**Genetic barcoding technology**

**“ClonTracer DNA barcode library” application protocol**

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**Contents:**

1. **CLONTRACER LIBRARY VIRUS PREPARATION**
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| **Day -1** | **Plating HEK293T cells for transfection** |  |  |  |  |
|  | Plate 2.5 x106 cells of HEK293T per 10 cm-plate in 15.5 ml ***antibiotics free***-growth medium (10% FBS). Use BD BioCoat™ Collagen I 100 mm culture dishes. |  |  |
|  |  |  |  |  |  |  |
| **Day 0** | **Transfection** |  |
|  | **I.** |  | **Per 10 cm-plate** |
|  |  |   | **Required amount (ug)** |
|  |  | **pRSI9-Barcode-ClonTracer** | **2.4** |
|  |  | **pCMV-VSV-G envelope vector** | **0.6** |
|  |  | **Delta VPR (Delta 8.9) packaging vector** | **2.4** |
|  |  | **Total** | **5.4** |
|  |  |  |  |  |
|  | **II.** |  | **Per 10 cm-plate** |
|  |  |   | **Volume (ul)** |
|  |  | **TransIT®-293 Transfection Reagent** | **16.2** |
|  |  |  |  |  |
|  | **III.** |  | **Per 10 cm-plate** |
|  |  |   | **Volume (ul)** |
|  |  | **Opti-MEM® Reduced Serum medium** | **Up to final volume of 600.00** i.e. 600 – (Total volume of pDNA) – 16.2  |
|  |  |  |  |  |  |  |  |

1. Warm TransIT®-293 Transfection Reagent (Mirus) to room temperature and vortex gently before using.

2. Place Opti-MEM® Reduced Serum medium (Invitrogen) in a sterile tube.

3. Add plasmid DNA (shown in Table I above).

4. Pipet gently to mix completely.

5. Add TransIT®-293 Reagent to the diluted pDNA mixture.

6. Pipet gently to mix completely.

7. Incubate at room temperature for 15-30 min.

8. Add 600 ul TransIT-293®-DNA complex dropwise to different areas of the plate.

9. Gently rock the plate to evenly distribute TransIT-293®-DNA complex.

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| **Day +1** | **Changing medium** |  |  |
|  | Change medium with 6 ml fresh growth medium per 10 cm plate |  |  |  |
|  |  |
| **Day +4** | **Harvesting virus** |  |
|  | 1. Harvest medium from each plate, combine & filter through cellulose acetate membrane filter (pore size: 0.45 µm).
 |  |  |
|  | 1. Aliquot into sterile tubes and store in the -80 freezer.
 |  |  |

1. **Optimizing the conditions for viral infection:**
* **The number of cells to seed**
* **The optimal concentration of puromycin for selection**
* **The optimal ratio of the concentration of virus to the number of seeded cells to achieve 10% infectivity**

1. **Determining the optimal number of cells to seed & optimal puromycin concentration for selection**

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| --- | --- | --- | --- | --- | --- |
| **Day -1** | **Plating cells in 6 well plates** |  |  |  |  |
|  | Plate 0.2x106, 0.4x106, 0.6x106 and 0.8x106 cells in 2 ml growth medium per well (if the doubling time of the cell line is not known). Prepare two or three 6-well plates per condition to have replicates. |  |  |
| **Day +1** | **Adding puromycin-containing medium** |  |  |
|  | Replace medium with 2 ml growth medium containing puromycin ranging from 0 – 4 µg/ml per well.

|  |  |  |
| --- | --- | --- |
| 0 µg/ml | 1.0 µg/ml | 2.0 µg/ml |
| 2.5 µg/ml | 3.0 µg/ml | 4.0 µg/ml |

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|  |  |
| **Day +4** | **Determining puromycin concentration by CellTiter-Glo assay or cell counting** |  |
|  | Choose the lowest puromycin concentration that caused approximately 100% cell killing. **Determining the optimal number of cells to seed**Compare cells in the wells with 0 µg/ml puromycin under the microscopy and choose the condition where the cells have not reached the complete confluency on Day +4.  |  |  |
|  |  |  |  |

1. **Determining the viral infection efficiency of the cell line**
* ***Either with pRSI9 reference virus or ClonTracer Library virus***

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| --- | --- | --- | --- | --- | --- |
| **Day -1** | **Plating cells in 6 well plates** |  |  |  |  |
|  | Plate cells in 2 ml growth medium per well. Prepare two-three 6-well plates per condition to have replicates. |  |  |
| **Day 0** | **Viral infection** |  |  |
|  | Add various amounts of virus stock per well in 1 ml of growth medium supplemented with 8 µg/ml polybrene (Millipore). Make sure not to add virus into the wells marked as “No virus”. Incubate cells with virus for 24 h.

|  |  |  |
| --- | --- | --- |
| 5.0 µl**No puromycin** | 2.0 µl | 4.0 µl |
| 6.0 µl | 8.0 µl | **No virus** |

