

Hanna LAB reprogramming protocol:

Protocol for mouse Mbd3 wild-type (+/+) secondary MEF or secondary adult fibroblast reprogramming following Mbd3 or Chd4 siRNA (Rais et al. Nature 2013).

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We have recently described that combined action of 1) adequate and robust OKSM induction 2) Mbd3 depletion and 3) Naïve 2i/LIF and KSR (contain Vitamin C and Albumax) containing conditions dramatically promote mouse naive iPSC induction. In controlled and optimized secondary OKSM transgenic driven reprogramming and hypomorphic Mbd3 expression, up to 100% synchronized iPSC can be achieved from mouse cells within 8 days (Rais et al. Nature 2013).

Very high reprogramming efficiencies 40-80% can also be achieved in OKSM transgenic Mbd3^{+/+} wild-type MEFs or adult fibroblasts by siRNA for Mbd3 or Chd4 (Extended Data Figure 6b – Rais et al Nature 2013). Here we provide detailed reprogramming protocol that entails the use of mouse E12.5-E13.5 or tail tip (TTFs) secondary fibroblasts (Mbd3^{+/+}) retaining robust and optimized M2rtTA and TetO driven OKSM expression.

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- Fibroblast cell line sources for reprogramming:**

We use cell lines validated to express adequate stoichiometry of OSKM, Namely:

- 1) **Rosa26-m2rtTA (+/- or +/+), mCol STEMCCA-OKSM (+/+)** as previously generated and described Stadtfeld et al. Nature Methods 2009. Strains were obtained through Jackson Laboratories #011001. Oct4-GFP or Nanog-GFP reporters were introduced when relevant and as indicated.

- 2) **Rosa26-m2rtTA (+/+), mCol-OSKM (+/+)** as previously generated and described Carey et al. Nature Methods 2009. Strains were obtained through Jackson Laboratories #011004. Oct4-GFP (knock-in or Transgene) or Nanog-GFP knock-in reporters were introduced when relevant and as indicated.
- 3) NGFP1 iPSC cell line – Hanna et al. Cell 2009 (TetO OSKM factors, Nanog-GFP knock-in reporter).

Optional: We recommend using constitutively labeled nuclear-mCherry lines to control for cell survival between samples and calculate reprogramming efficiency by fraction of Oct4 or Nanog/GFP out of total mCherry cells (or colonies).

*** siRNA mix Preparation and Transfection / per well in a 6 well plate**

To knock down mouse Mbd3 we use Stealth siRNAs mix (Set of 3) MSS237238, MSS-275658, MSS-275659 (Invitrogen). We now use only MSS-237238 alone.

To knock down mouse Chd4 (also known as Mi2B) we use Stealth siRNAs mix (Set of 3) MSS-200894, MSS-200895, MSS-200896 (Invitrogen).

A) Mix in one vile:

150ul Opti-MEM

9µl RNAiMAX reagent

B) Mix in second vile:

150ul Opti-MEM

We start from a 20microM siRNA stock. From that we take 1.5 µl for each siRNA.

When a mix of 3 siRNAs is used – total is 4.5µl (3 X 1.5 µl).

OR

When only one siRNA is used – we take 4.5µl from the siRNA starting stock.

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

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

Protocol option 1:

Cells were initially reprogrammed in 5% O₂ incubators, and moved to 20% O₂ days 6-10 after reprogrammed colonies appear.

- 1) Seeding 5,000-10,000 cells at day -1 per well in a 6 well plate (pre-coated with 0.2% gelatin and irradiated DR4 MEFs).
- 2) Day 0: Reprogramming was initiated by applying mouse ES medium containing 15% FBS (heat inactivated – ES qualified), Human LIF (5-20ng/ml – notably it is more potent than mouse LIF), Doxycycline 2µg/ml (optional: 10-50µg/ml L ascorbic acid).
- 3) Day 2: Apply Mbd3 or Chd4 siRNA transfection as indicated **above** and leave for 12 hours.
- 4) Day 3: Switch to mouse ES medium containing 15-20% KSR, Human LIF (20ng/ml), and Doxycycline 2µg/ml + 2i (1µM PD0325901 and 1µM CHIR99021) until the end of reprogramming process (day 7-10).
- 5) Day 4: Re-transfect with Mbd3 or Chd4 siRNA transfection for 12 hours as indicated **above**.
- 6) Optional: Day 6: Re-transfect with Mbd3 or Chd4 siRNAs. Transfection for 12 hours as indicated **above**.

Assay is terminated after 7-10 days post Dox initiation. Colonies can be picked and re-seeded in naïve mESC medium independent of DOX.

Protocol option 2:

Cells were initially reprogrammed in 5% O₂ incubators, and moved to 20% O₂ days 6-10 after reprogrammed colonies appear.

- 1) Seeding 5,000-10,000 cells at day -2 per well in a 6 well plate (pre-coated with 0.2% gelatin and irradiated DR4 MEFs).
- 2) Day 0: Apply Mbd3 or Chd4 siRNA transfection as indicated **above** and leave for 12 hours.
- 3) After 12 hours: Reprogramming was initiated by applying mouse ES medium containing 15% FBS (heat inactivated – ES qualified), Human LIF (5-20ng/ml – notably it is more potent than mouse LIF), Doxycycline 2µg/ml (optional: 10-50µg/ml L ascorbic acid).
- 4) Day 2: Apply Mbd3 or Chd4 siRNA transfection as indicated **above** and leave for 12 hours.
- 5) Day 3: Switch to mouse ES medium containing 15-20% KSR, Human LIF (20ng/ml), and Doxycycline 2µg/ml + 2i (1µM PD0325901 and 1µM CHIR99021) until the end of reprogramming process (day 7-10).

- 6) Day 4: Re-transfect with Mbd3 or Chd4 siRNA transfection for 12 hours as indicated **above**.
- 7) Optional: Day 6: Re-transfect with Mbd3 or Chd4 siRNAs. Transfection for 12 hours as indicated **above**.

Assay is terminated after 7-10 days post Dox initiation. Colonies can be picked and re-seeded in naïve mESC medium independent of DOX.