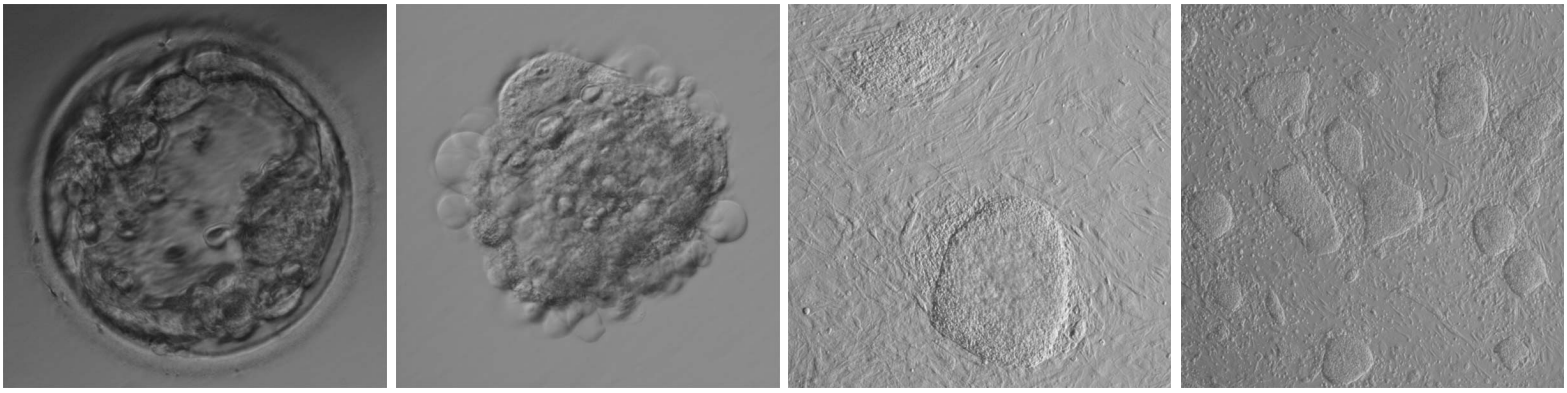
Human Embryonic Stem (HUES) Cell Collection



Instruction Manual
Version 1.0

HUES (Human Embryonic Stem) Cell Collection

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Derivation of Embryonic Stem Cells from Human Blastocysts

Publication

Cowan, C.A. et. al, Derivation of Embryonic Stem Cells from Human Blastocysts (2004), NEJM 1997; 336(23):1650-1656.

Derivation

Supernumerary frozen human cleavage stage or blastocyst embryos produced by *in vitro* fertilization for clinical purposes were donated after informed consent and institutional review board approval. Embryos were cultured to the blastocyst stage and the zona pellucida removed by digestion with acid tyrodes, followed by immunosurgery using rabbit anti-human RBC antibodies and a guinea pig sera complement. (cover image depicts the derivation of HUES-12).

Characterization

The cell lines presented here are similar to other reported human embryonic stem (hES) cells with a high ratio of nucleus to cytoplasm, prominent nucleoli, and compact colony morphology. The hES cell lines were found strongly positive for a number of molecular markers of undifferentiated pluripotent human stem cells, including octamer binding protein-3/4 (Oct-3/4), stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase. These results are consistent with the molecular characteristics reported for existing hES cell lines.

Expansion

17 of the human embryonic stem (HUES) cell lines that were derived in our hES facility have been expanded to distribution numbers. With the exception of HUES-3, all lines were expanded before passage twenty two. A number of the lines were expanded before passage 15. All cell lines are verified to be viable after thaw and competent to grow and undergo enzymatic passaging, if using the protocol and reagents included in this instruction manual. The following table lists each line, its passage number, and karyotype[1].

Available lines

Cell Line	Passage	Karyotype
HUES-1	19	46,XX
HUES-2	16	46,XX
HUES-3	26	46,XY
HUES-4	21	46,XY
HUES-5	12	46,XX,inv9
HUES-6	17	46,XX
HUES-7	15	46,XY
HUES-8	21	46,XY
HUES-9	18	46,XX,inv9
HUES-10	10	46,XY
HUES-11	13	46,XY
HUES-12	13	46,XX
HUES-13	15	46,XY
HUES-14	16	46,XX
HUES-15	12	46,XX
HUES-16	15	46,XY
HUES-17	15	46,XY

[1] Routine karyotype analysis of hES cell lines is warranted (see Maintenance and Passaging p.9)

