Human embryonic stem cell (hESC) H1 maintenance in mTeSR1 / matrigel. (TIER 1) ver. June 29, 2018

NOTE: THIS DOCUMENT FOCUSES ON THE SPECIFIC PROTOCOLS TO BE USED TO STANDARDIZE WORK DONE BY THE 4DN CONSORTIUM. SPECIFICS OF H1 CAN BE FOUND IN THE CELL LINE STATUS DOCUMENT ON THE CELLS WIKI PAGE.

WE HIGHLY RECOMMEND THAT FIRST TIME USERS OF hESCs CONSULT THE STEM CELL TECH MANUAL FOR STEM CELL CULTURE AT THE FOLLOWING LINK:

https://docs.google.com/viewer?a=v&pid=sites&srcid=NGRudWNsZW9tZS5vcmd8NGQtbnVjbGVvbWUtd2lraXxneDozYzczMTczOGQ2MTg3ODM4

The hard copy of the manual at the link above is also provided when you order mTeSR1.

YOU WILL BE REQUIRED TO SUBMIT AUTHENTICATION INFORMATION ALONG WITH YOUR DATA. FOR H1, THIS WILL GO EASIEST FOR YOU IF YOU FOLLOW CAREFULLY THE PROTOCOLS LISTED HERE FROM STOCKING THE CELLS WHEN YOU RECEIVE THEM TO EXPANDING THEM FOR EXPERIMENTS. YOU WILL BE ASKED TO SUPPLY THE FOLLOWING INFORMATION:

1) Did you stock your cells per this SOP?
2) Did you deviate at all from the growth SOPs listed here and, if so, how?
3) Photographs of cell morphology at the time of harvest for each dataset submitted.
4) Passage number AND days in culture since original thaw of vial from WiCell. No data from passage >+30 (since the original stock) will be accepted.
5) Karyotype information at time of harvest if more than 10 passages from thaw. This can be derived from sequencing data, or it can be done in house, or through a service (e.g. WiCell).

Materials and Reagents:

NOTE: there are negotiated discounted prices for the 4DN PIs for mTeSR with StemCell Tech and for reserved lot for Matrigel with Corning. The quotation numbers will change annually.

mTeSR Media: When ordering mTeSR media from StemCell Tech, PIs should reference BOTH the name of the PI and the quote number on each purchase order (**Q-98760** is the mTeSR quote number. This quote is for the entire consortium with the quantity of 1,000. In order to place an order, you need to call StemCell Technologies, giving the quote number and your necessary quantity).

Matrigel: Matrigel is supplied by Corning. Every 6 months, 4DN cell working group will reserve a unified lot for the consortium after inquiring how much each member needs, then you order what you reserved during the given window from either Corning directly or your favorite

distributor. If you are new and would like to be contacted for this lot reserve, please contact "Takayo Sasaki" <sasakit@bio.fsu.edu>.

mTeSR™1 (500 mL) : STEMCELL Technologies Catalog #85850
· mTeSR™1 Basal Medium (#85851) 400 mL Store at 2 - 8°C. Stable until expiry date (EXP) on label.
· mTeSR™1 5X Supplement (#85852) 100 mL Store at -20°C. Stable until expiry date (EXP) on label.
ReLeSR™ : STEMCELL Technologies Catalog # 05872/05873
Corning® Matrigel® hESC-qualified Matrix : Corning Catalog #354277

Tissue culture-treated cultureware : Corning Catalog #353046 or equivalent
Cryostor CS10 : STEMCELL Technologies Catalog #07930/07931
DMEM/F-12 : STEMCELL Technologies Catalog # 36254 or equivalent
D-PBS (without Mg2+ and Ca2+): STEMCELL Technologies Catalog # 37350 or equivalent

Y-27632 (ROCK inhibitor) - only used for applications in which cells are brought down to a single cell suspension. **NOT** necessary for routine passage and thawing stocks of H1.
STEMCELL Technologies Catalog#72302/72304/72307 or equivalent

