HENSM= Human Enhanced Naïve Stem cell Media

02-09-2021; Updated on 04/09/2023

Original HENSM was published in: Bayerl et al. Cell Stem Cell 2021 https://doi.org/10.1016/j.stem.2021.04.001 **and used in** Oldak et al. Nature 2023

Simplified and standardized HENSM was published in: Viukov et al. Stem Cell Reports 2022 https://doi.org/10.1016/j.stemcr.2022.09.008

---

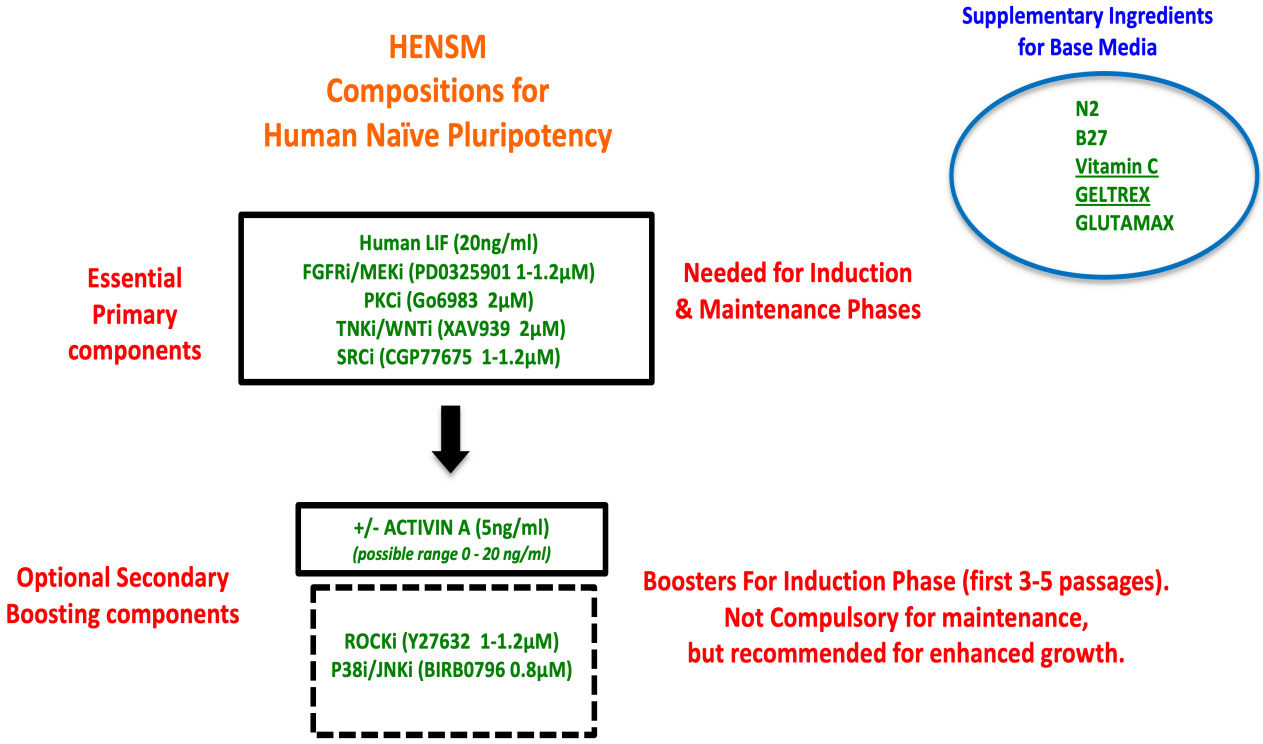
i) In HENSM conditions TNKi/WNTi, PKCi and SRCi consolidate human naïve pluripotency further from previously described NSHM conditions (Gafni et al. Nature 2013). ACTIVIN A/NODAL signaling promotes human naïve pluripotency induction, opposite to what is observed in rodents where it destabilizes rodent naïve pluripotent state. ACTIVIN A can be totally omitted or titrated down once stable human naïve lines are established (after 3-5 passages). HENSM(PD17) is a variant of HENSM that relies on FGFRi PD173074 to replace MEK and SRC inhibitors in HENSM.

ii) 0HENSM & tHENSM - allows obtaining naïve condition with titrated down or no ERKi conditions when NOTCHi/RBPJi is added.

