T cell Expansion with Xuri™ systems

Isolation and Cultivation Protocol

PBMC isolation

Preparation

Use freshly collected buffy coats (ideally <24 hours).

CAUTION Handling blood: Take necessary precautions when handling blood and follow local risk assessment guidelines.

Procedure

1. Transfer buffy coat from collection bag into a sterile bottle by cutting the appropriate tubing.

   All manipulations should be performed in a Class II biological safety cabinet using good aseptic technique.

2. Dilute buffy coat 1:1 in sterile phosphate buffered saline (PBS) supplemented with 2% heat-inactivated (HI) human serum and mix gently with swirling.

Ensure PBS with 2% HI human serum and Ficoll-Paque™ PREMIUM (1.077 g/mL) are at ~18 to 20°C prior to starting. If total volume is less than 100 mL following the 1:1 dilution, add extra PBS (with 2% HI human serum) to make up this volume.

3. Prepare 4 × 50 mL centrifuge tubes with 15 mL of Ficoll-Paque PREMIUM (1.077 g/mL). Carefully layer 25 mL of diluted blood sample onto the top ensuring that mixing of layers does not occur. Repeat for all 4 tubes.

Work quickly but carefully, do not leave layered gradients for long periods of time as this will cause layers to mix prior to centrifugation, leading to poor recoveries.

4. Centrifuge at 400 × *g* for 30 minutes and 18 to 20°C with maximum acceleration and no brake.

5. Remove tubes from the centrifuge carefully to prevent mixing of layers. Under aseptic conditions, slowly aspirate off plasma and platelet fraction without disturbing the white mononuclear cell layer at the interface.

6. Carefully withdraw mononuclear cell layer using a Pasteur pipette and transfer into fresh 50 mL tubes. The harvested volume should not be greater than 10 mL in a 50 mL tube. Add PBS with 2% HI human serum to make a total volume of 50 mL.

Ensure that minimal amounts of Ficoll-Paque PREMIUM (1.077 g/mL) are removed with the mononuclear cell layer to improve cell yield following the washing step.

7. Centrifuge tubes at 400 × *g*, 18 to 20°C for 10 minutes with the brake on. Repeat this step by carefully re-suspending the pellet in PBS with 2% HI human serum to 50 mL. Aspirate off supernatant and re-suspend each pellet in 50 mL of complete medium.

8. Pool all tubes into a sterile plastic bottle and take a sample for cell counting. Determine total number of viable cells recovered from the preparation.

Viable cell counts can be determined using various methods. Many are based on the trypan blue exclusion assay.

9. Pipette 25 mL of cell suspension onto a 150 mm Petri dish (tissue culture plastic) and then add further 10 mL of complete medium to give a total volume of 35 mL.

This step is required to remove monocytes from the preparation. Typically this will require ~8 Petri dishes.

10. Incubate Petri dishes at 37°C with 5% CO_2 for 30 to 60 minutes to allow monocytes to adhere to the plastic.

Check for adherence of monocytes using a light microscope between 30 and 60 minutes before moving on to the harvesting step.

11. Use remaining medium on the plates to gently wash the surface of the petri dish and then harvest non-adherent cells into 50 mL tubes. Add further 15 mL of complete medium to each dish recovering as many non-adherent cells as possible. Harvest into 50 mL tubes.

12. Centrifuge at 400 × *g* at room temperature for 5 minutes with the brake on.

13. Re-suspend and pool cells into a single 50 mL tube in 50 mL volume of complete medium.

14. Determine viable cell counts using method of choice.

15. Centrifuge at 350 × *g* at room temperature for 5 minutes with the brake on.

At this stage, cells can be frozen using a suitable freezing mix (either including serum or a commercially available DMSO based cryopreservation medium (e.g. HyCryo Stem)) following a standard protocol. To avoid damage or loss of cells we recommend transferring cells directly into static culture as described below.

Cultivation in static culture

Preparation

Prior to initiating static culture, determine the number of flasks required to seed at the correct density described in the procedure. Ensure that enough complete medium is prepared and pre-warmed to support the equivalent of two T225 flasks for each Xuri Cell Expansion System W5.