Equipment :
• Vertical laminar flow hood certified for Level II handling of biological materials
• Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO2 in air
• Low speed centrifuge (e.g. Beckman GS-6) with a swinging bucket rotor
Note: All centrifugation protocols described in this manual can be performed with the brake on
• Pipette-aid (e.g. Drummond Scientific) with appropriate serological pipettes
• Micropipette (e.g. Eppendorf, Gilson) with appropriate tips
• Inverted microscope with a total magnification of 20X to 100X (e.g. Olympus CKX31)
• Isopropanol freezing container (e.g. Nalgene, Fisher; Catalog #1535050)
• -150°C freezer or liquid nitrogen (LN2) vapor tank
• -80°C freezer
• -20°C freezer
• Refrigerator (2 - 8°C)

Preparing mTeSR1 media:
Use sterile techniques to prepare complete mTeSR™1 medium (Basal Medium + 5X Supplement). The following example is for preparing 500 mL of complete medium. If preparing other volumes, adjust accordingly.
Note: Thaw supplements or complete medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw in a 37°C water bath.
1. Thaw mTeSR™1 5X Supplement and mix thoroughly.
Note: Once thawed, use supplement immediately or aliquot and store at -20°C for up to 3 months. Do not exceed the shelf life of the supplement. After thawing the aliquoted supplement, use immediately. Do not re-freeze.

2. Add 100 mL of mTeSR™1 5X Supplement to 400 mL of mTeSR™1 Basal Medium. Mix thoroughly.
Note: If not used immediately, store complete mTeSR™1 medium at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C for up to 6 months. Do not exceed the shelf life of the individual components. After thawing the aliquoted complete medium, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.
If prepared aseptically, complete mTeSR™1 medium is ready for use but the medium can also be filtered using a 0.2 µm low-protein binding filter, if desired.

Preparing Matrigel coated dishes:

Corning® Matrigel® hESC-qualified Matrix should be aliquoted and frozen. Consult the Certificate of Analysis supplied with the Corning® Matrigel® for the recommended aliquot size ("Dilution Factor") to make up 24 mL of diluted matrix (for lot # 6011002, it is recommended ot aliquot 255 uL in 50 mL conical tube and add 25mL DMEM/F12 to make working solution) . If your culture scale is small and cannot use coated dishes from 24mL working solution before the expiration, you can make smaller aliquots of Matrigel. However, Corning does not support diluting Matrigel before aliquots (with any dilution factor) as dilution destabilizes Matrigel. Make sure to always keep Corning® Matrigel® on ice when thawing and handling to prevent it from gelling. Also, pipette tips and conical tubes to make aliquots, culture vessels to be matrigel-coated need to be pre-chilled (see https://docs.google.com/viewer?a=v&pid=sites&srcid=NGRudWNsZW9tZS5vcmd8NGQtbnVjbGVvbWUtd2lraXxneDozYzczMTczOGQ2MTg3ODM4 for the detailed instruction on handling matrigel) .

*Note: **Use tissue culture-treated cultureware** (e.g. 6-well plates, Corning Catalog #353046).*

1. Thaw one aliquot of Corning® Matrigel® on ice.
2. Dispense 24 mL of cold DMEM/F-12 into a 50 mL conical tube and keep on ice.
3. Add thawed Corning® Matrigel® to the cold DMEM/F-12 (in the 50 mL tube) and mix well. The vial may be washed with cold medium if desired.
4. Immediately use the diluted Corning® Matrigel® solution to coat tissue culture-treated cultureware. See Table 1 for recommended coating volumes.
5. Swirl the cultureware to spread the Corning® Matrigel® solution evenly across the surface.
Note: If the cultureware's surface is not fully coated by the Corning® Matrigel® solution, it should not be used for human ES or iPS cell culture.
6. Incubate at room temperature (15 - 25°C) for at least 1 hour before use. Do not let the Corning® Matrigel® solution evaporate.
Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the Corning® Matrigel® solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 1 week after coating. Allow stored coated cultureware to come to room temperature (15 - 25°C) for 30 minutes before moving onto the next step.
7. Gently tilt the cultureware onto one side and allow the excess Corning® Matrigel® solution to collect at the edge. Remove the excess Corning® Matrigel® solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
8. Immediately add mTeSR™1 medium (e.g. 2 mL/well if using a 6-well plate).