HENSM conditions are most efficient thus far in naïve conversions and in long-term maintenance without transgene transfections or compulsory feeder cells (MEFs), and without extremely high propensity for chromosomal abnormalities or immediate and global loss of imprinting or oocyte inherited memory.

- Human naïve cells expanded in HENSM can give rise also to human TE, TSCs, naïve PrE cells, Extra-Embryonic Mesoderm Cells (ExEM) and human PGCs as we show in Bayerl et al. Cell Stem Cell 2021, as well as to human SEM and human Blastoids formation in Oldak et al. Nature 2023 (i.e. via Kagawa et al. Nature 2022 blastoid induction protocol but from cells expanded in HENSM conditions).

The image below summarizes our HENSM approach and ingredients of:
(1) Primary basic components (to be used for induction and maintenance)
(2) Secondary boosting Components (that can be used to boost induction in the first 3-5 passages and not compulsory for maintenance).



Enhanced **NHSM** Composition = **HENSM** ***(feeder and feeder free compatible)*:**

Primary Cytokines + inhibitors:

- 1:1 mix of Neurobasal (Invitrogen 21103-049) and DMEM/F12 (Invitrogen 21331) – 470ml
- Pen-strep **5ml** (Biological Industries 03-033-1B)
- GlutaMAX - **5m**l (Invitrogen 35050061)
- NEAA – **5ml** (Biological Industries 01-340-1B)
- Sodium Pyruvate- **5ml** (Biological Industries 03-042-1B)
- **10ml** B27 supplement: Invitrogen 17504-044 **or** NCS21 supplement by Capricon Scientific or in-house made
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration) (1 vial)
- Geltrex (Invitrogen A1413202/A1413302) - **1ml** rapidly in media (**0.2% final conc.**)
- **5ml** Commercial N2 supplement (Invitrogen 17502048) or in house made from 5 parts below:
  - %Insulin (Sigma I-1882) - 5mg insulin per bottle (10µg/m final concentration)
  - % Apo-transferrin (Sigma T-1147), 50 µg/ml final concentration
  - % Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
  - % Putrescine (Sigma P5780), 16 µg/ml final concentration
  - % Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml.

Essential Primary Basic components (needed for both induction and maintenance**):**

- 1) LIF (in house produced or *Peprotech 300-05*) – **20ng/ml** final (1 vial=50µL)
- 2) WNTi-TNKi = *XAV939 (Sigma X3004)* - **2µM** final (**1 vial = 50**µL)
- 3) PKCi Go6983 (*Axon 2466*) – **2µM** final (1 vial=50µL)
- 4) FGFRi*/ MEK1/2i / ERK1/2i - PD0325901 (*Axon 1408*) – **1-1.2µM*** final (1-1.2 vial=50-60µL)
- *5)* SRCi CGP77675 *(Axon 2097)* – **1-1.2µM** (1-1.2 vial=50-60µL)

Secondary Boosters (Optional for maintenance, and **boosters** for **induction** phase (first 3-5 passages)):

- 6) +/- ACTIVIN A – (Peprotech 120-14E) – 2.5 - **5 -** 10 - 20 **ng/ml** final**
- 7) ROCKi Y27632 (*Axon 1683*) – **1.2µM** (60µL include upon assembling media)***
- 8) P38i/JNKi BIRB0796 (*Axon 1358*) – **0.8µM** final (0.4 vial = 20µL)

- ***This constitutes the enriched original HENSM version. The Hanna lab prefers to use it for both induction and maintenance of our naïve lines and was used for both human blastoid and SEM formation in Oldak et al. Nature 2023.***

*** *When passaging cells, you should use additional freshly added Y27632 ROCKi 5µM for first 24h after splitting, as it further enhances cloning and survival efficiency.*

