Hanna LAB reprogramming protocol:

Protocol for deterministic, synchronized and highly efficient (up to 100%) mouse iPSC generation, via secondary OKSM reprogramming of Mbd3$^{flox/-}$ derived somatic cells that are hypomorphic for Mbd3 protein expression. (Rais et al. Nature 2013).

Assembled: Yoach Rais, Asaf Zviran, Nofar Mor (Rais et al. Nature 2013)

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We have recently described that combined action of **1**) adequate and robust OKSM induction **2**) controlled Mbd3 depletion and **3**) Naïve 5% O2, 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 in naïve pluripotency promoting conditions and controlled hypomorphic Mbd3 expression, up to 100% synchronized iPSC can be achieved from multiple mouse cell types within 8 days (Rais et al. Nature 2013).

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Section 1: Establishing a deterministic mouse secondary reprogramming system

1) **Critical:** Starting cell lines with optimal Mbd3 depleted protein levels. **Mbd3$^{flox/-}$ ES cells were previously described in Kaji et al. Nature Cell Biology 2006, and kindly provided by Dr. Brian Hendrich (Cambridge University, UK).**
Important!!!: We use the Mbd3 3flox/- allele (described Figure S1-c in Kaji et al. Nature Cell Biology 2006), as cells derived form these ESCs have 50-90% reduction in Mbd3 protein levels in comparison to wild-type cells. Using clones that show less than 50% reduction in Mbd3 expression will yield a mild phenotype (Avoid a different Mbd3 flox/- ES cell allele described in Figure s1-a in Kaji et al. Nature Cell Biology 2006, which are not used in our study). On the contrary, starting with somatic cells that already have 100% loss of Mbd3 long before (more than 2 days

before) starting of reprogramming will also hamper reprogramming, because Mbd3 is critical for somatic cell proliferation.

2) Optional:

a) Introducing pluripotency reporters: The ES line can be used to introduce knock-in or transgene reporter for Oct4 or Nanog.
b) 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).

2) ES lines can be injected into host mouse blastocysts according to standard protocols, and secondary MEFs or other somatic cells can be derived and expanded in vitro.

3) Generation of Primary iPSC lines: E12.5 MEFs are generated according to standard protocol and expanded in 5% O2. Cells were transduced with the following optimized reprogramming vectors to ensure high and adequate induction of OKSM reprogramming factors:

1) **STEMCCA-TetO-OKSM (kindly provided by Gustavo Mostoslavsky, Boston University). (Use of adequate OKSM induction is important as well and we recommend using this vector).**
2) **FUW-Ubi-M2rtTA-LoxP (Addgene #20342) lentiviral vectors**

4) Apply Doxycycline 1-2µg/ml in mouse Reprogramming ESC medium and iPSCs appear at very high efficiency within 8 days. Make up to 10 independent sub-clones primary iPSC lines for further evaluation and experimental testing (see below).

Section 2: Utilizing a deterministic mouse secondary reprogramming system

Important comments and reminders:

1) $Mbd3^{flox/-}$ cells have sufficient residual levels that allow adequate differentiation and high contribution to all lineages in chimeric mouse embryo and adult animals. Yet Mbd3 levels are low enough not to interfere with OSKM activatory potential.

2) Different primary iPS lines have different integration pattern of OKSM, and thus different induction levels in a variety of cell lines derived from each clone. Thus it is recommended to test each primary iPSC line (generated in step 4 in the previous section) for optimality of reprogramming in each desired cell type (NPCs, B cells, TTFs etc.). For example, in our experience 70% of primary iPSC clones generated, will

be optimal for secondary MEF or NPC reprogramming as they will often induced adequate OKSM levels in these cells.

Secondary reprogramming of mouse Fibroblasts – Option 1:

1) Seed secondary transgenic cells at day -1 on plates already pre-coated with 0.2% gelatin and irradiated DR4 MEFs or irradiated HFFs in 5% O2 hypoxia conditions.
   - Irradiated DR4 feeder cell plating density: 2* 10^6 per 10c"m dish, 2X10^6 per total of 6 wells in a 6 well dish. Total of 2X10^6 MEFs divided into 96 wells of a single gelatinized plate.
   -Reprogramming MEF donor cell plating density: For 6 well plate: 100-1000 cells per well (not more than 1000 cells); For 10 c"m TC dish- 1000-10,000 cells per plate); per 96 well plate: sort 1 cell per well (mCherry can be used to correct for plating survival efficiency).
2) Day 0: Reprogramming was initiated by applying mouse ES medium containing 15% FBS (heat inactivated – ES qualified), Human LIF (10-20ng/ml – notably it is more potent than mouse LIF), Doxycycline **1**µg/ml. (optional 10µg/ml L-Ascorbic acid - up to 50µg/ml can be used).
3) Day 2-3: Switch to mouse ES medium containing 15-20% KSR, Human LIF (10-20ng/ml), and Doxycycline **1**µg/ml + 2i (1microM PD0325901 and 3microM CHIR99021) until the end of reprogramming process (day 7-10).
4) Replace media every 48 hours.
5) Assay is terminated after 7-10 days post Dox initiation. Colonies can be picked and re-seeded in naïve ESC medium independent of DOX in 20% O2 conditions.

Secondary reprogramming of mouse Fibroblasts – Option 2:

1) Seed secondary transgenic cells at day -1 on plates already pre-coated with 0.2% gelatin and irradiated DR4 MEFs or irradiated HFFs in 5% O2 hypoxia conditions.
   - Irradiated DR4 feeder cell density: 2* 10^6 per 10c"m dish, 2X10^6 per total of 6 wells in a 6 well dish. Total of 2X10^6 MEFs divided into 96 wells of a single gelatinized plate.

   -Reprogramming MEF donor cell density: For 6 well plate: 100-1000 cells per well (not more than 1000 cells); For 10 c"m TC dish- 1000-10,000 cells per plate); per 96 well plate: sort 1 cell per well (mCherry can be used to correct for plating survival efficiency).

2) Day 0: Reprogramming was initiated by applying mouse ES medium containing 15% FBS (heat inactivated – ES qualified), Human LIF (10-20ng/ml – notably it is more potent than mouse LIF), 10µg/ml L-Ascorbic acid (up to 50µg/ml), TGFb inhibitor A83-1 0.5µM and Doxycycline **1**µg/ml.

3) Day 2-3: Switch to mouse ES medium containing 15-20% KSR, Human LIF (10-20ng/ml), and Doxycycline **1**µg/ml + 2i (1µM PD0325901, 3µM CHIR99021, TGFbi A83-1 0.5µM, ROCKi Y27632 5µM) until the end of reprogramming process (day 7-10).

4) Replace media every 48 hours.
5) Assay is terminated after 7-10 days post Dox initiation. Colonies can be picked and re-seeded in naïve ESC medium independent of DOX in 20% O2 conditions.