CELL guidance systems

Pluripro®

hPSC Culture System

Version 5.1

Updated April 2012

Components of culture system

The Pluripro® hPSC culture system requires the purchase of two products: Pluripro® medium and Pluripro® MATRIX, which are used in combination to enable stable, homogeneous expansion of human pluripotent stem cells (hPSCs). These products can also be purchased together as a trial kit.

Product	Catalogue No	Storage
Pluripro® media, 500ml	MK01	-20°C
Pluripro® MATRIX, 50ml	M04	-20°C
Pluripro® Trial kit: media 250ml, MATRIX, 20ml	T01	-20°C

Additional reagents

1. **Antibiotics, if required (all formulations are antibiotic-free)**

Storage conditions

Minimize freeze-thaw cycles. On first thawing of Pluripro® medium, aliquot the medium into individual volumes, each sufficient for a maximum of 7 days use. Immediately return all aliquots that will not be used within the next 7 days to storage at -20°C. Thawed aliquots of Pluripro® media can be stored at 4°C for up to 7 days.

Pluripro® MATRIX should be thawed at room temperature or 37°C prior to first use and can subsequently be kept for up to 28 days at 4°C.

Protocol

The Pluripro® culture system enables robust, trouble-free culturing of hPSCs. However, even if you are very experienced, there will be aspects of this hPSC culture system that may not be familiar. Please take time to carefully read through these notes to ensure optimal performance. Technical support: tech@cellgs.com

Coating culture surface with Pluripro® MATRIX

Pluripro® MATRIX is supplied at a ready-to-use concentration. Plastic culture ware should be coated with the MATRIX solution at a volume to surface area ratio of 0.1ml/cm^2. If cells detach during culture, the most likely reason is inadequate coating with MATRIX.

1.1 Calculate the amount of Pluripro® MATRIX required for the surface area of your culture dish. Use 0.1ml/ cm^2. It is essential that an adequate coating of matrix is achieved.

1.2 Add Pluripro® MATRIX and agitate to ensure the entire surface is covered. If plating cells in small vessels (e.g. 96 well plates), ratios higher than 0.1/cm^2 will be required to achieve complete coverage.

1.3 Incubate the vessels at 37°C for at least 3 hours or, alternatively, overnight at 4°C.

1.4 Remove and discard excess MATRIX immediately before plating hPSC suspension. Do not allow coated vessels to dry out prior to plating of cell suspension. Coated plates can be stored at 4°C for up to 10 days if sealed with Parafilm to prevent drying out. If plates have been refrigerated, allow the coated vessels to return at least to room temperature before use. Coated vessels can be warmed rapidly to 37°C in a humidified incubator if necessary.

Aliquoting of Pluripro® media

Pluripro® medium is supplied as a complete product which does not require any supplements. It is not necessary to add any growth factors. Add antibiotic supplements only if required. On first use of the product, prepare aliquots and store as described above in Storage Conditions. When thawed, medium may be stored for up to one week at 4°C. Medium should be pre-warmed to 37°C before use.

2.1 Calculate the weekly Pluripro® medium requirements, aliquot and then pre-warm sufficient volume of medium for first day.

Medium Change Frequency: Generally it is recommended to change the culture medium daily using a volume of 0.3ml Pluripro® medium for each cm^2 culture surface. If 48 hour medium changes are necessary, the volume used should be increased to 0.45ml/cm^2. Multiple 48 hour feeding cycles are supported by the medium. However, for best results, we recommend that culture change should be performed daily. **48 hour feeding should not be used when establishing culture for a new hPSC line.**

Establishing culture in Pluripro® medium: Transfer of pluripotent cells into Pluripro® medium

Pluripro® medium allows stable, confluent culture of hPSCs. Cultures can be transferred from either MEF or hFF based feeder culture or directly from feeder free systems such as mTeSR® or StemPro®. Establishing cells in Pluripro® medium should require no more than 3 passages. The Pluripro® culture system works well with single cell passage combined with confluent culture.

Cell culture dishes, coated with Pluripro® MATRIX as described above, should now have been prepared and be ready for use. Pluripro® medium should be warmed to 37°C.

