

THE CULTURE OF PRIMARY HUMAN CELLS AN INSTRUCTION MANUAL

This manual has been developed by scientists at TCS CellWorks as a concise set of basic instructions for the culture of all primary cells sold by TCS CellWorks.

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1. Introduction

Normal primary and early passage cells differ from immortalised cell lines in a variety of ways. From a practical point of view, their culture is more fastidious, and specialised culture media and careful culture technique are required to ensure their survival and normal expression of cell markers.

Normal primary cells are genetically programmed to have a finite lifetime. In practice, this means that, depending on the cell type, they can be cultured *in vitro* for a maximum of 50-60 doublings before becoming senescent. Well before this time they may begin to lose surface markers or change their morphology.

The protocols that follow will enable cells to be cultured through at least 15 population doublings (approximately three passages depending on the split ratio), using recommended media and reagents. Because the medium is optimised to be used without feeder layers or matrix components, the cells can be added to the pre-equilibrated medium without pretreating flasks*. In most cases, a confluent monolayer can be obtained within 7-10 days.

It is important that all the instructions are followed scrupulously in order to obtain satisfactory and reproducible results.

* Except human dermal microvascular endothelial cells

2. Materials Supplied.

2.1 Cell Kits with cryopreserved cells

A Cell Kit contains everything required for successful cell culture: cells, basal medium, medium supplements and subculture reagents. The components have been optimised together to provide a complete integrated system. Use of medium or subculture reagents from other sources may give sub-optimal results and cell performance cannot be guaranteed in these circumstances. The standard Cell Kit contains cryopreserved cells but cells can be shipped as proliferating cultures if required.

For Cell Kits with cryopreserved cells, all components are shipped on dry ice, except for the basal medium which should not be frozen. Basal medium is shipped using cold packs to maintain the temperature near 2-8°C.

Note that cryopreserved cells will survive satisfactorily in dry ice for the duration of shipment only. Beyond this time period, they will quickly lose viability and they should either be used immediately or immediately transferred to liquid nitrogen for long term storage. Storage at -70°C is not suitable.

Each Cell Kit contains -

1 vial of cryopreserved primary cells (usually 500,000 cells)

1 bottle of basal medium (500 ml)

1 bottle of medium supplement

1 Passage Pack™ for subculture containing 100 ml each of BSRS, trypsin (0.025%)/EDTA (0.01%), and trypsin blocking solution (TBS)

Components may be bought as a complete kit or separately.

On receipt of your Cell Kit, please unpack immediately, check the components are complete and correct, and store as indicated in the table below:

Storage Instructions for Cell Kit Components

Component*	Storage conditions	Shelf life
Cryopreserved cells	Transfer <u>immediately</u> to liquid nitrogen.	Indefinite
Proliferating cells	Transfer immediately to 37°C	Use immediately. See page 11 for further instructions
Basal medium	4°C in the dark	4-6 months
Supplements	-20°C	1 year
Supplemented medium	4°C in the dark	Usually 4 weeks
Subculture reagents	-20°C. If thawed on receipt aliquot and store at -20°C	1 year

* Details of individual media and supplement components are given in the batch specific analysis sheets provided with the products.

2.3 Proliferating cells

If required, cells can be provided as proliferating cultures in the form of monolayers in tissue culture flasks. In this case, the cells are shipped in flasks completely filled with medium, and in conditions designed to maintain the temperature at 25-37°C. In the case of proliferating cells, the medium in the initial Cell Kit will have been used at TCS CellWorks to set up your culture using the procedures documented later in this manual. (You will be charged for this medium and an additional sum of £20 per T25 flask to cover the labour of setting up the cells). If you intend to grow the cells on further, you will need to buy additional medium and subculture reagents.

Proliferating cells should be transferred to 37°C and treated immediately as detailed on page 11.

Warning

1. All cells and culture reagents in the pack are approved for in vitro research use only. They should not be used for therapeutic or diagnostic procedures.

2. Although the cells test negative by PCR for HIV-1, Hepatitis B and Hepatitis C DNA, no test procedure can guarantee the absence of known and unknown infectious agents. Consequently, all products of human origin should always be considered potentially biohazardous and appropriate precautions should be taken.

(See for example Grizzle, W.E. & Potts, S.S. (1988) Guidelines to avoid personnel contamination by infectious agents in research laboratories, J. Tissue Culture Methods 11; 4.