-****Although not compulsory, supplementation of ACTIVIN A during initial phases of primed to naïve reversion (5-10-20ng/ml), greatly enhances efficiency of naïve PSC clone recovery both in feeder containing and feeder free conditions for the first 3-5 passages, and then it can be totally omitted or titrated down to 5ng/ml or 2.5ng/ml. *For many lines-PD0325901 concentration increase from 1 to 1.2 µM is preferable to remove heterogeneity. Alternatively, FGFRi PD170374 at 0.05-0.1 µM final concentration can be added in addition to ERKi PD032590 and SRCi used. Alternatively, FGFRi PD170374 at 0.5-0.75 µM final concentration can be added instead of any ERKi PD032590 and SRCi CGP77675 in HENSM termed HENSM (PD17) – see next page).***

- *Adding 1mM VPA (Tocris, 2815) for the first 48h of HENSM media induction from primed PSCs can boost efficiency of conversion and upregulate further naïve pluripotency markers, but it is not an essential step in HENSM protocol.*

- *Change media every 24-48h. Media is stable for 10 days in 4C.* -Cells should be ideally expanded on plates coated with 1% GELTREX (Thermo A1413202/ A1413302) or 1% Matrigel or Biolaminin511 (5µg/ml) or MEF/gelatin coated plates. - Cells are expanded in 5% O2, but also possible in 20% O2.
- TryplE (1X) is optimal for single cell passaging (3ml per 10c"m dish, 1ml per 6 well - for 4-6 min at 37C). Rapid 0.05% trypsinization (0.5-1 min @ 37C) is optimal for passaging as small clumps. Longer 0.05% trypsinization for 3-5 minutes @37 is optimal for microinjection into as the cells come out less sticky. Centrifuge @4 min x 1300RPM

Standardized and simplified HENSM Composition = ***(feeder and feeder free compatible)*:**

Primary Cytokines + inhibitors:

- 1:1 mix of Neurobasal (Invitrogen 21103-049) and DMEM/F12 (Invitrogen 21331) – 470ml
- Pen-strep **5ml** (Biological Industries 03-033-1B)
- GlutaMAX - **5ml** (Invitrogen 35050061)
- NEAA – **5ml** (Biological Industries 01-340-1B)
- Sodium Pyruvate- **5ml** (Biological Industries 03-042-1B)
- **10ml** B27 supplement: Invitrogen 17504-044 **or** NCS21 supplement by Capricon Scientific or in-house made
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration) (1 vial)
- Geltrex (Invitrogen A1413202/A1413302) - **1ml** rapidly in media (**0.2% final conc.**)
- **5ml** Commercial N2 supplement (Invitrogen 17502048) or in house made (from 5 components)

- 1) LIF (in house produced or *Peprotech 300-05*) – **10ng/ml** final
- 2) WNTi-*TNKi = XAV939 (Sigma X3004)* - **2µM** final (**1 vial = 50**µL)
- 3) PKCi Go6983 (*Axon 2466*) – **2µM** final (1 vial=50µL)
- 4) MEK1/2i PD0325901 (*Axon 1408*) – **1.2µM** final (1.2 vial=60µL)
- *5)* SRCi CGP77675 *(Axon 2097)* – **1µM** (1 vial=50µL)
- 6) ACTIVIN A – (Peprotech 120-14E) – **5 ng/ml** final (1 vial).
- 7) ROCKi Y27632 (*Axon 1683*) – **1µM** (50µL include upon assembling media)***

- This simplified HENSM recipe was standardized after testing and optimizations on over ~30 different human established naïve human iPSC and ESC lines in the original HENSM conditions. Has been validated for TE/TSC formation.
*- *** When passaging cells, you should use additional freshly added Y27632 ROCKi 5µM for first 24h after splitting, as it further enhances cloning and survival efficiency.*

- *For many lines- PD0325901 concentration increase from 1 to 1.2 µM is preferable to remove heterogeneity. Alternatively, FGFRi PD170374 at 0.05-0.1 µM final concentration can be added in addition to 1µM ERKi PD032590 used.
-ACTIVIN A range can be used up to 20ng/ml.

- Adding 1mM VPA (Tocris, 2815) for the first 48h of HENSM media induction from primed PSCs can boost efficiency of conversion and upregulate further naïve pluripotency markers, but it is not an essential step in HENSM protocol.

