml **Promega Catalog #**E2692

Plasmids (quantify the concentration of each plasmid via Qubit or Nanodrop)

- Bxb1
- Gene of interest library
- attB-mCherry
- pMax-GFP
- WT GOI
- Empty vector (no GOI inserted)

Equipment

- Biological Safety Cabinet

- Incubator
- Aspirator
- Pipettes
- Serological pipettor
- Centrifuge

	A	B	C	D
	Set up for Tube B - Fugene dilution			
	Sample	**volume of OptiMEM (uL)**	**volume of Fugene 6 (uL)**	**Note**
	controls	115	5	Should be used for all controls - WT, pMax-GFP, attB-mCherry, no transfection control, no GOI insert Each control will occupy one well of a 6-well plate
	Library	724.5	31.5	Library transfections will occupy all 6 wells in 3 plates, so this mix should be made 3 times

	A	B	C	D	E
	Set up for Tube A - plasmids for 6-well plate transfection				
	Plasmid	**Concentration (ng/uL)**	**DNA volume (1120ng/rn)**	**Volume of BXB1 (80 ng/rn)**	**OptiMEM to120µl/rn**
	TSC2_Lib 2 (1/3)	629	11.22	6.3	738
	TSC2_Lib 2 (2/3)	629	11.22	6.3	738
	TSC2_Lib 2 (3/3)	629	11.22	6.3	738
	TSC2_WT		3.07	1	116
	EV		2.13	1	117
	pKam 05		3.47	1	116
	Pmax_GFP		1.15	1	118

	A	B	C	D	E
	No transfection	0	0	1	119

Safety warnings

Use BSL-2 precautions.

Before start

- Be sure that the HEK293T-LLP-iCasp9-blast cell line (Matreyek et al 2020) expresses BFP at a level of at least 98% before beginning a transfection (see Purity Sorting HEK293T LLP iCasp9 Cells).
- VAMP-seq library transfections can be scaled up to 10cm^2 culture dishes. Here we detail the protocol for transfection in 6-well plates.
- Allow Fugene 6 to come to room temperature before use.

Day -2

1 Two days before the planned transfection, passage HEK293T-LLP-iCasp9-blast culture and seed 2 T-175 flasks (more or less, depending on the number of cells needed).

> **Note**
>
> Do not add doxycycline or blasticidin to the D10 media.

Day 0

15h 30m

2 In the morning (or night before), lift and plate cells at 50% confluence in 4 6-well plates. Let the plates sit in the BSC for 00:30:00 after plating, before carefully placing them in the incubator.

30m

2.1 At least 7-8 hours after plating the cells, transfection can begin.

> **Note**
>
> Cells should mostly be adhered to the plate with minimal cell division.

2.2 For a 6-well plate, aim to plate 500,000 - 600,000 cells per well.

3 Set up two sets of 1.7-mL microcentrifuge tubes for each sample (2 per well)

3.1 Label tubes with the sample/control name and A or B for the corresponding tube.

4 To the set of B tubes, add OptiMEM first and Fugene 6 second as outlined in the table below.

	A	B	C	D
	Set up for Tube B - Fugene dilution			
	Sample	**volume of OptiMEM (uL)**	**volume of Fugene 6 (uL)**	**Note**

	A	B	C	D
	controls	115	5	Should be used for all controls - WT, pMax-GFP, attB-mCherry, no transfection control, no GOI insert Each control will occupy one well of a 6-well plate
	Library	724.5	31.5	Library transfections will occupy all 6 wells in 3 plates, so this mix should be made 3 times

4.1 It is very important to add Fugene 6 **second** in this step.

4.2 When adding Fugene 6 to the OptiMEM, do NOT touch tube walls with the pipette tip.

4.3 Mix by pipetting gently up and down.

5 Allow the B tubes to incubate at Room temperature for at least 00:05:00

5m

6 To the set of A tubes, add OptiMEM first, Bxb1 plasmid second, and sample plasmid last as outlined in the table below. Mix by pipetting or a short vortex.