Prepare medium: commercially available lymphocyte medium with 5% heat-inactivated human serum or RPMI 1640 with 10% heat-inactivated human serum; and 2 mM L-Alanyl-L-Glutamine.

Prepare Xuri IL-2 by reconstituting in sterile water for injection (WFI) to a final concentration of 1 mg/mL*. Using the biological activity quoted in the Xuri IL-2 certificate of analysis for the current lot, add reconstituted Xuri IL-2 to the medium to a final dilution of 200 IU/mL. Medium after addition of supplements described above and Xuri IL-2 is subsequently referred to as 'complete medium'.

*See instructions for use for Xuri IL-2 for more detailed guidance and working stock preparation.

Procedure

1. Rapidly thaw mononuclear cell vials in a 37°C water bath for approximately two minutes or until only a sliver of ice remains.
2. Transfer cells into 9 mL of complete medium for every vial thawed.

The complete medium does not require pre-warming for this step.

3. Centrifuge tubes at 350 × *g* for 5 minutes at room temperature.
4. Aspirate supernatant and re-suspend cell pellet in 25 mL of complete medium and repeat centrifugation step. Re-suspend pellet in 5 mL of complete medium for every vial thawed.
5. Determine total viable cell number for the whole preparation and from this determine the number of T cells.

Use data obtained from the population composition assay for the cell batch used and described above and confirm phenotype of T cell subsets by flow cytometry or other method of choice. Alternatively if this information is not available assume that 50% of the total cell number are T cells.

6. Determine number and volume of Dynabeads Human T-Activator CD3/CD28 required for the preparation.

Number of beads = 3 × number of T cells.
Volume of beads (µl) = Number of beads / 10^5.

To account for donor and preparation differences, instead of assuming that T cells account for 50% of the total mononuclear cell number, a more accurate way to determine this percentage is by flow cytometry for the expression of T cell surface marker CD3. This can then be used to adjust the starting density of viable mononuclear cells required.

7. Mix beads and add required volume to a 15 mL tube. Prepare beads following manufacturer's instructions. Re-suspend beads in 3 mL of complete medium.
8. Supplement complete medium with 200 IU/mL of Xuri IL-2.
9. Culture cells in static flasks for 3 to 5 days at 37°C with 5% CO_2.

Cultivation in Xuri Cell Expansion System W5

Preparation

A 2 litre perfusion cell bag requires an addition of at least 5 × 10^7 viable mononuclear cells to provide a minimum starting density of ~2.5 × 10^7 T cells.

Following static culture, T cells require transferring into a cell bag prior to further cultivation in the Xuri Cell Expansion System W5. A small Labtainer (500 mL) is suitable for transferring cells between static culture vessel and CellBag for the Xuri W5 using tube welding.

Prepare fresh medium and Xuri IL-2 as described above.

For perfusion culture in Xuri Cell Expansion System W5 over the full period the use of Xuri IL-2 at 200 IU/ml might not be sufficient to support maximum expansion. An evaluation of Xuri IL-2 in the range of 200 IU/ml to 400 IU/ml is recommended.

Procedure

1. Take complete cell bag assembly kit and hang feed and waste bags on separate hooks of an IV pole. Insert plastic bars on the cell bag into the metallic clips at the end of the Xuri W5 tray.

Check that the cell bag assembly includes both the feed bag and waste bag attached. Ensure that the cell bag is firmly positioned so that it doesn't move following inflation.

2. Run silicon tubing into the upper and lower peristaltic pump. Follow arrows on the front to ensure that fluid flows from feed bag to the bioreactor as well as from filter to the harvest bag.
3. Attach heater to outlet filter and CO_2 line to inlet valve.

Heating the outlet filter prevents the formation condensation which can cause clogging.

4. Turn on flow of CO_2 from main CO_2 line. Make sure pressure from the main CO_2 line is between 0.75 and 1.0 bar. Turn on CO_2 mixing unit using the switch on the back panel. Attach MIX IN and MIX OUT tubing lines on the front of the unit. Press PUMP ON and CO_2 ON buttons on the front of the unit. Their LED lights will light up. Increase flow rate to 0.2 L/mL and percentage CO_2 to 5.0. Allow cell bag to inflate.