- Change media every 24-48h. Media is stable for 10 days in 4C.-Cells should be ideally expanded on plates coated with 1% GELTREX (Thermo A1413202/ A1413302) or 1% Matrigel or Biolaminin511 (5µg/ml) or MEF/gelatin coated plates. - Cells are expanded in 5% O2, but also possible in 20% O2.

- TryplE (1X) is optimal for single cell passaging (3ml per 10c"m dish, 1ml per 6 well - for 4-6 min at 37C). Rapid 0.05% trypsinization (0.5-1 min @ 37C) is optimal for passaging as small clumps. Longer 0.05% trypsinization for 3-5 minutes @37 is optimal for microinjection into as the cells come out less sticky. Centrifuge @4 min x 1300RPM

Simplified and standardized HENSM was published for human TE and TSC derivation in: Viukov et al. Stem Cell Reports 2022
https://www.cell.com/stem-cell-reports/fulltext/S2213-6711(22)00457-X

HENSM (PD17) *(feeder and feeder free compatible)*:

Primary Cytokines + inhibitors:

- 1:1 mix of Neurobasal (Invitrogen 21103-049) and DMEM/F12 (Invitrogen 21331) – 470ml
- Pen-strep **5ml** (Biological Industries 03-033-1B)
- GlutaMAX - **5ml** (Invitrogen 35050061)
- NEAA – **5ml** (Biological Industries 01-340-1B)
- Sodium Pyruvate- **5ml** (Biological Industries 03-042-1B)
- **10ml** B27 supplement: Invitrogen 17504-044 **or** NCS21 supplement by Capricon Scientific or in-house made
- **5ml** N2 supplement (Invitrogen 17502048)
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration) (1 vial)
- Geltrex (Invitrogen A1413202/A1413302) - **1ml** rapidly in media (**0.2% final conc.**)

Primary components (needed for both induction and maintenance**):**

- 1) LIF (in house produced or *Peprotech 300-05*) – **20ng/ml** final (1 vial=50µL)
- 2) WNTi-TNKi = *XAV939 (Sigma X3004)* - **2µM** final (**1 vial** = **50**µL)
- 3) PKCi Go6983 (*Axon 2466*) – **2µM** final (1 vial=50µL)
- 4) FGFRi- PD173074 (*Axon 1673*) – **0.5-0.75*** final
- 5) ACTIVIN A – (Peprotech 120-14E) – **5 ng/ml** final**

- This constitutes a HENSM version in which FGFRi can replace MEKi, SRCi and P38i.

- **ACTIVIN A (5ng/ml) is compulsory for these conditions in feeder free conditions to maintain cell growth rate. (Up to 20ng/ml ACTIVIN A can be used).

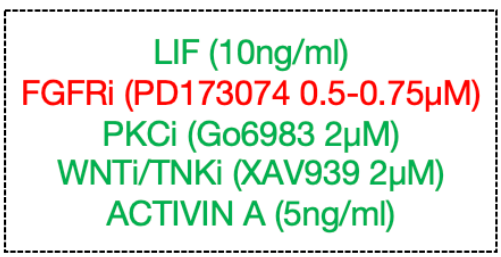
-When passaging cells, you should use additional freshly added Y27632 ROCKi 5µM for first 24h after splitting, as it further enhances cloning and survival efficiency.

-*For some lines- PD173074 reducing concentration from 0.75µM to 0.5µM allows faster growth while maintaining naïve pluripotency.

- Change media every 24-48h. Media is stable for 10 days in 4C. -Cells should be ideally expanded on plates coated with 1% GELTREX (Thermo A1413202/ A1413302) or 1% Matrigel or Biolaminin511 (5µg/ml) or MEF/gelatin coated plates. - Cells are expanded in 5% O2, but also possible in 20% O2.