3.1 Manually remove obviously differentiated regions from the source population to leave a clean, undifferentiated culture. Transferred cells should be >95% pluripotent. Ideally, aim for 100% pluripotency by manually dissecting differentiated cells from the input culture. **Lower levels of pluripotency in cells transferred into the Pluripro® system will result in failure to establish the system.**

3.2 Dissociate cells enzymatically using either TrypLE™ or Accutase.
For single cell passaging, good results have been obtained with TrypLE™. If passaging as single cells (see below), a survival agent such as a **Rho kinase inhibitor** (e.g. Y27632, 10µM), added for the first 24 hours, will aid re-plating.

3.3 Following dissociation, add 2-3 mls of Pluripro® medium and then centrifuge the cells at 250-300g for 3 minutes to pellet the cell suspension.

3.4 Aspirate and discard the supernatant.

3.5 Gently resuspend cells in Pluripro® medium and plate in the pre-coated culture vessel at a density of 80-100K cells/cm^2. This relatively high density culture in the first plating using Pluripro increases success rates. **Seeding in the first passage at densities below 80K/cm^2 will cause the cells to differentiate and result in failure to establish the system.** Density can be reduced in subsequent plating (see below). If the cells have clumped together, then gently pipette the suspension up and down as necessary to dissociate.

3.6 Transfer the culture vessel immediately to a 37°C humidified incubator and allow the cells to become adherent. Do not disturb for 24 hours.

3.7 If cluster passage is preferred, use Collagenase to dissociate to clusters.

Passage of cells cultured in Pluripro® medium

Cell culture vessels (dishes or flasks) should be prepared beforehand by coating with Pluripro® MATRIX as described above. Pluripro® medium should be warmed to 37°C before use. Cells should be maintained in 6 well plates for the first few passages and any larger areas of differentiated material (that have developed from partially differentiated cells carried over from the previous culture system) should be manually removed by scraping prior to subsequent passage. Once clean culture has been achieved (which should take no more than 3 passages), the cell culture can be transferred to a flask-based format if required. Subsequently, maintenance in Pluripro® medium will ensure they will remain as a clean and homogeneous population of pluripotent cells.

Once established in Pluripro®, cells should be seeded at a density of 60K/cm^2 to enable a 4-5 day passage interval. If the culture appearance is suboptimal, it may be necessary to increase the seeding density and shorten the passage interval. Cells should be passaged before they reach a density of 300K/cm^2

4.1 Aspirate medium from the cells

4.2 Wash the cell layer with D-PBS

4.3 Cells may be passaged as single cells or as clusters. Use either protocol A or B.

(A) Single cell passage:

4.3.1 **Add TrypLE™ Select** (Invitrogen) to the cell culture vessel in sufficient volume to cover the cell layer.

4.3.2 **Incubate for 5 minutes at 37°C** by which time all cells should have detached. If necessary, agitate or tap the cell culture vessel to detach any remaining cells.

4.3.3 **Add 1ml/5cm^2 Pluripro® medium.**

If good results are not achieved then consider the use of a survival agent such as Y27632 at 10µM concentration for the first 24 hours of each passage, added when reseeding cultures. There is no positive effect of continuing to add a survival agent during routine medium change. It should only be added when reseeding cultures.

(B) Cluster passage:

4.3.1 **Add either collagenase or accutase to the cell culture vessel.** Use a minimal volume, just sufficient to cover the cell layer.

4.3.2 **Incubate at 37°C for 3-10 minutes** checking frequently to monitor the dissociation state.

4.3.3 **As cells begin to dissociate, aspirate enzyme and then add 0.2ml/cm^2 Pluripro® medium and gently wash clusters from the Pluripro® MATRIX.** Any residual cells still attached can be released using a cell scraper.

4.4 **Centrifuge the cell/cluster suspension at 300xg for 3 minutes** to pellet the suspension.

4.5 **Resuspend in Pluripro® medium, gently pipette up and down to dissociate clumps as necessary.**

4.6 **Re-seed at a density of approximately 60K cells/cm^2 on a pre-coated culture vessel**. For cluster passaging this will approximate to a 1:4 ratio split from a confluent culture.

Single cells passaged in Pluripro® medium have an appearance distinct from the normal, tightly packed colony morphology. Post plating, the cells are very adherent, flattened and spread out (Figure 1). **This morphology is characteristic of hPSC in single cell state and is expected.** The hPSCs will quickly reach confluence and then begin to pack up tightly and form the more familiar hPSC with characteristic morphology (Figure 2).

Pluripro® culture system trouble-shooting

If any difficulty is experienced during culture with Pluripro® medium, please do not hesitate to contact tech@cellgs.com. Our team of experienced stem cell scientists will respond quickly and will be happy to assist you.

