HENSM= Human Enhanced Naïve Stem cell Media

28-04-2021
HANNA LAB – Weizmann Institute of Science

Published in: Bayerl et al. Cell Stem Cell 2021

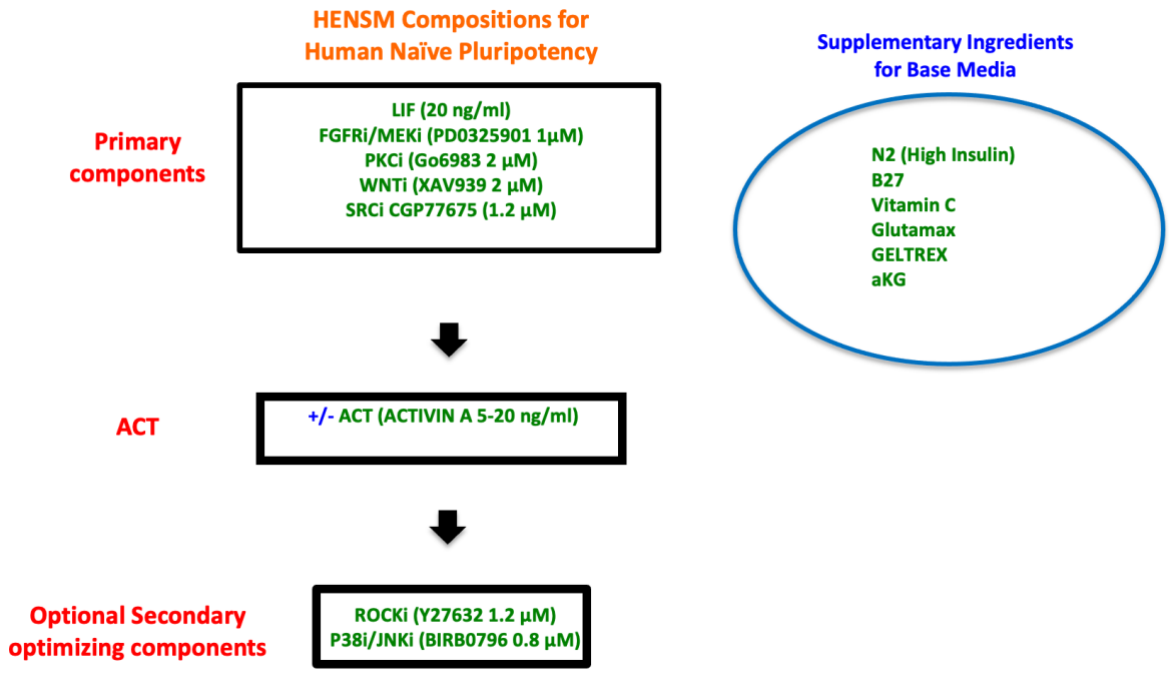
i) In HENSM conditions TNKi/WNTi, SRCi and PKCi consolidate human naïve pluripotency further from previously described NSHM conditions (Gafni et al. Nature 2013). ACTIVIN A/NODAL signaling can promote inducing human naïve pluripotency, opposite to what is observed in rodents where it destabilizes the rodent naïve pluripotent state.

ii) 0HENSM & tHENSM - allows obtaining naïve condition with titrated 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 feeders or transgene transfections and without high propensity for chromosomal abnormalities or immediate and GLOBAL loss of imprinting or oocyte inherited memory.

The image below summarizes our HENSM approach and ingredients of (1) Primary base Components, and (2) Secondary Optimizing Components that can be used to boost, consolidate and accelerate acquisition of naïve features of human pluripotent cells.

Summary of HENSM and HENSM-ACT conditions:



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

HENSM or HENSM ACT

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)
- **10ml** B27 supplement: Invitrogen 17504-044 **or** Xenofree A1486701 **or** in-house made
- N2 comp.% - Insulin (Sigma I-1882) - 5mg insulin per bottle (10microg/ml final concentration)
- N2 comp.% - Apo-transferrin (Sigma T-1147), 50 µg/ml final concentration
- N2 comp.% - Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
- N2 comp.% - Putrescine (Sigma P5780), 16 µg/ml final concentration
- N2 comp.% - Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml.
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration) (1 vial)
- Geltrex (Invitrogen A1413202/A1413302) - add **1ml** rapidly in media (**0.2% final conc.**)
- Alpha-KG (Dimet2-oxoglutarate; Sigma 349631; add **60µL**) – **0.8 mM** final

Primary Cytokines + inhibitors:

- 1) LIF (in house produced or *Peprotech 300-05*) – **20ng/ml** final (1 vial=50µL)
- 2) WNTi: *TNKi = XAV939 (Sigma X3004)* - **2µM** final (**0.5 vial** = **50**µL) or IWP2 - **2µM** final
- 3) FGFRi/MEKi/ERKi PD0325901 (*Axon 1408*) – **1µM** final (1 vial=50µL)
- 4) PKCi Go6983 (*Axon 2466*) – **2µM** final (1 vial=50µL)
- 5*)* SRCi CGP77675 *(Axon 2097)* – **1.2µM** (1.2 vial=60µL)

- 6)Optional: ACTIVIN A – (Peprotech 120-14E) – 5-20ng/ml final (termed **HENSM-ACT**)*.