- TryplE (1X) is optimal for single cell passaging (3ml per 10c"m dish, 1ml per 6 well - for 4-6 min at 37C). Rapid 0.05% trypsinization (0.5-1 min @ 37C) is optimal for passaging as small clumps. Longer 0.05% trypsinization for 3-5 minutes @37 is optimal for microinjection into as the cells come out less sticky. Centrifuge @4 min x 1300RPM

The image below summarizes our HENSM (PD17) approach, in which FGFR/MEK/ERK inhibition is based on PD173074 (PD17) (which replaces MEKi, SRCi and P38i used in original HENSM):



Enhanced **NHSM** Composition with low or no ERKi:

tHENSM / 0HENSM

Primary Cytokines + inhibitors:

- 1:1 mix of Neurobasal (Invitrogen 21103-049) and DMEM/F12 (Invitrogen 21331) – 470ml
- Pen-strep **5ml** (Biological Industries 03-033-1B)
- Glutamax - **5ml** (Invitrogen 35050061)
- NEAA – **5ml** (Biological Industries 01-340-1B)
- Sodium Pyruvate- **5ml** (Biological Industries 03-042-1B)
- **10ml** B27 supplement: Invitrogen 17504-044 **or** NCS21 supplement by Capricon Scientific or in-house made
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration) (1 vial)
- Alpha-KG (Dimet2-oxoglutarate; Sigma 349631; **add 60µL**) – 0.8 mM final (1 vial)
- Geltrex (Invitrogen A1413202/A1413302) - add **1ml** rapidly (**0.2% final conc.**)
- **5ml** Commercial N2 supplement (Invitrogen 17502048) or in house made from 5 parts below:
  - %Insulin (Sigma I-1882) - 5mg insulin per bottle (10µg/m final concentration)
  - % Apo-transferrin (Sigma T-1147), 50 µg/ml final concentration
  - % Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
  - % Putrescine (Sigma P5780), 16 µg/ml final concentration
  - % Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml.

Essential Primary Basic components (needed for both induction and maintenance**):**

- 1) LIF (in house produced or *Peprotech 300-05*) – **20ng/ml** final (1 vial=50µL)
- 2) WNTi - *TNKi = XAV939 (Sigma X3004)* **– 2µM** final (**1 vial** = **50**µL)
- 3) NOTCHi/RBPJi DBZ (Axon 1488) – **0.15µM** final (0.5 vial - **25µL**)
- 4) PKCi Go6983 (*Axon 2466*) – **2µM** final (1 vial= 50µL)
- 5) SRCi CGP77675 *(Axon 2097)* – **1.2µM** (1.2 vial=60µL)
- 6) FGFRi/MEKi PD0325901 (*Axon 1408*) – 0µM (for **0HENSM**) or 0.33 µM final (for **tHENSM**)

Optional Secondary Boosters (Optional for maintenance, and rather **great boosters** for **induction** phase (first 3-5 passages)):

- 7) ROCKi Y27632 (*Axon 1683*) – **1-1.2µM** (50-60µL include upon assembling media)**
- 8) P38i/JNKi BIRB0796 (*Axon 1358*) – **0.8µM** final (0.40 vial = 20µL)

- 9) +/- ACTIVIN A (Peprotech 120-14E) – 2.5 - **5** – 10 - 20ng/ml final*.

** **When passaging cells, you can use additional freshly added** Y27632 ROCKi 5µM for first 24h after splitting, as it further enhances cloning and survival efficiency.

- ***Change media every 24h for better results, but 48h is possible!!***
- **Fully assembled media is stable for up to *10* days at 4C.**
- ***t**HENSM: ERKi PD0325901 is 0.33µM. 0HENSM no-ERKi: PD0325901 =0µM*

- ***Although not compulsory, supplementation of ACTIVIN A during initial phases of primed to naïve reversion (10ng/ml), greatly enhances efficiency of naïve PSC clone recovery both in feeder containing and feeder free conditions for the first 3-5 passages, and then it can be omitted or titrated down to 5ng/ml.***
- *Adding 1mM VPA (Tocris, 2815) for the first 48h of HENSM media induction from primed PSCs can boost efficiency of conversion, but it is not an essential step in HENSM protocol.*

- Cells should be ideally expanded on plates coated with 1% GELTREX (Thermo A1413202/ A1413302) or 1% Matrigel or Biolaminin511 or MEF/gelatin coated plates.
- Cells are expanded preferably in 5% O2, but also possible in 20% O2.
- TryplE (1x) is optimal for single cell passaging (3ml per 10c"m dish, 1ml per 6 well - for 4-6 min at 37C). Rapid 0.05% trypsinization (0.5-1 min @ 37C) is optimal for passaging as small clumps. Longer 0.05% trypsinization for 3-5 minutes @37 is optimal for microinjection into as the cells come out less sticky. Centrifuge @4 min x 1300RPM