Storage, Growth Conditions and Technique Notes

Shipping and Storage

hES cells and mitotically inactivated MEFs (PMEF*i*) have been supplied in 90% fetal calf serum and 10% dimethyl sulphoxide frozen stocks. Each hES vialcontains ~ 4 x 10^5 cells and is sufficient for plating one 35 mm tissue culture treated plate (1 well of a 6- well plate). Each PMEF*i* vial contains ~4 x 10^6 cells and is sufficient for plating 6 wells of a 6-well plate. Vials are shipped on dry ice. Upon receipt, store hES vials in N_2(l) and PMEF*i* vials at -80°C.

***Successful thaws and splits have been carried out on Specialty Media's hygromycin and neomycin resistant, pre-treated MEFs. (Not supplied)**

Growth/ Atmospheric Conditions		
	Ambient Temperature :	37 ± 0.5°C
	CO_2 concentraion :	5.1 ± 0.6% CO_2
	Relative Humidity :	85 – 100 %

General Technique

Tissue Culture

General tissue culture techniques should be observed when working with hES cells. All protocols should be carried out using sterile/aseptic technique in an appropriate tissue culture room and under a laminar flow hood. Additionally, our hES facility has taken an added precaution to reduce airborne particulate by installing air handling and filtration equipment to produce a pseudo-clean room. Gloves are worn when handling all reagents and material that come in contact with cells. (This includes opening of incubators.) All workspaces are thoroughly cleaned with 70 % isopropanol before and after use.

Media/Materials

All media and reagents are filtered sterilized prior to first use with a 0.2µm filter. Media bottles and TC materials should be sprayed down with 70% isopropanol before being placed in the hood.

Cell Handling

We recommend thawing no more than two samples at a given time to ensure for easy handling. The handler should take care not to leave cultures at room temperature and low CO_2 for long periods of time. All centrifugation of live cells is done at 500-600 x *g* for 5 min at room temperature.

Methods, Media Preparation

Media preparation

Both MEF and hES media are prepared under sterile/aseptic conditions. After preparation, media is filter sterilized at 0.2 μm.

When preparing hES growth media, a general consistency will be important. It is advisable to work with the same lot numbers of reagents whenever possible. It is especially important to work with lots of serum replacement which meet the criterion presented in Appendix CAT- 1.

Growth factors should be the last reagents added to the media. It is important to note that bFGF requires a protein carrier when being re-suspended. DO NOT try to re-suspend bFGF in minimal media.

MEF media

MEF media	**560.5 ml**
DMEM	500 ml
PenStrep	5.5 ml
FBS	55 ml

hES media

hES media	**651.5 ml**
KO-DMEM	500 ml
PenStrep	6.5 ml
Gluta-MAX™	6.5 ml
NEAA	6.5 ml
2-mercaptoethanol	0.65 ml
KO Serum Replacement	65 ml
Plasmanate	65 ml
bFGF[2] (~10 ng/mL final)	0.54 ml
hLIF[3] (~12 ng/mL final)	0.78 ml

Freezing media

Freezing media is a mixture of 90% fetal calf serum and 10% dimethyl sulphoxide. Filter sterilize at 0.2 μm and store at 4°C.

Gelatin preparation

Prepare a 0.1% solution of gelatin in milli-Q (tissue culure grade) water. Ensure that the gelatin is thoroughly dissolved by heating. Autoclave or filter sterilize the solution at 0.2 μm before use.

[2] Re-suspend in 1 ml of DMEM plus 0.1% BSA (or 1 ml pre-made hES media – protein carrier)
[3] Optional

Methods, Gelatin Coating and Preparation of MEF Plates

Gelatin Coating

Prior to plating any cells, coat tissue culture treated plates with 0.1% gelatin (prepared with milli-Q water) for a minimum of 30 minutes at 37°C. Use 1.5 ml or 4 ml gelatin solution to coat a 35 mm or 10 cm plate respectively. Aspirate off the gelatin solution just before thawing MEFs.

MEF Preparation

A reliable feeder layer of mouse embryonic fibroblasts (MEF) is important for the survival and growth of hES cells. With any HUES cell line, you will receive vials of pre-treated, mitotically inactivated MEFs (PMEF*i*), for the thaw and for one split. Each vial contains enough PMEFs (~4 x 10^6 cells) to cover one 6-well tissue culture treated plate. If extensive passaging/expanding is to take place, it is advisable to derive, expand and inactivate your own MEFs (see Appendix MEF-1 for details on deriving and expanding).