Thawing:

Note: thawing conditions are WiCell, not StemCell Tech, so we deviate from the StemCell Tech manual here to ensure that the cells are thawed precisely as per WiCell.
NOTE: Before thawing hESCs from WiCell, check the certificate of analysis for the line from WiCell to acquire the recommended number of wells one vial should be thawed into, which can vary across lots. For our current WA01 lot WB35186, it is recommended to thaw 1 vial into 3 wells of 6 well plate).
IMPORTANT: Passage cells at least twice before re-freezing after a thaw.

Human ES and iPS cells should be thawed into previously Matrigel coated cultureware. If prepared Matrigel™ coated plate has been stored at 4°C, allow to warm to room temperature for one hour.

1. Have all tubes, warmed mTeSR™1 (15 - 25°C) and DMEM/F-12 , and pre-coated cultureware ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.
Note: Do not warm mTeSR™1 in a 37°C water bath.
2. Remove excess plating medium from prepared Matrigel™ coated plate from wells which will receive cells, and add 1.5 ml mTeSR™1 Medium to every well that will receive cells.
3. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials).
4. Remove the pluripotent stem cell vial from the liquid nitrogen storage tank.
5. Roll the vial between your gloved hands until the outside is free of frost. This should take between 10-15 seconds.
6. Quickly thaw cells in a 37°C water bath by gently shaking the vial. Remove the vial when a small frozen cell pellet remains.
7. When only a small ice crystal remains, remove the vial from the water bath and wipe it with 70% ethanol or isopropanol.
8. Transfer the cells gently into a sterile 15ml conical tube using a 1ml - 5ml pipette.
9. Slowly, add 11ml of mTeSR™1 Medium drop-wise to cells in the 15ml conical tube. While adding the medium, gently move the tube back and forth to mix the pluripotent stem cells. This reduces osmotic shock to the cells.
10. Centrifuge cells at 200 x g for 5 minutes at room temperature (15 - 25°C).
11. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed.
12. Re-suspend the cell pellet in 0.5ml mTeSR™1 Medium for every well that will receive cells (number of wells receiving cells is based on the thaw recommendations found in the certificate of analysis which is included in the shipping packet insert). **For example:** When the thaw recommendation is to thaw 1 vial into 4 wells, re-suspend the pellet in 2ml, 0.5ml will be plated into each well.
13. Very gently pipette cells up and down in the tube a few times.
14. Slowly add 0.5ml of the cell suspension drop-wise into each well.
15. Place plate gently into the incubator and gently shake the plate back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent cells from pooling in the center of the well.
Note: While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells across the well.
16. The next day, remove the spent medium and debris using a sterile 5ml serological pipette, and transfer it into a prepared well of a second 6-well plate. This will serve as the backup for the thaw and should be given to a second technician if possible. This backup should be maintained with separate medium and in a separate incubator. (At least Kaufman lab and Gilbert lab found that there were no cells that would attach later to the plate in the spent media, but this is what WiCell insisted.)
17. Add 2.0ml of mTeSR™1 medium to the first original well/plate. Place both plates

gently into an incubator overnight.
18. Feed cells daily as described below (without ROCK inhibitor) until ready to be passaged or harvested.

Feeding Pluripotent Stem Cells

1. Observe the pluripotent stem cells using a microscope. If they require passaging,

follow the passaging protocol below.

2. If they do not require passaging, aspirate the spent medium with a sterilized Pasture pipette. If feeding more than one plate, use a different pipette for each plate in order to reduce risk of contamination.
3. Add 2.0ml of mTeSR™1 Medium to each well. After pipettes are used once, they must be disposed of to reduce the contamination potential. Return the 6-well plate to the 37°C incubator.
4. Repeat procedure daily until cells require passaging.

