Hanna LAB reprogramming protocol

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Protocol for human secondary or BJ fibroblast reprogramming with Stemgent mRNA OSKML Reprogramming System and MBD3 siRNA

We provide here in reprogramming protocol that entails the use of different fibroblasts cell lines (secondary ES derived early passage fibroblast-like cells, human BJ, BJ-SV40, BJ-ERAS and BJ-hTert transgenic fibroblasts). The protocol entails combinations of MBD3 knockdown and OSKML (OCT4, SOX2, KLF4, MYC and LIN28) transfection that are conducted together or separately as indicated.

With this protocol iPSCs are attainable after 6-10 days of initiation (versus typically more than 20 days and higher number of OSKML transfection rounds when Mbd3 is not depleted).

For robust program that applies also to other primary human cell types, please see unpublished protocol on Hanna Lab Website.

*MBD3 siRNA Transfection / 6 well plate

To knock down MBD3 we use Stealth siRNAs (Set of 3) HSS147580, HSS147581, HSS182377 Cat. # 1299003 from Invitrogen. **We have similar experience if we use a mix of 3 or only HSS147581 sequence.**

Day before seed 200,000 BJ fibroblasts in each well of 6 well plate

A) Mix in one vile:

150ul Opti-MEM

9ul RNAiMAX reagent

B) Mix in second vile:

150ul Opti-MEM

When using siRNA mix of 3then : 3 x1.5 ul siRNA (20microM concentration of our starting siRNA stock)

OR

When using only one siRNA then: 1 x4.5 ul siRNA (20microM concentration of our starting siRNA stock)

A+B) Mix the two viles and incubate for 5 min RT

Add 250ul (~75pmol siRNA) to the targeted well (without changing the medium) for 12hours hours.

#Stemgent OSKML mRNA transfection

Pretreat target Cells with B18R Protein

Comments: The B18R protein must be present in the culture medium at a concentration of 200 ng/ml during every transfection. A 2-4 hour pretreatment with the B18R protein is required prior to each OSKML mRNA transfection.

Day 0 to pre-suppress the cells' interferon response.

Reprogramming is carried out in (**A**) Pluriton™ Reprogramming Medium + 5microM ROCKi (for the first 2-3 days) and then in (**B**) NHSM medium (Version **3**, **2** or **1** already including 5microM ROCKi as indicated in our in house Human naive protocol (AVAILABLE on our WEBSITE)).

1. Add 10 ml of Medium to a sterile 100 mm dish.

2. Incubate the medium for 2 hours at 37°C and 5% CO2 to equilibrate the medium to the proper oxygen tension.

3. Just prior to use, thaw one vial of Pluriton™ Supplement and one vial of B18R protein on ice.

4. Add 4 µl of the supplement and 4 µl of the B18R protein to the medium to generate Pluriton™ Reprogramming (**used in days 0-2 of reprogramming**) **or** supplemented WIS-NHSM medium (**used starting from day 2 of reprogramming**).

Medium (with B18R protein):

5. Aspirate the target cell medium from each of the 4 wells to be transfected.

6. Add 2 ml of Pluriton™ or NHSM Reprogramming Medium (both with B18R protein) to each of the 4 wells.

7. Incubate the cells for 2 hours at 37°C and 5% CO2 5-20% O2 prior to transfecting (we reprogram in hypoxia 5% O2 conditions for the first 7 days – maintenance of human naïve PSCs we prefer 5% O2, but also 20% O2 is compatible).

Prepare mRNA transfection Complex

Prior to each daily transfection. The RNAiMAX® transfection reagent must first be diluted in Opti-MEM® medium, before combining with the diluted mRNA cocktail to generate the mRNA transfection complex.

TUBE 1: (for 4 well in 6 well plate)

200 µl Opti-MEM

50 µl mRNA Cocktail (3:1:1:1:1:1 for the Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP mRNAs total of 5ug mRNA ~1.25ug for each well)

250 µl Total

TUBE 2:

225 µl Opti-MEM

25 µl RNAiMAX

250 µl Total mRNA Transfection Complex

Using a 1000 µl RNase-free, aerosol-barrier pipet tip, pipet gently but thoroughly to mix the RNAiMAX with the Opti-MEM (Tube 2).

Tube1+Tube2:

6. Transfer the entire contents of Tube 2 to the mRNA cocktail solution in Tube 1 to generate the mRNA transfection complex and pipet gently 3 to 5 times.

7. Incubate the mRNA transfection complex at room temperature for 15 minutes to allow the mRNA to properly complex with the transfection reagent.

OSKML + MBD3si iPSC Protocol 1:

- Grow fibroblasts in 5% O2 also at early stages of the reprogramming, and then are moved to 20% O2 at days 7-10 (when 20% O2 maintenance conditions are opted).

1) Seeding 200,000 cells at day -1 per well in a 6 well plate (pre-coated with gelatin/vitronectin coated plates).
2) OSKML transfection at day 0 morning.
3) Mbd3 siRNA transfection at day 2 morning.
4) Re-transfect with Stemgent OSKML mRNA at day 2 evening.
5) Mbd3 siRNA transfection at day 4 morning.
6) Re-transfect with Stemgent OSKML mRNA at day 4 evening.
7) Optional: Mbd3 siRNA transfection at day 6 morning.
8) Optional: Re-transfect with Stemgent OSKML mRNA at day 6 evening.

- Consider adding low-density irradiated MEFs after initiation of reprogramming starting from day 3 (on top of the already ongoing reprogramming cultures).
- First colonies appear after 7-10 days post first transfection, subsequently picked and re-seeded on feeders with human naïve medium WIS-NHSM.

OSKML + MBD3si Protocol 2:

1) Seeding 200,000 cells at day -2 at 200,000 cell per well of 6 well plate, and then apply MBD3 siRNA transfection at day -1.

2) At day 0 apply OSKML mRNA transfection.

3) At day 1 apply OSKML mRNA transfection.

4) At day 2 apply Mbd3 siRNA transfection.

5) At day 3 apply OSKML mRNA transfection.

6) Optional: At day 4 apply OSKML mRNA transfection.

- Consider adding low-density irradiated MEFs after initiation of reprogramming starting from day 3 (on top of the already ongoing reprogramming cultures).
- First colonies appear after 7-10 days post first transfection, subsequently picked and re-seeded on feeders with human naïve medium WIS-NHSM.