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ASSESSING RTTA ACTIVITY (Support Protocol 6)

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iPSCs

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Protocol status: Working
We use this protocol and it's working

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Attachments

PDF

fernandopulle2018.pd...

1.7MB

Materials

- Cell line of interest and appropriate culture medium
- pBI-MCS-EGFP plasmid (Addgene, cat. no.)

pBI-MCS-EGFP **addgene Catalog #**16542

- Doxycycline (2 mg/ml in PBS; 1000×; Sigma, cat. no. D9891)

Doxycycline hyclate **Sigma Catalog #**D9891

- Fluorescent microscope
- Additional reagents and equipment for general iPSC culture (**Basic Protocol 1**) and transfection (**Basic Protocol 2**)

Safety warnings

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

1 Prepare four wells of the cell line of interest by EDTA split (Basic Protocol 1).

Note

For at least the first time performing this protocol, include positive and negative control cells in order to compare with cells of interest.

2 1 to 2 days after plating, follow **Basic Protocol 2** (steps 6 to 9) for two of the wells in order to transfect with the pBI-MCS-EGFP plasmid.

2.1 For each transfection, add 100 µL of Opti-MEM and 3 µg of total DNA to one 1.5-ml microcentrifuge tube and vortex for 00:00:02 to 00:00:03. In a second tube, combine 100 µL of Opti-MEM and 10 µL of Lipofectamine Stem reagent, and vortex for 00:00:02 to 00:00:03.

Note

For TALEN-mediated insertion to the AAVS1 or CLYBL locus, such as for the hNGN2 (Addgene, **cat. no. 105840**) and hNIL (Addgene. **cat. no. 105841**) differentiation cassettes, use a 2:1:1 ratio of 1.5 µg donor construct with 0.75 µg of each of the site-specific TALENs.

For AAVS1: 0.75 µg of pTALdNC-AAVS1_T2 (Addgene, **cat. no. 80496**) and 0.75 µg of pTALdNC-AAVS1_T1 (Addgene, **cat. no. 80495**) per transfection.

For CLYBL: 0.75 µg of pZT-C13-R1 (Addgene, **cat. no. 62197**) and 0.75 µg of pZT-C13-L1 (Addgene, **cat. no. 62196**) per transfection.

2.2 Combine the contents of the two tubes and vortex again for 00:00:02 to 00:00:03. Incubate this mixture for 00:10:00 at Room temperature.

2.3 Retrieve the cells plated in steps 3 to 4, and, using a P200 pipet tip and repeat pipettor, add 200 µL of the complete transfection solution from step 6 **dropwise**, evenly across the surface of the well. Return the cells to the incubator overnight.

2.4 24:00:00 after transfection, aspirate transfection medium and replace with fresh E8. If applicable, evaluate transfection efficiency by fluorescence microscopy.

Note

All cells transfected with the hNGN2 (Addgene, **cat. no. 105840** or **110492**) and hNIL constructs (Addgene, **cat. no. 105841** or **105842**) will transiently express mCherry for **3 to 4 days**, while only those cells with transgene insertion will maintain **stable expression** of mCherry for longer periods of time.
See **Basic Protocols 3** and **4** for options for enrichment and clonal isolation.

3 One day later, aspirate medium and replace with fresh E8. In one trans- fected well and one un-transfected well, supplement medium with 2 µg/ml doxycycline.

Note

If cells are more than 30% confluent when transfected, doxycycline may be added at the time of transfection in order to permit imaging the next day.

4 One day later, aspirate medium, rinse twice with PBS, and image with a fluorescent microscope.

Note

eGFP typically has a half-life of 24 hr, so fluorescence should persist for at least 2 to 3 days after doxycycline treatment.

5 Since transfection is heterogeneous, only a subset of cells should express GFP. In addition, levels of expression may vary by the number of plasmid copies delivered to each cell. However, in cells with active tetracycline transactivator, the average intensity of GFP will be notably higher in the well exposed to doxycycline than in the well which was not. Some leaky expression is expected due to the sequence of the tetracycline operator in this plasmid, so un-transfected cells thus serve as a further negative control.