Comments, explanations, FAQs and tips:

- To convert your already established primed hESCs and iPSCs, simply apply the media and start passaging the cells poly-clonally, as the naïve pluripotent cells take over within 2-5 passages. Acquisition and consolidation of most naïve features occurs within 10-14 days. **To get experience for the cells by new users, we recommend converting 2-3 lines simultaneously on feeder and feeder-free conditions.**

- Naive HENSM cells were routinely assessed by FACS to confirm high expression of the naive pluripotency cell surface marker CD77, which is totally absent on human primed PSCs.

- For many lines- PD0325901 concentration increase from 1 to 1.2 μM is preferable to remove heterogeneity and boost naïve marker expression. Alternatively, FGFRi PD170374 at 0.05-0.1 μM final concentration can be added in addition to 1μM ERKi PD032590 used. Alternatively, FGFRi PD173074 at 0.5-0.75 μM final concentration can be added instead of any ERKi PD032590 and SRCi CGP77675 and P38i in HENSM.

- Human PSC lines show variability in their ability to tolerate FGF pathway inhibition to obtain naivety while maintain reasonable growth rate. Some PSC lines may require small modifications in the HENSM protocol in order to avoid very low proliferation and expansion rate (typically involves a slight increase in ACTIVIN A dose used and/or a decrease in FGFR/MEK inhibitor used).

- Different batches of B27 can produce some notable differences in the level of expression of naive pluripotency markers and cell growth rate of the same PSC line. One can consider using a different B27 batch or B27-like supplement from a different vendor (e.g. NCS21 supplement by Capricon Scientific - https://www.capricorn-scientific.com/en/shop/ncs21-neuronal-supplement-50x-serum-free~p1196).

- Adding VPA 1mM (Tocris, 2815) for the first 48h of HENSM media induction can boost efficiency of primed PSC conversion and enhance naïve pluripotency marker upregulation levels further, but it is not an essential step for any of the lines tested so far.

- Supplementing HENSM conditions with additional FGFRi PD173074 0.5-1 μM generated more rapidly human naïve PSCs capable of random X inactivation upon priming as observed so far in WIBR2 29-8 and 29-9 reporter X cell lines, but the growth rate was very low in these conditions due to combined high levels of MEKi and FGFRi inhibitors used.

- TrypLE 1X solution (# 12604 Invitrogen) is used for passaging**,** we apply 3ml on 10c"m dish for 4-6 minutes at 37C, then aspirate the TrypLE, add 5ml PBS and resuspended the cells. Cells are detached by gentle pipetting.

-Human PSCs expanded/maintained in naïve-like RSeT media (commercialized version of NHSM media – Gafni et al. Nature 2013) acquire the expanded milieu naïve qualities faster (within 1-2 passages) than when starting from conventional primed cells (by 4-5

passages). RSeT medium (Stem Cell Technologies, 17148311) (RSeT medium (2-component) is a commercialized version of NHSM medium), and was assembled according to the manufacturer's instructions, including dissolving 250 µl Matrigel or Geltrex in 500 ml medium as instructed; https://www.stemcell.com/products/rset-medium-2-component.html), and expanded on Matrigel or Geltrex or Cultrex-coated plates and passaged with TrypLE every 3–5 days.

-Go6983 has red fluorescence signal, so pre-washing of cells before analysis should be applied. (Remarkably, we note it down-regulates Mbd3-Gatad2a expression in mouse pluripotent cells and during reprogramming).

- Xenofree B27 supplement (Invitrogen A1486701) can be used instead of BSA containing B27 by Invitrogen 17504-044.

-- ** On Feeder cells, the cells are tolerant to PERMANENT 1-5 *µM* Y27632 ROCK inhibition (basically indefinitely without differentiating). - Permanent high concentration >2.5 *µM* of Y27632 ROCKi CANNOT be used in feeder free condition maintenance. Thus, use 1-*2 µM* freshly or permanently added Y27632 is recommended (but not essential).
- ** After splitting, *Y27632 5-10 µM (total conc.) can be used only 24h after splitting the cells.*
- ** During iPSC reprogramming continuous Y27632 5*µM* ROCKi is recommended (at least during first 7 days).