3. Cell Culture Procedures

3.1. Preparation of Medium

Quick tips -

- Only warm the amount of medium required at any one time, or the shelf life of the medium will be adversely affected.
- Always equilibrate medium in the CO₂ incubator, not in a waterbath: CO₂ equilibration is as important as temperature equilibration.

Please note down lot numbers of medium and supplements for reference in case if any problems.

- 1 Thaw supplements rapidly at 37°C.
- 2 Transfer supplements and basal medium to a sterile field.
- 3 Wipe all caps with alcohol (ethanol or isopropanol) before opening. Mix contents by gentle inversion.
- 4 Transfer contents of supplement vials to the basal medium using a sterile Pasteur pipette. Use some of the medium to rinse out the vials. Be sure to add *all* of the recommended quantity of supplements.
- 5 Replace cap on medium bottle and mix contents gently but thoroughly. Write the date on the bottle: once supplemented the medium has a shelf life of 4 weeks.
- 6 Calculate how many flasks you will need (see table on next page) and aliquot medium into flasks -
5 ml per flask for T25
15 ml per flask for T75

Normally do not plan to seed all the cells into one flask: usually 4-8 T25s, or 2-3 T75s can be used, depending on the cell type.

- 7 Place the flasks in a humidified incubator at 5% CO₂ and 37°C, for about 30 min to equilibrate. This step is important to ensure a gentle recovery of the cells after thawing.

Number of viable cells per flask -

Cell type	Minimum seeding density	Total cells seeded/flask	
		T25	T75
Large vessel endothelials	2500/cm ²	62,500	187,500
Microvascular endothelials	5000/cm ²	125,000	375,000
*Other cell types	See below	87,500	262,500

* Please refer to specific data sheets for each cell type for more detailed seeding density information.

3.2. Thawing and seeding cells

Quick tips -

- Cells have been cryopreserved in medium supplemented with cryoprotectant
- Check you have everything to hand before you begin.
- Work fast but carefully - the whole process from liquid nitrogen to culture flasks should take no longer than 4-5 minutes. Experience has shown that speed at this stage is essential for good recovery of cells.
- Do not centrifuge out cryoprotectant - this can cause more damage than leaving it in until cells have adhered. However, to prevent toxicity, the cryoprotectant should be diluted at least 1/25 with medium.
- Always change the medium after 24 hours to remove residual cryoprotectant.

Before you start

- 1 Adjust temperature of waterbath to 37°C.
- 2 Check you have a micropipette, sterile tips and wipes ready.
- 3 Check volume of cells you will use per flask (see table in section 3.1. *Preparation of Medium*) and set the micropipette accordingly.
- 4 Check that the medium is equilibrated for both temperature and CO₂.
- 5 Have beaker of dry ice or Dewar of liquid nitrogen available for transferring cryovial.

Once you are ready

- 1 Remove cells from liquid nitrogen and transfer them on dry ice or in liquid nitrogen to a sterile field. Wipe the vial with ethanol or isopropanol, loosen the cap slightly for about 10 seconds to release any pressure and retighten.
- 2 Transfer vial immediately to the 37°C waterbath. Dip the bottom half of the cryovial in the water, making sure not to submerge the cap. Swirl gently but rapidly for 1-2 minutes until only a small piece of ice is left.
- 3 Wipe the vial dry and transfer the cells on ice to the hood.
- 4 Rinse the outside of the vial with alcohol, particularly around the cap area. Wipe to remove the excess. Open the vial and gently pipette the suspension up and down to disperse the cells.
- 5 Determine the number of viable cells per ml by removing 20µl cell suspension from the vial, diluting with 20µl trypan blue and counting the viable cells using a haemocytometer
- 6 Transfer pre-equilibrated culture flasks to the hood.
- 7 Pipette the requisite volume of cells carefully onto the culture flask in an arc on the surface of the medium so that the cells are spread out over the surface. Immediately disperse the cells in the medium in the flask, by gentle swirling. Uneven cell growth may occur if the cells are not thoroughly dispersed in this way.

Note: in the frozen state the colour of the medium in the cryovial may appear yellowish, but usually turns pink or orange-pink after thawing. Please note the thawed colour in case of future problems.

- 7 Examine the cells microscopically to ensure that a good transfer and distribution of cells has been achieved in each flask.
- 8 Return cells to the CO₂ incubator for seeding to take place. Do not disturb the culture for the next 16 hours or so while the cells attach.