	A	B	C	D	E
	Set up for Tube A - plasmids for 6-well plate transfection				
	Plasmid	**Concentration (ng/uL)**	**DNA volume (1120ng/rn)**	**Volume of BXB1 (80ng/rn)**	**OptiMEM to120µl/rn**
	TSC2_Lib 2 (1/3)	629	11.22	6.3	738
	TSC2_Lib 2 (2/3)	629	11.22	6.3	738
	TSC2_Lib 2 (3/3)	629	11.22	6.3	738

	A	B	C	D	E
	TSC2_WT		3.07	1	116
	EV		2.13	1	117
	pKam 05		3.47	1	116
	Pmax_GFP		1.15	1	118
	No transfection	0	0	1	119

7 Add the DNA mixture from Tube A TO the Fugene mixture in Tube B and mix by pipetting.

Note

⚠ VERY IMPORTANT ⚠ to add the DNA to the transfection mixture

8 Allow the transfection mixture (now in Tube B) to incubate at Room temperature for at least 00:15:00 but no more than 00:30:00 . 45m

9 Remove the plated cells from the incubator and label the wells for each control and the plates for library transfection.

10 Add the transfection mixture from Tube B to the culture media in a dropwise manner to the appropriate well. Swirl gently to mix.

Note

Make sure the pipette tip is submerged in media but not touching the flask.

11 Place the plates in a 5% CO_2, 37 °C incubator for 48:00:00 . 2d

Day 2 (48 hours after transfection)

2d

12 Remove plates from the incubator.

13 Lift cells and split the controls 1:5. Combine the library wells by plate into an appropriately sized culture flask.

13.1 This is growth dependent. If the library cells are slow growing, can put 6 wells into one T175 flask (3 flasks total). If the cells are faster-growing, combine 3 wells into one T175 flask (6 flasks total).

13.2 Cultures in T175 flasks should each contain a volume of 30mL D10 media.

14 Add [M] 2 mg/mL doxycycline to each sample and swirl gently to mix.

14.1 Add 2 µL for each control (in a 6-well plate)

14.2 Add 30 µL to each library T175 flask.

15 Incubate in a 5% CO_2, 37 °C incubator for 48:00:00 .

2d

Day 4 (96 hours after transfection)

1d

16 Prepare a portion of all cultures for flow cytometry. Passage the controls if necessary and re-plate. Re-plate the libraries without expanding the culture.

17 On the cytometer, set the laser parameters and gates for live cells and single cells.

18 Create a scatterplot with BFP fluorescence on the x axis and mCherry expression on the y axis.

19 Create a gate for the BFP-negative and mCherry-positive cells (in the top-left corner of the graph).

20 Collect data for an appropriate number of events (50,000 events) and note the percentage of single cells that are within the BFP-negative, mCherry-positive gate. This is the transfection efficiency.

21 After recording data for all controls and library samples, these cells may be discarded (aspirate into the bleach trap).

22 To the library flasks, add 30 µL of [M] 10 nanomolar (nM) AP1903 (Rimiducid) and swirl gently to mix.

22.1 AP1903 kills cells that express iCasp9. In this case, cells that express iCasp9 were not successfully recombined, and do not contain a variant from the library.

22.2 This step enriches the number of recombined cells by killing those which did not successfully recombine during transfection.

23 Incubate at 5% CO_2, 37 °C for 24:00:00 .

1d

Day 5

24 Check the library flasks. If there is a lot of cell debris, aspirate the media from the flask and replace it with 30 mL D10 media and 30 µL doxycycline ([M] 2 mg/mL).

> **Note**
>
> Do not add more AP1903.

25 Return the flasks to the incubator.

Days 6-8 (or when cells have reached confluence)

26 Continue to check on the library flasks for the next few days. Split the controls as necessary while the library flasks grow.

27 When library cultures have reached 90-100% confluence, they are ready to sort by fluorescence-activated cell sorting.

Fluorescence-activated cell sorting (FACS) - 2 days total

10m

28 Prepare half of the library flasks and half of the control cultures for flow cytometry (see the Purity sorting HEK293T LLP iCasp9 cells protocol for preparation).

29 Prepare several (5-8) sets of 4 recovery tubes for the sorted cells. Label each set with numbers 1-4 to represent the bins that the sorter will separate cells into.

- Add 2 mL D10 media to each recovery tube.

30 On the flow cytometer software, open your experiment and create tubes within that experiment for the controls and variant library.