- Supplementing naïve media with 0.2% Geltrex (Invitrogen A1413202/A1413302) or Growth Factor Reduced Matrigel (BD- FAL356231) [add 1ml in assembled media (0.2% final concentration)] has a dramatic influence on boosting naïve features and homogeneity among colonies and different PSC lines. We find Geltrex is slightly more favorable than Matrigel (particularly in no ACTIVIN conditions).

- Unlike in rodents where WNT signaling and nuclear Beta Catenin promotes naïve pluripotency, in humans this leads to an opposite effect. Thus, we use WNTi in HENSM versions: 1) Porcupine inhibitors (like IWP2 – 2µM final) are potent WNT inhibitors in human PSCs. 2) The use of inducer/stabilizer of AXIN complex (abbreviated as AXINs) through inhibition of Tankyrase (TNKi) (e.g., IWR1 5µM final concentration or XAV939 2µM final) induces cytoplasmic WNT/beta-catenin retention at the expense of its nuclear localization effects, and thus reduces mesodermal gene expression patterns, reduces dependence on exogenous FGF2, and boosts epithelial signature and pluripotency gene expression. XAV939 or IWP2 yield better outcome than IWR1 in HENSM conditions also in terms of cell growth rate and less toxicity, and most prominently in ACTIVIN free conditions.

- Alpha-KG (Dimet2-oxoglutarate; Sigma 349631; add 60µL) – 0.8 mM final is a booster for naïve human PSC induction and maintenance, although it tends to slightly slow down proliferation thus, we first try to avoid using it in HENSM.

- % Ready-made **N2 supplement (Invitrogen - 17502-048)** can be used instead of the 5 individual components (N2 comp. = Insulin, Apo-transferrin, Progesterone, Sodium selenite, Putrescine).

- Cells can be expanded on plates coated with 0.2% gelatin/irradiated mouse feeder cells (we use DR4 routinely). More optimal for our current gene targeting approaches and subsequent colony picking.

- Mitomycin C inactivated MEFs quickly die in NHSM and HENSM conditions and are NOT RECOMMENDED for use. Irradiated MEFs are much more resilient under these conditions.

- Media is relatively sensitive, no need for pre-heating (10 minutes at RT before use is enough, protect from light).

- Please make sure you use HUMAN LIF and NOT mouse Lif (Human LIF works both on mouse and human cells, Mouse Lif works only on mouse cells).

-Vial # indicated throughout the protocol is of course based on HANNA LAB internal aliquot stocks in our lab (which can change).

How should one approach adapting previously hESC/iPSC differentiation protocols?

We recommend to first test applying the differentiation protocol on human naïve cells, and/or simultaneously start with naïve cells, apply priming while the cells are adherent for 24-48-72 hours with primed human ESC medium or in N2B27 media with **2 µM** XAV939 and then apply your differentiation protocol.

Human Naïve Cell Handling Protocols:

-Freezing human naïve cells:

Solution 1: 20% DMSO and 80% FBS

Solution 2: Freshly made HENSM medium including additional **20µM final** Y27632 ROCKi.

Re-suspend cells in 1:1 solution mix and freeze in regular cryotubes at -80 in styro-foam boxes (for at least 1 day – up to 2 months). Then move vials to liquid nitrogen. No need to use special freezing devices throughout the process.

- Thawing human naïve cells:

Thaw vial at 37C and spin down in 10ml of 15% FBS supplemented DMEM (3 min at 1000RPM). Plate cells in HENSM containing **20µM** ROCKi.

- DNA electroporation in human naïve PSCs:

200-50ug DNA can be used for electroporation 10"cm confluent plate of human naïve PSCs harvested and trypsinized into single cells (0.05% trypsin or TrypLE).

Electroporation parameters on human naive PSCs on BIORAD Gene Pulser Xcell (with CE module): Square wave pulse protocol, Voltage: 250V, Pulse length 20ms, Number of pulses: 1, Pulse interval .0 (sec), 4mm cuvettes (BIORAD).