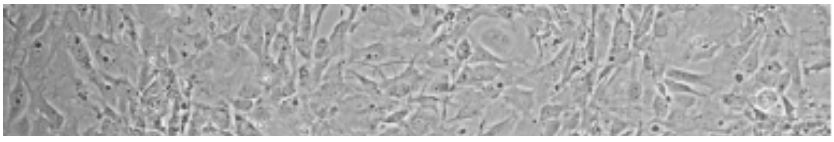
MEFs should be plated one to two days before thawing/passaging hES cells. **MEFs should not be used as a feeder layer for longer than 12-14 days.**

Plating MEFs

Pre-warm MEF media to 37°C. Aliquot 10ml of the media into a sterile 15 ml conical tube and set inside hood. Remove MEF vial from -80°C and immediately submerge the bottom half of tube in a 37°C water bath. It should take about 30-45 seconds before the cells are 80% thawed (small frozen portion left)[4]. Quickly bring the tube to the laminar flow hood, spray down with 70% isopropanol, and transfer cells to the 10 ml of pre-warmed media. It is recommended to wash the vial with an additional 1 ml of pre-warmed media (add to 15 ml conical).

Spin the 15 ml conical tube at 500-600 x *g* for 5 minutes. While cells are spinning, aspirate the gelatin solution from plates prepared earlier, and set inside hood. After spin is complete, carefully aspirate the media without disturbing the pellet. Resuspend the pellet in 12 ml (6 well plate) pre-warmed MEF media. Aliquot 2 ml of MEF solution in a drop wise manner into each of the six wells. Be sure to distribute the MEFs evenly about the well. Date the MEF plate, and place in a 37°C incubator overnight to allow MEFs to attach to plate.

After 6 hours MEFs will be attached to the plate. MEFs should be confluent. It is best to use MEF plates 24-48 hours after plating. DO NOT use a MEF plate that is over 4 days old.



Gamma irradiated pretreated MEFs (PMEF*i*)–24 hours after plating (10x)

[4] Thaw time will vary with volume and concentration (based on 1 ml @ 4 X 10^6cells/ml).

Methods, Thawing Human Embryonic Stem Cells

hES cells

The human embryonic stem cells you have received were frozen down at a ~1:6 split from a sub-confluent, 10 cm tissue culture dish. Most vials are frozen at a concentration of ~4 x 10^5 cells/ ml. It is recommended to thaw one vial into one 35 mm tissue culture dish, or one well of a 6-well plate.

Thawing/Plating hES cells
Part C

Before thawing, ensure that the MEF plate you have already prepared is properly plated and in good condition. DO NOT try to use a less than desirable plate, or one that is older than three days. It is recommended to pre-label all conical tubes and wells being used.

Pre-warm hES media to 37°C. Aliquot 10 ml hES media into a sterile and labeled 15 ml conical tube for each line. Remove hES vial/s from $N_2(l)$ and immediately submerge the bottom half of the tube in a 37°C water bath. It should take about 45-60 seconds before the cells are 80% thawed (small frozen portion left). Quickly bring the tube to the laminar flow hood, spray down with 70% isopropanol, and gently transfer cells to the 10 ml of pre-warmed media. It is recommended to wash the vial with an additional 1 ml of warmed media (add to 15 ml conical).

Centrifuge the 15 ml conical tube at 500-600 x *g* for 5 minutes. While cells are spinning, remove pre-plated MEFs from incubator. Under the hood, aspirate off the media from as many wells as you will be thawing into. Quickly, aliquot 1 ml pre-warmed hES media back into each well of the plate, being careful not to disturb the attached MEFs. Set the plate aside in the hood. After spin is complete, carefully aspirate the media without disturbing the pellet (if necessary, do not aspirate all media). Gently re-suspend each pellet in 1 ml of pre-warmed hES media. Add the hES solution, in a drop wise manner, to a MEF plate well which already contains 1 ml hES media. As with MEFs, best results are obtained if the drops are evenly distributed about the plate. Carefully return the plate to a 37°C incubator overnight to allow the hES cells to seed the MEFs.

Methods, Maintenance and Passaging Human Embryonic Stem Cells

hES cell maintenance

hES cell culture is demanding. With the exception thaw/split days, media should be changed every day. Additionally, a growth lag after a thaw or passage often occurs. For this reason, it is very important to observe the cells every day. Be sure to prepare MEF plates ~ 2 days in advance as splits may catch you by surprise.