3.3. Treatment the next morning (i.e. after 24 hours maximum)

- 1 Examine cultures to see if seeding has been successful. If the cells are seeded at the recommended densities, there may only be a small number of cells attached per high power field. Always use phase contrast microscopy to observe the cells, as they may easily be missed with ordinary illumination.

If at this stage the seeding looks lower than expected, don't panic. Keep following the instructions below. If after several days, you are still concerned, contact TCS Biologicals to discuss the TCS CellWorks Manual

problem.

- 2 Pre-equilibrate fresh medium in the CO₂ incubator as before, allowing 5 ml per T25 or 15 ml per T75.
- 3 Carefully aspirate old medium from the cells, and replace with new medium. Be careful to run the medium over a cell-free surface of the flask and never over the cell layer, as the cells may become dislodged.
- 4 Return the cells to incubator.

3.4. Maintenance of Cells in Culture

Quick tips -

- To prevent stalling, primary cells need to be fed regularly, at least every 48 hours.
- To prevent irreversible contact inhibition, do not allow cultures to become more than 80% confluent. If in doubt, subculture.
- If cultures were seeded unevenly, confluent patches may develop. A few cells in the centre of these patches may stall, but the rest will remain capable of division.

- 1 Within 24-48 hours of seeding, mitotic cells should become obvious in most fields. These will be more rounded and far more refractile than the rest of the cell population.
- 2 Feed the cells every other day using the following guidelines -

Approximate confluence	Amount of fresh medium per 5 cm²
<25%	1.0 ml
25-45%	1.5 ml
>45%	2.0 ml

- 3 If the cells are unlikely to become confluent over the weekend, the recommended amount of medium may be doubled on Friday so that it is not necessary to come in over the weekend. *Do not do this if the cells are >50% confluent as they will need daily feeding, extra medium and possibly splitting before Monday morning. If this is not done, the cultures may stall irreversibly.*
- 4 Subculture when the cells reach 60-80% confluence (if the recommended seeding densities are used, this usually takes 7-10 days, depending on the doubling time).

3.5. Subculture of Cells

Quick tips -

- Do not allow cells to reach confluence before subculturing.
- Treat cells as gently as possible at all stages.
- Cells are guaranteed for 15 population doublings after recovery from cryopreservation, or 10 doublings if received proliferating (this is approximately 3 and 2 passages respectively if the recommended seeding densities are used).
- Growth beyond the guarantee period is usually possible, depending on the cell type. However, growth rate, expression of cell markers, biological response and function may deteriorate with age.
- The recommended trypsin concentration is 0.025%. Trypsin at a higher concentration than this is detrimental to the cells.

3.5.1. Trypsinisation

Cell Kits contain a set of subculture reagents – Buffered Saline Rinsing Solution (BSRS), 0.025% trypsin/0.01% EDTA, and trypsin blocking solution (TBS).

Volumes given are for T25 flasks. Increase the amounts accordingly for T75 flasks.

- 1 Subculture while the cells are still actively dividing.
- 2 Bring subculture reagents to room temperature before use.
- 3 Aspirate old medium from the flasks to be subcultured.
- 4 Rinse the cell layer gently with 5 ml BSRS.
- 5 Add 3 ml trypsin/EDTA (0.025% trypsin/0.01% EDTA) to the cell layer ensuring that the entire surface of the cell sheet is covered and set a timer.
- 6 After about 3 minutes, examine the cells microscopically to monitor trypsinisation.

Please note that higher concentrations of trypsin or longer exposure may change the cells irreversibly.

- 7 When about 90% of the cells have rounded up, tap the flask sharply to detach them. If they do not detach, leave them a little longer, but no more than 5-6 minutes in total from when the timer was set.
- 8 Add 3 ml TBS and transfer the cells to a centrifuge tube. Rinse the flasks with a further 2 ml TBS or BSRS to collect residual cells and add to the other cells in the tube.
- 9 Centrifuge harvested cells for 5 min at 220 g.
- 10 Aspirate the supernatant and gently mix cells with 1-2 ml fresh medium.

- Count the cells using a haemocytometer and estimate yield and viability. Typical yields from one T25 are of the order of 5×10^5 , depending on the percentage confluence at harvesting.