Preparing N2 stock components in-house:

Insulin (Prospec Bio CYT 270 = 1000mg)
• Prepare a 25mg/ml stock solution by dissolving 1000mg insulin in 40ml 0.01M HCl overnight at 4C. Sterile filter and store in 200µl individual aliquots in -80C (use 1 vial per 500ml bottle).

Apo-Transferrin (ATF Prospec PRO-325 - 5000MG)
• Prepare a 100mg/ml stock by dissolving 5000mg A-T in 50ml dH20 overnight at 4C. Sterile filter and store in-80C. Make 250µl aliquots (use 1 vial per 500ml bottle)

Progesterone (Sigma P8783, 25g)
• Prepare a 0.6mg/ml stock by dissolving 6mg Progesterone into 10ml Ethanol. Sterile filter and store in -80C. Make 17µl individual aliquots (use 1 vial per 500ml bottle)

Putrescine (Sigma P5780, 25g)
• Prepare a 160mg/ml stock by dissolving 1.6g Putrescine into 10ml dH20. Sterile filter and store. Make 50µl individual aliquots and store in -80C (use 1 vial per 500ml bottle)

Sodium Selenite (Sigma S5261, 25g)
• Prepare a 1.5mM stock by dissolving 2.59mg Na Selenite in 10ml dH2O. Make 10µl individual aliquots and store in -80C (use 1 vial per 500ml bottle)

Preparing B27 supplement in-house (AKA B22):

See Hanna lab website for our in-house protocol:

https://www.weizmann.ac.il/molgen/hanna/sites/molgen.hanna/files/users/user52/HANNA-LAB-B22-B27-PROTOCOL-V3.pdf

Hanna Lab - Human NHSM and HENSM iPSC microinjection protocol

1. Dissect oviducts of hormone primed and mated B6D2F1 females X B6D2F1 males, and extract zygotes (as routinely done with mouse micro-manipulation in our lab).
2. Culture zygotes for 2 days in KSOM medium droplets (Zenith Biotech KSOMaa Evolve cat # ZEKS-050) covered with mineral oil at 37C 20% O2 or 5%O2 incubator, until they develop to morula stage.
3. Grow human naïve cells to 70-90% confluence in NHSM/NHSM+TNKi or HENSM/HENSM-ACT medium.
4. The day before cell harvesting add 20μM Y27632 ROCKi to the cells (in case not continuously used in the (E)NHSM medium).
5. Trypsinize the cells for ~ 5 minutes with 0.05% trypsin, shake and pipette thoroughly to yield one cell suspension. Stop the reaction with DMEM+15% FBS and centrifuge at 1000RPM for 4 Minutes. Aspirate and discard medium. 9 (**0.05% trypsinization for 5 minutes @37 is more optimal for microinjection than TryplE, as the cells come out less sticky after trypsinization)**
6. Resuspended cells in 900μl NHSM medium, add 100μl filtered FBS (to reduce stickiness of cells) and 20μM Y27632 ROCKi. Filter the cells through 40microN BD basket to reduce clumps. Keep on ice until and during injections!!! It is preferable to inject the cells as soon as they are harvested.
7. Inject 5 p53-/- or 10 WT human naïve cells to a mouse morula by using of Piezo (as routinely done with mouse ES injections). Include 20μM Y27632 ROCKi also in M2 media throughout the injection period. (any drop that has naïve PSCs during injection should have ROCKi to increase cell survival during the process). (We use 15micron Piezo needles 15-15-MS for both mouse and human naïve injections).
8. After injection, incubate for 3-4 hours in KSOM droplets supplemented with Y27632 20μM ROCKi covered with mineral oil.
9. After 3-4 hours transfer the morulas to KSOM droplets (without ROCKi) covered with mineral oil, incubate overnight. That way the morulas will develop into blastocyst (DO NOT LEAVE ROCKi for more than 4h as it will block blastocyst development).
10. The next day, most morulas should develop to blastocysts. Transfer 15-20 blastocysts to uterus of pseudo-pregnant B6D2F1 female mice.