Please note, we have observed karyotypic changes involving trisomy of chromosome 12 (in two cell lines; HUES-3 and 4) as well as other changes (additions to chromosome 2 in HUES-1) in the HUES cell lines. These karyotypic abnormalities are accompanied by a proliferative advantage and a noticeable shortening in the population doubling time. As chromosomal abnormalities may be commonplace in hES cell lines, we recommend frequent karyotypic analysis.

Day 1

24 hours after thawing the hES cell stocks, check the wells under 4x magnification. You may notice that there is quite a bit of debris, which is normal. Do not be alarmed. The debris is a mixture of dead cells. At this early stage, you should not expect to see many colonies. If the layer of dead cells completely covers the well, it is recommended to perform a partial media change.

Day 2-4

Typically, the first full media change takes place 48 hours after thawing the hES cells. 2 mls per well is recommended. Colonies may start to become visible as early as the second day post thaw. Continue changing the media every day.

Day 4-7

In our experience, cultures may require passaging as early as 4 days post-thaw and as late as 10 days post thaw (at this point the MEFs are old). The number of days before a split will depend on a number of factors including how well the thaw has been carried out. On average, expect that a culture will take seven days to become sub-confluent. For an example of how a given cell line performs, please contact us.

Do not be discouraged if your thaw does not recapitulate the images in the appendix. We have noticed that there is some inconsistency in thawing, and that some lines behave more reliably than others. It is not uncommon to see cultures which exhibit ragged, flat colonies, or in the extreme case, a monolayer of cells. DO NOT give up on your thaw. It is common for the cellular morphology of HUES cell lines to improve after one or two passages.

hES cell passaging

After 4-7 days post-thaw, your cells should be near confluence. A sub-confluent hES cell culture is generally split at a 1:3 ratio. It is important to split your cells before they differentiate. If the colonies begin to turn a brownish color prior to reaching sub-confluence, it may be necessary to split the cells early and at a 1:2 ratio. Likewise, the split may be adjusted if the cells are too dense (evenly distributed and touching one another).

Methods, Maintenance and Passaging Human Embryonic Stem Cells, Cont'd

1:3 split

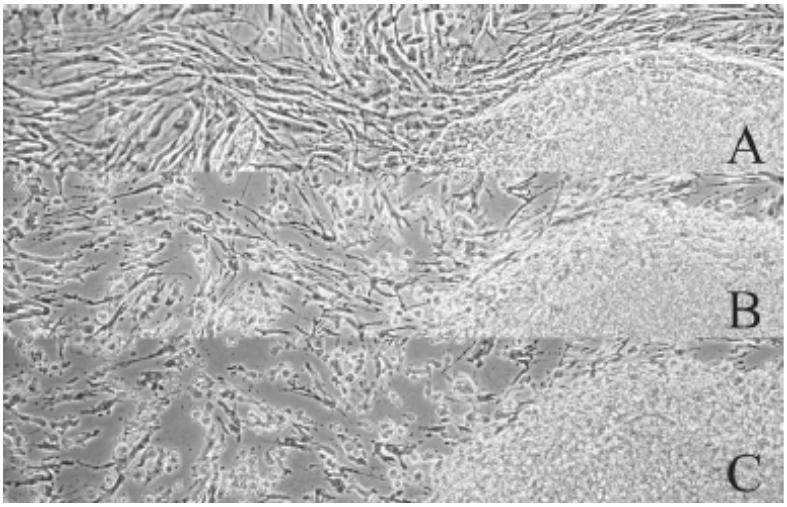
Before splitting, ensure that you have three wells of pre-plated MEFs. Allow MEFs to attach for at least 24 hours, but no more than three days.

Pre-warm hES media and 0.05% Trypsin/EDTA to 37°C. Under the hood, prelabel one sterile 15 ml conical tube. Remove MEF plate from incubator. Under the hood, aspirate off the media from three wells. Quickly, aliquot 1 ml prewarmed hES media back into each well of the plate, being careful not to disturb the attached MEFs. Set the plate aside in the hood.

The following steps should be done quickly to minimize the amount of time that cells are not in hES media.

Carefully aspirate the hES media from the culture to be split. Gently wash the well with 1X phosphate buffered solution (PBS). Aspirate the PBS. Add 0.3 ml 0.05% Trypsin/EDTA to the well. Replace lid, and observe the cells under 4x magnification. The MEFs surrounding the hES colonies should begin to retract.