3.5.2. Reseeding

- Prepare fresh flasks, by adding 5 ml fresh growth medium per T25 (15 ml per T75), and pre-equilibrating for at least 30 min in the CO₂ incubator.
- Calculate the number of cells to use per flask using the same formula given for the initial seeding -

Number of viable cells per flask -

Cell type	Minimum seeding density	Total cells seeded/flask	
		T25	T75
Large vessel endothelials	2500/cm ²	62,500	187,500
Microvascular endothelials	5000/cm ²	125,000	375,000
*Other cell types	3500/cm ²	87,500	262,500

- Add the cells to the fresh medium and mix gently.
- Examine the flasks under the microscope to ensure that the cells have been successfully transferred to all flasks.
- Return the flasks to the incubator, and incubate overnight in the usual way.
- The next day, check that reseeded has been successful and change the medium as before, pre-equilibrating the fresh medium first in the CO₂ incubator.
- Follow the feeding schedule given in section 3.4. *Maintenance of cells in culture.*

* **NB** Please refer to specific data sheets for each cell type for more detailed seeding density information.

3.6. Proliferating Cell Cultures

Proliferating cell cultures are delivered as established sub-confluent monolayers in culture flasks. The flasks are transported packed with warm packs to maintain a temperature of 25-37°C. The culture flasks are filled completely with medium to prevent mechanical damage to the monolayer at the air/liquid interface during shipping.

- 1 Examine the cells microscopically on receipt. Some cell detachment and stress is normal following transportation. However, please contact TCS Biologicals if this seems unusually severe, or if you have any other concerns at this stage.
- 2 Estimate the % confluence microscopically. The cultures are grown at TCS Biologicals for 4-6 days from recovery, until they are well established. However, the % confluence on receipt may vary.
- 3 Wipe the external surface with either ethanol or isopropanol to decontaminate the surface of the flasks.
- 4 Place cultures in a 37°C, 5% CO₂ incubator for about 4 hours to recover before opening the flasks.
- 5 Prepare fresh medium as outlined on page 5, and pre-equilibrate the appropriate volume in the CO₂ incubator. Never warm the whole bottle of medium as this can shorten its shelf life.
- 6 In a sterile field, open the culture flasks carefully and remove the medium. Be particularly careful to remove all liquid around the neck area of the flask, and from the caps. If necessary, change the caps. This area is a prime site for microbial infection to develop following transportation.
- 7 Add the appropriate volume of fresh pre-equilibrated medium (see table on page 8) to the flasks, and return the cells to the incubator.
- 8 Examine the cultures daily, feed as outlined on page 8, and subculture when they reach 60-80% confluence (see subculture instructions).

4. Trouble shooting

Medium in cryovial appears yellow after thawing - continue to follow the instructions but contact TCS Biologicals if after several days the cell morphology is abnormal or the cells do not seem to be proliferating.

Seeding after 24 hours is much lower than you expected - continue to follow instructions but contact TCS Biologicals if after several days the cells do not seem to be proliferating. It is essential that the medium is changed after 24 hours regardless of the apparent seeding efficiency.

Not all the cells lift off the flask with the trypsin - it is common for a few to remain stuck down. However, the majority should lift easily using the instructions given. If they do not, this may be either because the trypsin has lost its potency, is cold, has been inhibited, or because the cells have become over-confluent. Always use fresh trypsin which has been warmed to room temperature, and always rinse the cell layer with HBSS before adding trypsin. Never incubate the cells with trypsin for longer than 5-7 min maximum (preferably no more than 5 minutes).

Yield after harvesting is lower than expected - this may be caused by an error in estimating % confluence. If the cells have been growing well, reseed at the recommended densities and continue as usual.

Viability is low after subculture or cells do not start to divide again after subculture - this may be due to trypsinising conditions, or the % confluence at harvesting. Make sure the trypsin used is fresh and at the recommended concentration. Never leave the trypsin on for more than the recommended time. Always neutralise the trypsin, then gently spin and resuspend the cells in fresh medium for reseeded. Treat the cells extremely gently at all times. If cultures are too confluent, they may stall irreversibly. Also, once the cells have reached 40-50% confluence, feeding should be daily and the amount of medium should be doubled to prevent irreversible stalling (see table on page 8). Make sure the cells are clearly underconfluent at the time of harvesting.

Cells do not express recommended cell markers - the cells provided are tested for specific cell markers as outlined in the Certificate of Analysis. This is usually done in early passage but after a pure culture is obtained. Some cell types will lose characteristic markers with repeated passage even although the morphology remains normal. Loss of markers does not necessarily mean that the cells have been overgrown by a contaminant cell type.

5. Cell culture process overview