31 Remove the cap and load the flow tube containing cells into the sample loader.

1. Press “Load” on the software.
2. Analysis should begin automatically, or press “Acquire data”.

32 Gate for single cells by gating in the following order:

a. FSC-A vs SSC-A to gate for live cells
b. FSC-A vs FSC-H to gate for single cells in the forward direction
c. SSC-A vs SSC-H to gate for single cells in the side direction

33 Create a graph that shows BFP fluorescence on the x-axis and mCherry on the y-axis.

- Adjust the BFP laser setting if needed so that the main population (with strong BFP fluorescence) is centered over 10^5.
- Adjust the mCherry laser setting if needed so that the main population (high mCherry fluorescence) is centered over 10^5 on the y axis.

34 Create a gate for the BFP-negative and mCherry-positive cells (in the top-left corner of the graph). This will be the sorted population. Note the percentage of the single cell population that expresses mCherry. Click on “Record data”.

35 Create a graph from the BFP-negative, mCherry-positive cells that shows the ratio of GFP to mCherry on the x-axis and select “histogram” on the y-axis.

- This graph will set the gates for sorting the cells.

36 Record data for all controls before beginning sorting.

37 Load a library tube in the sample holder and record data for this tube.

38 Create 4 gates on the GFP:mCherry graph - they should each contain ~25% of the total population shown on the graph. Label them Bin 1-4 from left to right.

39 Install the recovery tubes in the sorter tray with Bin 1 on the left and Bin 4 on the right.

40 In the software's experiment dashboard, open the "Cytometer settings", then open a sort layout.

41 Choose the stall which contains the recovery tube for Bin 1, and select the gated population labeled as Bin 1 to sort into the recovery tube. Repeat for the remaining 3 bins.

42 Click on the "Sort" button in the sort layout window.

43 As the flow cytometer sorts the cells, pay attention to the volumes in both the original flow tube and the recovery tube. Do NOT let the original flow tube run empty, or the recovery tube overflow. To stop the sorter, click on "Pause" in the sort layout window - this does not stop the sorter from pulling from the original flow tube. Click on "Stop Acquisition" or "Unload" if it is necessary to change the tube on the sample loader.

- Record the number of cells sorted into each Bin for each set of recovery tubes to keep track of the total number of cells sorted.

44 Sort cells into the prepared recovery tubes until the total number of cells needed is reached.

45 Remove tubes from the cytometer and follow instructions for post-sorting cleanup given by the manager of the flow cytometer.

46 Return the sorted cells to the tissue culture room. Centrifuge the recovery tubes at 300 x g, 00:05:00. Look for a small cell pellet at the bottom of the tubes.

5m

47 Aspirate the media from the recovery tubes, being very careful not to disturb the pellet.

48 Resuspend the cells from each Bin in a total of 1mL D10 media.

- Ex: Combine all recovery tubes from Bin 1 by resuspending in 1mL divided between the recovery tubes and combining them in one tube after resuspension.

49 Plate the cells from each sorted Bin into a T75 tissue culture flask with 9 mL D10 media (plus the 1mL of resuspended cells).

> **Note**
>
> Should have 4 T75 flasks labeled by bin number.

50 Add 10 µL doxycycline to each flask and swirl to mix.

51 Check there are cells in the flask by looking at each flask under the microscope.

52 Place the T75 flasks into the incubator (5% CO_2, 37 °C).

53 Repeat steps 28-48 the next day for the remaining half of the cultures.

54 Plate the cells sorted on this second day of sorting in the same flasks as the first day (e.g. Bin 1 from sorting day 2 is added to the Bin 1 flask from day 1 of sorting).

55 Incubate at 5% CO_2, 37 °C until cells are 100% confluent.

> **Note**
>
> Check the cells daily.

56 When cells are confluent, lift the cells in each flask.

57 Centrifuge the cultures at 300 x g, 00:05:00 . 5m

58 Aspirate the supernatant, do not disturb the pelleted cells.

59 Freeze the pellets in labeled 15-mL centrifuge tubes until ready to extract genomic DNA from cells.

Protocol references

Citations

Matreyek KA, Stephany JJ, Chiasson MA, Hasle N, Fowler DM. An improved platform for functional assessment of large protein libraries in mammalian cells. Nucleic Acids Res. 2020 Jan 10;48(1):e1.
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