Culture of hSPCs in Pluripro® is extremely robust and stable. However, colony based systems are not as clean and the transition from colony-based culture to clean culture in Pluripro® requires attention to detail. The following list is a sample of problems that we have anticipated may arise.

1) **Cells detach during or at the end of the culture period**

    a. Cells will detach if there is insufficient matrix coverage or if the cells have become too dense at the end of the passage. In order to correct or prevent such an occurrence the following steps can be taken

        i. Use a longer coating period with matrix solution: Leave the culture vessel overnight at 37°C to after coating to maximise matrix absorption.

        ii. Increase matrix volume to 2ml/10cm^2 then, once culture is established, titrate back to determine an appropriate level for that cell line.

        iii. Passage sooner, before cells become too dense. Densities above 300K cells/cm^2 are too dense and passaging times/seeding densities should be modified to maintain densities below this threshold.

2) **Cultures difficult to establish as clean homogenous populations**

    a. The input quality of the cells being used to establish the culture is critical since cells which are already committed to differentiation will proceed to do so regardless of the medium environment. These cells will grow at slower rates than pluripotent cells and will eventually be eliminated from culture. However, it is important to seed with a starting population that is at least 90% pluripotent. Any islands of differentiated cells that develop during passage 1 and 2 should be excised. If differentiated cells are still present at passage 3, please contact us for advice.

    b. Maintaining a high culture density and shortened passage time will help to establish clean undifferentiated culture in Pluripro® medium. Seeding densities should be >80 K/cm^2 on the first plating and subsequently maintained above 60K/cm^2 at passage. Harvest densities at the end of passage should be approximately 250-300K/cm^2.

3) **Excessive cell death during passage**

    a. Some cell lines will tolerate single cell passage. However, many require additional support at the single cell stage. If excessive cell death occurs, it can be resolved by one of the following

        i. the inclusion of Rho kinase (ROCK) inhibitor e.g. Y27632 at a concentration of 10µM during the **first 24 hours of each passage** (on seeding).

        ii. Increasing the matrix volume used for coating to 2ml/10cm^2.

        iii. Inclusion of Laminin 511 or 521 in the matrix, coating at 5µg/10cm^2.

4) **Differentiation or deterioration in cell viability during culture.**

   a. This can be due to insufficient medium change intervals; leaving cells in old, exhausted medium will cause rapid deterioration. Follow changing intervals described in the protocol.

5) **Cells clump after enzymatic passage**

   a. Cells left too long under enzymatic treatments can clump and require vigorous pipetting to return to normal suspension. To prevent such an occurrence, do not routinely leave cells exposed to enzymes for prolonged periods of time and add diluting medium prior to aspirating enzyme treated cells.

Figures

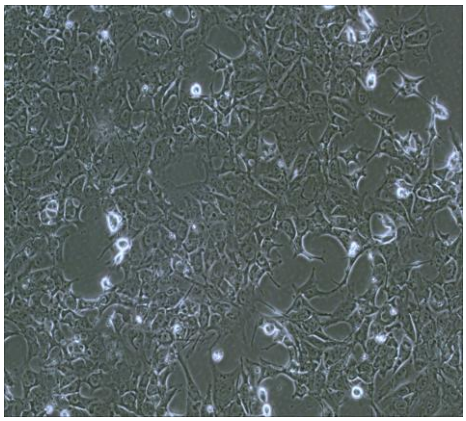

Fig 1 Single cells post-plating in Pluripro® medium

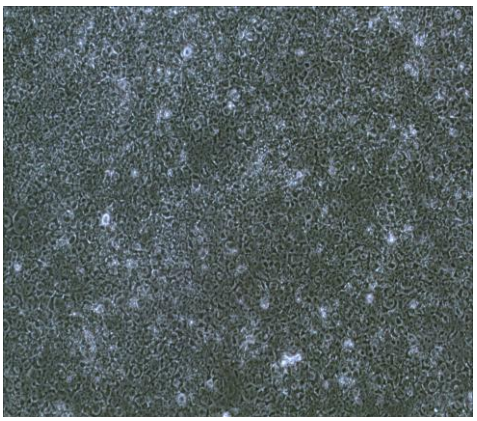

Fig 2. Cells at confluence in Pluripro® medium

Technical Support tech@cellgs.com