Inflation of the cell bag takes approximately 20 minutes.

5. Turn on the Xuri W5 unit by pressing the switch on the back panel. Press the MENU button to view set values and change parameters. Use arrow UP/DOWN buttons to change settings and press ENTER to confirm each.

Make sure that you tare the weight of the empty cell bag before the addition of medium.

6. Press the PAGE button to display current weight readings of the instrument and press the PAGE button again to return to main screen.

Set point (SP) is the desired weight of medium in the cell bag. It should read 1000 for a 2 L bag and 5000 for a 10 L bag. Process value (PV) is the current net weight displayed in grams.

7. Open clamps on the connecting-feed bag and waste bag lines and clamps on a 3-way adapter that connects cell bag to the feed bag and waste bag.

The other clamps on the 3-way adapter should remain closed.

8. Add medium to the cell bag using the FEED-IN button. For a 2 L bag this should be at least 200 mL and for a 10 L bag this should be at least 300 mL.

The amount of media added will be reflected in the change of the PV weight value.

9. Press the TEMP button to turn the heating plate on. The TEMP LED should light up. Press the UP/DOWN arrows to set the temperature to 37°C.

10. Press the ROCK button to initiate rocking. The ROCK LED should light up. Press the UP/DOWN arrows to set the rocking rate of between 10 and 15 rpm and an angle of 4 to 6.

11. Allow media in the bag to warm and equilibrate approximately 2 hours before inoculating with cell preparation.

12. Aseptically transfer cells cultures from T225 flasks into a small Labtainer and determine total volume accumulated. Mix gently and take an aliquot for viable cell counting. Determine volume of cell suspension and additional complete medium required so that final cell density of the culture is 5×10^5 cells/mL.

13. In the tissue culture hood, attach a piece of sterile weldable extension tubing to the Labtainer containing the cells. Close clamp on the feed-in line from the media bag.

14. Using a suitable tube welder, connect bag containing the cells to the C-flex tubing on the feed-in line.

15. Press the FEED-IN button to pump cells into the cell bag. Using the tube welder, re-connect media bag to the C-flex tubing on the feed-in line and discard empty cell bag.

16. Open media bag clamp and flush residual cells through the line into the cell bag by pumping complete medium from the feed bag. Top up cell bag with complete medium to give final density of 5×10^5 cells/mL.

17. When required number of cells is reached harvest cells cells via harvesting line into desired container.

PBMC isolation Process Requirements

Item	Supplier	Catalogue Code
Ficoll-Paque PREMIUM	GE	17-5442-02
HyClone™ Dulbecco's PBS (w/o Ca, Mg, Phenolred)	GE	SH30378.02
HyClone RPMI 1640	GE	SH30096.01
For cryopreservation (optional): for example: HyCryo Stem Cryopreservation Media	GE	SR30002.02
Human Serum*		

Static culture Process Requirements

Item	Supplier	Catalogue Code
Commercially available lymphocyte medium or RPMI 1640 for example: HyClone RPMI 1640	GE	SH30096.01
HyClone SG-200 (stable L-Alanyl-L-Glutamine)	GE	SH30590.01
Xuri IL-2, 10 µg	GE	29-0627-89
Xuri IL-2, 1 mg	GE	29-0627-90
2 L CellBag™ (BioClear™ 10, Perfusion, DO)	GE	28-9376-52
Dynabeads™ T cell expander CD3/CD28	Life Technologies	111.41D
Human Serum*		

Cultivation in Xuri Cell Expansion System W5 Process Requirements

Item	Supplier	Catalogue Code
Commercially available lymphocyte medium or RPMI 1640 for example: HyClone RPMI 1640	GE	SH30096.01
HyClone SG-200 (stable L-Alanyl-L-Glutamine)	GE	SH30590.01
Xuri IL-2, 10 µg	GE	29-0627-89
Xuri IL-2, 1 mg	GE	29-0627-90
2 L CellBag (BioClear 10, Perfusion, DO)	GE	28-9376-52
Labtainer (500 mL)	Thermo Fisher Scientific	SH30658.14
Human Serum*		

Human Serum* With a broad variety of different human serum products available, it is recommended to evaluate the suitability for the application.

Legal

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