<u>Passaging hESCs grown in mTeSR1:</u>

The following instructions are for passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.
1. At least one hour before passaging, coat new plates with Corning® Matrigel®.
2. Aliquot sufficient mTeSR™1 and warm to room temperature (15 - 25°C).
Note: Do not warm mTeSR™1 in a 37°C water bath.
3. Wash cells with 1 mL of phosphate-buffered saline (PBS) without Ca2+ and Mg2+ and aspirate.
Note: There is no need to remove regions of differentiated cells.
4. Add 1 mL of ReLeSR™ and aspirate ReLeSR™ within one minute, so that colonies are exposed to a thin film of liquid. [NOTE: enzymatic dissociation, such as Dispase, is not reccomended as it creates additional stress on the cells and causes fewer cells to survive the passage. hESCs do not like to be singletons, and will show increased karyotype changes when passaged this way. There are many versions of non-enzymatic dissociation reagents, all of which are based on EDTA. For uniformity across the consortium, please use ReLeSR.]
5. Incubate at 37°C for 5 - 7 minutes.
Note: Optimal dissociation time may vary depending on the cell line used; when passaging a cell line with ReLeSR™ for the first time, the optimal dissociation time should be determined.
6. Add 1 mL of mTeSR™1.
7. Detach the colonies by placing the plate on a plate vortexer (e.g. Multi-MicroPlate Genie, 120V, Scientific Industries Model SI-4000, at 1200 rpm) for 2 - 3 minutes at room temperature. Alternatively, hold the plate with one hand and use the other hand to firmly tap the side of the plate for approximately 30 - 60 seconds.
8. Transfer the detached cell aggregates to a 15 mL tube using a 5 mL serological pipette. Cell aggregates should be appropriately sized for plating (mean aggregate size of approximately 50 - 200 μm).
Note: If you wish to plate cell aggregates directly from your passaged well (i.e. without transferring into a tube), pipette the aggregate mixture up and down once using a 5 mL pipette. This will ensure breakup of any large aggregates that may still be present.

9. Plate the cell aggregate mixture at the desired density onto pre-coated wells containing mTeSR™1. If the colonies are at an optimal density, the cultures can be split every 5 days using 1 in 10 to 1 in 20 splits (i.e. cell aggregates from 1 well can be plated in 10 to 50 wells). If the colonies are too dense or too sparse, at the next time of passaging adjust the split ratio accordingly). There are details of passaging, including pictures, in the StemCell Tech manual at the link provided at the beginning of this SOP. **IMPORTANT FOR AUTHENTICATION:** Keep track of your passages and keep them close to every 5 days as possible.
10. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to side motions to evenly distribute the cell aggregates. Do not disturb the plate for 24 hours.
Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.
11. Perform daily medium changes using mTeSR™1 and visually assess cultures to monitor growth until the next passaging time.

Table 1. Recommended Volumes for matrigel, media, cell dissociation reagents

CULTUREWARE	VOLUME OF Matrigel/PBS/ReLeSR	VOLUME OF mTeSR1
6-well plate	1 mL/well	2 mL/well
100 mm dish	6 mL/dish	10 mL/dish
T-25cm2 flask	3 mL/flask	5 mL/flask
T-75cm2 flask	8 mL/flask	15 mL/flask

Cryopreserving hESCs:

Cells will be fragile for the first passage out of thaw. Wait to passage +2.

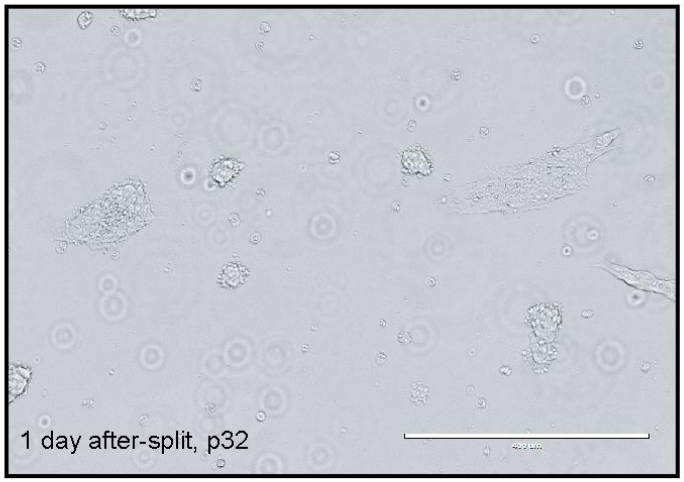
Freezing:

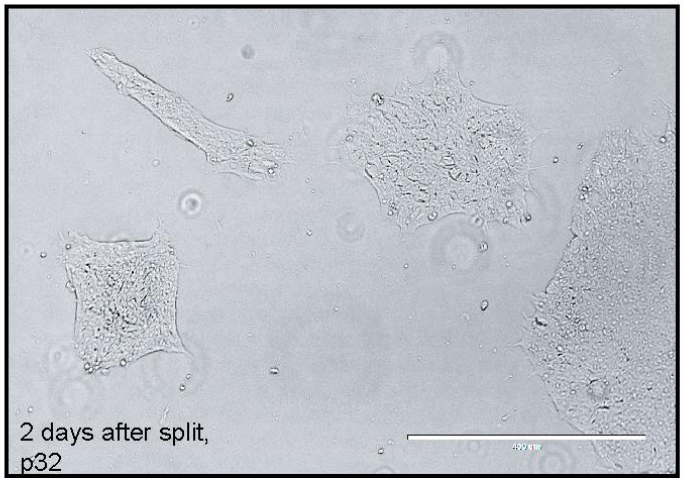
Note: Passage cells at least twice before re-freezing after a thaw.
Note: Wipe down the outside of the bottle with 70% ethanol before opening.
The following are instructions for cryopreserving cultures grown in mTeSR™1 medium in 6-well plates using CryoStor CS10. Cultures should be harvested and frozen at the time they would normally be ready for passaging. Each vial should contain the cell aggregates from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.
1. Passage cells using enzyme-free passaging protocols until step 8 of passaging protocol.
2. Centrifuge at 300 x g for 5 minutes at room temperature (15 - 25°C).
3. Gently aspirate the supernatant taking care not to disrupt the cell pellet.
4. Gently resuspend the pellet with 1 mL per well harvested of cold (2 - 8°C) CryoStor CS10 using a serological pipette. Minimize the break-up of cell aggregates when dislodging the pellet.
5. Transfer 1 mL of cell aggregates mixture into each labeled cryovial using a 2 mL serological pipette.
6. Freeze cell aggregates using either:

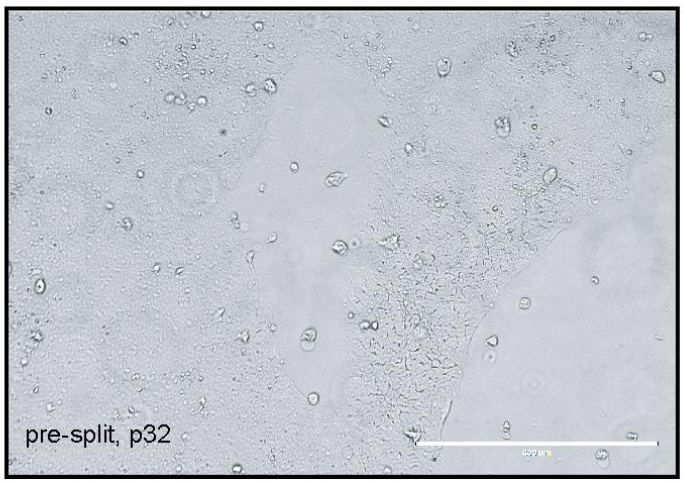
· a standard slow rate controlled cooling protocol that reduces temperatures at approximately -1°C/min, followed by long-term storage at -135°C (liquid nitrogen) or colder. Long-term storage at -80°C is not recommended.
· a multi-step protocol where cells are kept at -20°C for 2 hours, followed by -80°C for 2 hours, followed by long-term storage at -135°C (liquid nitrogen) or colder.
· in an iso-propanpol container or styrofoam sandwich at -80°C for 24 hours followed by long-term storage at -135°C (liquid nitrogen) or colder.

Example image of cell morphology and growth next page (credit: Pauk Kaufman lab)

WA01 H1 cells pre-split, 1 day after split and 2 days after split, 10x mag on inverted scope (note out of plane floating cells. Cells were cultured on matrigel mTeSR media in accordance to SOP 4DN protocol







PYRAMID METHOD TO STOCK hESC LINES

(UPON RECEIPT OF YOUR VIAL, THIS IS THE FIRST THING YOU NEED TO DO)

Principle: Mammalian cells are constantly changing. They are continually under selection for cells that grow fastest under whatever conditions they are grown in, and this can lead to the accumulation of genetic and epigenetic changes. Cells need to be kept at as early a passage as possible. This is done by keeping an original stock, or "layer 1 stock" that is almost never touched, a secondary layer or "layer 2 stock" that is needed infrequently, and a "working stock", that you go back to every so often. All cells that come into the laboratory should be stocked in this way, and labeled as such. Layer 1 and Layer 2 stocks should be kept in separate places, working stocks can be kept in a more accessible area.