Trypsinization
Figure 1



When the MEFs are sufficiently rounded and the borders of the hES colonies are rough, return the plate to the hood. Add 2 ml pre-warmed hES media to the trypsinized cells. Gently pipette up and down, washing the bottom of the well, until the MEF monolayer has completely detached (monolayer is sticky and may remain in one piece). Transfer the cell suspension to the 10 ml conical tube. Wash the well one more time with an additional 1 ml hES media, transferring it to the same conical tube. At this point the trypsin should be adequately inactivated.

Pipette the solution 5-7 times with an automated pipette. Aliquot 1 ml of the hES solution drop wise, making sure to distribute the drops evenly about the well. Without shaking the plate, carefully return the cells to a 37°C incubator overnight to let hES colonies seed.

hES cells coming out of a split should behave similarly to those coming out of a thaw. The maintenance guidelines presented in the previous section can also be followed for splits.

MEF Derivation Protocol

Mouse preparation

Mouse embryos are harvested at 12.5 days post-coitum, from natural ICR matings. (ICR mice can be purchased through Taconic)

Collection of embryos

Before collection, gelatin coat 150mm tissue culture treated plates (Part A). We prepare one plate per 1.5-2 embryos. Generally expect 12 embryos per mouse (6-8 plates). Additionally, prepare three non-tissue cultured 10cm plates per mouse with sterile 1X PBS.

Pre-warm MEF media and 0.05% Trypsin to 37°C.

Euthanize ICR females and remove uterus with embryos and place into 1X PBS. Under a microscope in a laminar flow hood remove embryos from their deciduas, dissect and discard the internal organs (intestines, liver, heart, etc.) and transfer the cleaned embryos to a dry, sterile 10 cm Petri dish.

Using a sterile blade, mince the embryos. Add 10ml warm 0.05% trypsin per 10-14 embryos. Pipette up and down until the small pieces of embryo are homogenized. Transfer the solution to a 50 ml conical tube. Incubate at 37°C for 1 minute. Pipette the solution 5-10 more times.

Plating primary MEFs

Add 40 ml pre-warmed MEF media to solution. Spin for 10 minutes at 500-600 x *g*, at room temperature. Aspirate media and then re-suspend pellet with 30 ml pre-warmed MEF media. Plate 1.5-2 (~5 ml solution) embryos per 150 mm gelatin coated plate. The final volume of the plates should be 20 ml. Place plates in 37°C, 5% CO_2 incubator.

When plates reach confluence, split them 1:3 to 1:4 to a new tissue culture treated (non-gelatin coated) 150 mm plate. Incubate at 37°C and 5% CO2 until cells are again confluent.

Freezing primary MEFs

Pre-warm MEF media and 0.05% trypsin to 37°C. Place freezing media (Media Preparation, p.7) on ice. Aliquot 20 ml pre-warmed MEF media into a 50 ml conical tube (1 tube:3 plates).

Remove plates, three at a time, from incubator. Aspirate media from plate. Wash once with 5 ml 1X PBS. Add 5 ml trypsin to each plate. Allow cells to detach. Transfer trypsin solution to 50 ml conical tube. With an additional 5 ml trypsin, wash all three plates, and add the solution to the 50 ml conical. Perform a final wash with 10 ml fresh, pre-warmed MEF media. Again, adding the solution to the 50 ml conical tube. Spin cells at 1000 x *g* for 5 min. Gently re-suspend pellet in 9 ml freezing media (1:3 split). Aliquot 1 ml (~3 x 106 cells) per labeled, cryogenic vial. Freeze at -1°C / minute[5]. Store in $N_2(l)$.

[5] In the hES facility, vials are placed in styrofoam, at -80°C overnight.

MEF Expansion

MEF expansion

One vial of primary (passage 1) MEFs will expand to a 150 mm plate in ~4-7 days. Expect ~10 x 10^6 cells (~3, 6-well plates) for every expanded 150 mm plate.

Pre-warm MEF media to 37°C. Aliquot 10 mls MEF media into a 50 ml conical tube, per vial to be thawed. Remove vial/s from $N_2(l)$ and immediately submerge the bottom half of the tube in a 37°C water bath. It should take about 45-60 seconds before the cells are 80% thawed (small frozen portion left). Quickly bring the tube to the laminar flow hood, spray down with 70 % isopropanol, and gently transfer cells to the pre-warmed media. Spin down the cells at 500-600 x *g*. Resuspend pellet in 5 ml pre-warmed media per tube thawed. Prepare an appropriate number of 150 mm tissue culture treated plates by aliquoting 15 ml pre-warmed MEF media to each. Add 5 ml of MEF solution to each 150 mm plate, taking care to distribute cells evenly. Label plates (date, passage 2) and place in 37°C, 5% CO_2 incubator.