Secondary optional boosters:

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

** **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!!

- *Although not compulsory, supplementation of ACTIVIN A during initial phases of primed to naïve reversion, greatly enhances efficiency of naïve PSC clone recovery both in feeder containing and feeder free conditions. Later on, ACTIVIN A concentration can be tapered down or completely omitted.

- Fully assembled media is stable for up to *10* days at 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 (diluted into 0.8X – add 20ml PBS to the bottle with 80ml TrypleE) is optimal for single cell passaging (2ml per 10c"m dish, 0.75ml per 6 well - for 5-7 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

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

tHENSM / 0HENSM or tHENSM-ACT / 0HENSM-ACT

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)
- **10ml** B27 supplement: Invitrogen 17504-044 **or** Xenofree A1486701 **or** in-house made
- N2 comp.% - Insulin (Sigma I-1882) - 5mg insulin per bottle (10microg/ml final concentration)
- N2 comp.% - Apo-transferrin (Sigma T-1147), 50 µg/ml final concentration
- N2 comp.% - Progesterone (Sigma P8783), 0.02 µg/ml final concentration;
- N2 comp.% - Putrescine (Sigma P5780), 16 µg/ml final concentration
- N2 comp.% - Sodium selenite (Sigma S5261), add 5 µL of 3 mM stock solution per 500ml.
- L-ascorbic acid 2-phosphate (Sigma - A8960) (50 µg/ml final concentration) (1 vial)
- Geltrex (Invitrogen A1413202/A1413302) - add **1ml** rapidly in media (**0.2% final conc.**)
- Alpha-KG (Dimet2-oxoglutarate; Sigma 349631; add **60µL**) – **0.8 mM** final

Primary Cytokines + inhibitors:

- 1) LIF (in house produced or *Peprotech 300-05*) – **20ng/ml** final (1 vial=50µL)
- 2) WNTi: *TNKi = XAV939 (Sigma X3004)* **– 2µM** final (**0.5 vial** = **50**µL) or IWP2 - **2µM** final
- 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)Optional: ACTIVIN A (Peprotech 120-14E) – 5-20ng/ml final (**tHENSM-ACT/0HENSM-ACT**)*.

Secondary optional boosters:

- 7) ROCKi Y27632 (*Axon 1683*) – **1.2µM** (60µL include upon assembling media)**
- 8) P38i/JNKi BIRB0796 (*Axon 1358*) – **0.8µM** final (0.40 vial = 20µL)
- 9) FGFRi/MEKi PD0325901 (*Axon 1408*) – 0µM (for **0HENSM**) or 0.33 µM final (for **tHENSM**)

** **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.**
- *tHENSM: ERKi PD0325901 is 0.33µM. 0HENSM no-ERKi: PD0325901 =0µM*
- **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 (diluted into 0.8X – add 20ml PBS to the bottle with 80ml TrypleE) is optimal for single cell passaging (2ml per 10c"m dish, 0.75ml per 6 well - for 5-7 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 2-3 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.**

- TrypLE 1X solution (# 12604 Invitrogen) should be diluted with PBS-/- – yielding **TrypLE 0.8X,** we apply 2ml on 10c"m dish for 5-7 minutes at 37C, then aspirate the TrypLE, add 5ml PBS and resuspended the cells. Cells are detached by gentle pipetting.

-GLUTAMAX ((Invitrogen 35050061) yields much more superior results and tissue culture stability than conventional L-Glutamine.

- % 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). We still recommend adding extra 3-5mg Insulin to 500ml Bottle when commercial N2 is used.

-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.**

-- ** 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 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.

- 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.

- supplementing HENSM conditions with FGFRi P173074 (0.5-0.75 μM) generated more rapidly human naïve PSCs capable of random X inactivation upon priming.

- 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 hours with primed human ESC medium, 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:

http://hannalabweb.weizmann.ac.il/wp-content/uploads/2014/10/Hanna-Lab-Protocol-B27-B22-Protocol.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.