Passage number should always be kept and should be written directly on the freezing vial. You should know the passage when you get the cell line. If not, record it as the passages since receiving (+1, +10, etc).

1) First, thaw your vial and expand cells up to enough material to bank back about 30-40 vials.
2) Freeze "layer 1", which is your "master cell bank"– all the dishes except 1 or 2 pooled together. Store them out of reach.
3) Thaw one to
    a) verify that they are viable, not contaminated, and have retained identity and karyotype.
    b) Work up your "layer 2" or "working stock"
3) Expand your "layer 2" working stock up to 30-40 vials again and repeat #2 above.
4) Freeze "layer 2" – the "working stock".
5) Thaw one to
    a) verify that they are viable, not contaminated, and have retained identity and karyotype.
    b) Use for your experiments

Work with the remainder until such time as you feel they may have changed (morphology is your best initial indicator), or up to 30 passages. If morphology changes or after 30 passages, go back to "layer 2" or "working stock". When the working stock gets low, you may have to go back to "layer 1" and repeat steps 3 through 5.

Additional protocols:

I. Preparing a Single-Cell Suspension for Downstream Applications:

Examples of applications: Flow cytometry, microfluidics, subcloning.
NOTE: WiCell would like to warn that **taking hESCs down to single cell suspension should be avoided unless absolutely necessary** as it drives cells toward an abnormal karyotype.
The following are instructions for preparing a single-cell suspension from cultures grown in mTeSR™1 medium in 6-well plates. If using other cultureware, adjust volumes accordingly. Cultures should be harvested and frozen at the time they would normally be ready for passaging.

1. Warm medium (DMEM/F-12 or mTeSR™1), ReLeSR Reagent, and PBS without Ca2+ and Mg2+, to room temperature (15 - 25°C) before use.
Note: Do not warm mTeSR™1 in a 37°C water bath.
2. Wash the well to be passaged with 1 mL of PBS without Ca2+ and Mg2+.
3. Aspirate the wash medium and add 1 mL of ReLeSR Reagent. Incubate at 37°C for 8 - 10 minutes.
Note: The incubation time may vary when using different cell lines or other cell passaging reagents.
4. Harvest cells by pipetting up and down with either a serological pipette or a 1 mL micropipette to ensure a single-cell suspension and transfer cells to a 15 mL conical tube. Rinse wells with an additional 2 - 4 mL of medium (DMEM/F-12 or mTeSR™1) and add the rinse to the tube containing the cells.
5. Centrifuge cells at 300 x g for 5 minutes.
6. Resuspend cells in appropriate medium for desired downstream applications.

Note: Addition of 10 µM ROCK inhibitor Y-27632 (Catalog #72302) is recommended when plating single cell suspensions of human ES or iPS cells as it has been reported to increase cell survival.

II. Weekend-Free Culture of hESCs in mTeSR™1: this is not for novices and deviates from the 5 day standards for keeping track of passage, but it may be very helpful as labs get more experienced.

ALSO SEE:

https://drive.google.com/file/d/0B548E2qA0rVAUUV2UWVzelY0cVk/view?usp=sharing

This protocol involves routine passaging of hESCs on Fridays and eliminates feeding on Saturdays and Sundays (when colonies are small).
1. Passage cells using enzyme-free passaging protocols until step 8 of passaging protocol.
2. Break up aggregates by gently pipetting up and down, to a relatively homogeneous size of about 80 - 150 µm in diameter.

3. Seed ~30 - 50 aggregates/cm2 (e.g. 300 -500 aggregates/well of a 6-well plate) into new wells.
4. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to side motions to evenly distribute the cell aggregates. Do not disturb the plate for at least 24 hours.
5. Refresh media again on Monday. Removal and replacement of media is not required on Saturday and Sunday as cell aggregates are small enough to grow and maintain pluripotency without fresh media.