Treating MEFs

Once confluent, MEFs must be mitotically inactivated either by mitomycin C treatment, or by γ-irradiation.

Mitomycin C inactivation

For mitomycin C treatment of plates, remove 15 ml of culture media/plate and transfer to a 50 ml conical tube. Add an appropriate volume of mitomycin C such that the final concentration is 10 μg mitomycin C / ml culture media. Mix solution well. Aspirate remaining 5 ml of culture media from each plate, and add back 15 ml of the mitomycin C containing media. Incubate at 37°C, 5% CO_2 for three hours.

Pre-warm MEF media and 0.05% trypsin to 37°C. Aliquot 20 ml pre-warmed MEF media into a 50 ml conical tube (1 tube: 3 plates).

Remove plates, three at a time, from incubator. Aspirate media from plate. Wash once with 5 ml 1X PBS. Add 5 ml trypsin to each plate. Allow cells to detach from plate. Transfer solution to 50 ml conical tube. Wash all three plates with 5 ml trypsin, add solution to 50 ml conical tube. Perform a final wash with 10 ml fresh, pre-warmed MEF media. Add solution to 50 ml conical tube. Count cells with a hemocytometer. Spin cells down at 500-600 x *g*. (continue on to plating or freezing)

γ -irradiation

An alternative to mitomycin C treatment is γ-irradiation. Prior to irradiation, trypsinize the MEFs as described above. Spin down, and re-suspend in a volume of MEF media that will be accommodated by the d-irradiator. Irradiate the MEFs for 25 minutes at 247.3 Rads/min for a total exposure of 6182.5 Rads. More than 25 minutes at room temperature and normal atmospheric conditions is not recommended. After irradiation, increase the volume of the MEF solution to 50 ml. Count cells with a hemocytometer. Spin cells down at 500-600 x *g*.

Plating fresh-treated MEFs

If there is immediate demand for inactivated MEFs, re-suspend pellet in warmed MEF media to a concentration of ~4 x 10^6 cells/ 12 ml if plating a 6-well plate (~4 x 10^6 cells/ 10 ml for a 10 cm). Follow directions for plating MEFs (p. 8)

Freezing pre-treated MEFs

If there is no immediate need for treated MEFs (PMEF*i*), they can be frozen at a concentration of ~4 x 10^6 cell/vial. Freeze in freezing media (Media Preparation, p.6) at a rate of -1°C/ minute. Store at -80°C.

Materials and Reagents

Materials / Catalogue no.

KO-DMEM	Invitrogen Gibco	CAT#10829
DMEM	Invitrogen Gibco	CAT#11965-092
FBS	Valley Biomedical, Inc.	CAT#BS-3033
•KO-Serum Replacement	Invitrogen Gibco	CAT#10828
Plasmanate	Bayer	CAT#NDC 0026-0613-20
bFGF	Invitrogen Gibco	CAT#13256-029
hLIF	Chemicon International	CAT#LIF1010
2-beta-mercaptoethanol	Gibco	CAT#21985-023
Non-essential amino acids (NEAA) (10mM or 100x)	Invitrogen Gibco	CAT#11140050
Pen/Strep (5000U/ml Penicillin G sodium and 5000ug/ml Streptomycin sulfate in 0.85% saline)	Invitrogen Gibco	CAT#15070-063
Glutamax-I (200mM or 100x)	Invitrogen Gibco	CAT#35050-061
Mitomycin	C Sigma	CAT#M-0503
0.05% Trypsin/EDTA	Invitrogen Gibco	CAT#25300-054
Dimethyl sulphoxide (DMSO)	Sigma	CAT#D2650
Gelatin	Sigma	CAT#G1890
Tissue Culture Treated Plates (6 well)	Corning	CAT#3506
Hygromycin resistant, pre-treated MEFs	Specialty Media	CAT#PMEF-H
Neomycin resistant, pre-treated MEFs	Specialty Media	CAT#PMEF-N

• Our facility uses KO-SR with an endotoxin level < 1.0 EU/ml and osmolarity > 460mOsm/kg