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|  |  |  |
| --- | --- | --- |
| 30.0 µl**No puromycin** | 12.0 µl | 24.0 µl |
| 36.0 µl | 48.0 µl | **No virus** |

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| **Day +1****Day +4** | **Adding puromycin-containing medium**Remove the virus-containing medium and replace with 2 ml puromycin-containing medium per well except for the wells marked as “No puromycin”. For “No puromycin” wells, remove the virus and add fresh growth medium. Select the cells in puromycin for 72 h. **Determining the virus amount to achieve approximately 10% cell survival** |  |
|  | Choose the virus volume in 1 ml growth medium that achieved approximately 10% surviving cells compared to “No puromycin” condition. We are targeting MOI of <0.1 to make sure that one cell is labeled with one unique barcode. Use either Cell-TiterGlo assay or cell counting.  |  |  |
|  | ◼ ***FACS cell sorting for TagRFP expression*** *can replace the puromycin selection step or can be done as an additional selection process. TagRFP (Excitation: 555 nm/Emission: 584 nm) was successfully sorted by using the 582/15 band pass filter on the BD FACSAria with the 561-nm (Yellow-Green) Laser.*  |  |  |

1. **Generation of the ClonTracer-Barcoded cell lines**
2. **Viral infection with ClonTracer library in the 150 mm-dish format**

***All the conditions that were determined in the previous section in 6-well plate format will be scaled up by 15 times considering the difference in cell culture surface.***

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| --- | --- |
| **Day -1** | **Plating cells in 150 mm-Nunclon™ surface cell culture dishes** |
|  | Plate cells in 30 ml growth medium per dish. Given that we are targeting 10% surviving cells after puromycin selection, assume that 1/10 of the seeded cells on Day -1 will be uniquely barcoded. Plate at least 15 times more cells than the required number of uniquely barcodes cells for the particular experiment. Remember that two dishes will be used as controls: Control #1: No virus infection + Puromycin selectionControl #2: Virus infection + No Puromycin **◼** For example, if 6x106 cells are plated per dish, assume that we will harvest 0.6x106 cells labeled with unique barcodes from this dish. If 6x106 uniquely barcoded cells are required for the in vivo protocol (i.e. subcutaneous injection of 6x106 cells per mouse), AT LEAST 12-15 dishes of cells should be infected simultaneously to ensure having the cell population of sufficient complexity. **◼ *If the infected cells will be selected by FACS cell sorting for TagRFP expression****, consider that FACS sorting will significantly decrease cell viability and determine the number of cells to plate accordingly.*  |
| **Day 0** | **Viral infection** |
|  | Add virus stock per dish in 15 ml of growth medium supplemented with 8 µg/ml polybrene (Millipore). Make sure not to add virus into *Dish #1* “No virus + Puromycin selection”. Incubate cells with virus for 24 h.

|  |  |  |
| --- | --- | --- |
| **Dish #** | **ClonTracer virus infection** | **Puromycin selection** |
| **1 (Control #1)** | **No** | **Yes** |
| **2 (Control #2)** | **Yes** | **No** |
| **3 - N** | **Yes** | **Yes** |

 |
| **Day +1****Day +4** | **Adding puromycin-containing medium**Remove the virus-containing medium and replace with 30 ml puromycin-containing medium per dish. For Dish #2 “Virus infection + No puromycin” dish, remove the virus and add 30 ml of fresh growth medium. Select the cells in puromycin for 72 h. **Harvest the ClonTracer-barcoded cells and expand up to 14 days to have enough cells for implantation**  |
|  | Trypsinize the ClonTracer-barcoded cells (if adherent cell lines) from *Dish #3-N* and combine them together. Count the number of cells and confirm that the MOI of approximately 0.1 was achieved (i.e. 10% surviving cells after puromycin selection compared to *Dish #2*). Based on % infected cells, calculate the number of individually infected (i.e. uniquely barcoded) cells on Day 0. Re-plate the combined pool of ClonTracer virus infected cells into T150 or T225 flasks and expand them preferentially for no more than 14 days until having the enough number of cells for the treatment experiment. We have confirmed that 14 days of cell expansion does not affect the clonal diversity and barcode representation significantly. |
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1. **Generation of the ClonTracer-barcoded cell lines containing the defined number of unique barcodes**

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| --- | --- |
| **Day -1** to**Day +1****Day +4** | **Follow the protocol as described above in Section 3.1.****Harvest the ClonTracer-barcoded cells, seed the particular number of cells & expand to have enough cells for experiments** |
|  | Harvest the ClonTracer-barcoded cells from *Dish #3-N* and combine them together. Count the number of cells and confirm that the MOI of approximately 0.1 was achieved (10% surviving cells after puromycin selection compared to *Dish #2*). Based on % infectivity, calculate the number of individually infected cells on Day 0. Seed the desired number of cells out of the combined ClonTracer-barcoded cell population into culture dishes or flasks of an appropriate surface area. Expand them (preferentially for no more than 14 days) until having the enough number of cells for the designed study.  |