T Cell Expansion with Xuri™ Systems

Isolation and Cultivation Protocol

PBMC isolation

Preparation

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

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

Procedure

1. Transfer the buffy coat from the collection bag into a sterile bottle by cutting the appropriate tubing. Follow appropriate handling procedures for other blood sources.

   All manipulations should be performed using good aseptic technique.

2. Dilute the buffy coat 1:1 in sterile phosphate buffered saline (PBS) supplemented with 2% heat-inactivated (HI) human serum and mix gently with swirling. If blood from a different source is used then dilution may not be required.

   Ensure PBS with 2% HI human serum and Ficoll-Paque™ PREMIUM (1.077 g/mL) are at room temperature prior to starting. If the 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 20 mL of Ficoll-Paque PREMIUM (1.077 g/mL). Carefully layer 25 mL of the 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 at room temperature with maximum acceleration and **no brake**.

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

6. Carefully withdraw the 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 45 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* at room temperature for 10 minutes with the brake on. Aspirate off the supernatant and re-suspend each pellet in 45 mL of PBS with 2% HI human serum.

8. Centrifuge at 400 × *g* at room temperature for 5 minutes with the brake on. Aspirate off the supernatant and re-suspend each pellet in 45 mL of PBS with 2% HI human serum. Repeat this step.

9. Re-suspend and pool cells into a single container in a 50 mL volume of PBS with 2% HI human serum or complete medium if proceeding to cultivate cells directly.

10. Take a sample for cell counting. Determine viable cell counts using the desired method.

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

11. Centrifuge at 350 × *g* at room temperature for 5 minutes if the cells are to be cryopreserved.

    At this stage, cells can be cryopreserved e.g. at a cell concentration of 2.5×10^7 cells/mL using a suitable freezing mix (either including serum or a commercially available DMSO based cryopreservation medium (e.g. HyClone™ HyCryo) following a standard protocol. Alternatively, transfer cells directly into static culture to avoid damage to the cells.

Initial cultivation in static culture

Preparation

Prior to initiating static culture, determine the number of flasks required to seed at the correct cell density described in the procedure. Culture media must be equilibrated to room temperature for at least 2 hours. Ensure that enough complete medium is prepared to support the equivalent of one to two T225 flasks for each Xuri Cell Expansion bioreactor system.

Prepare Xuri IL-2 by reconstituting in sterile water for injection (WFI) to a concentration of 1 mg/mL and dilute in PBS with 0.1% human albumin to a desired final concentration (the biological activity is quoted in the Xuri IL-2 certificate of analysis)*.

Evaluation of Xuri IL-2 in the range of 200 to 500 IU/mL is recommended.

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

Prepare complete Xuri T Cell Expansion Medium by adding 5% heat-inactivated human serum and Xuri IL-2 at 350 IU/mL. L-Alanyl-L-Glutamine is already present in the medium. Medium supplemented this way is subsequently referred to as ‘complete medium’.

To prepare complete medium in bottles, transfer of medium from bag can be done by different methods such as using a syringe, silicone tubing with a peristaltic pump or a pipeline dispenser.

Procedure

1. A 2L perfusion Xuri Cellbag bioreactor culture requires at least $3 \times$ to 5×10^7 viable mononuclear cells from an initial static culture to provide a minimum starting density of ~$1.5 \times$ to 2.5×10^7 T cells.
2. Rapidly thaw the cells by incubating the vials in a 37°C water bath for approximately two minutes or until only a sliver of ice remains.
3. Transfer cells into a centrifuge tube. Then, gently and drop-wise, add 9 mL of complete medium for every vial thawed.

4. Centrifuge tubes at 400 × *g* for 5 minutes at room temperature.
5. Aspirate the supernatant and re-suspend the cell pellet in 5 mL of complete medium for every vial thawed.
6. Repeat centrifugation step. Aspirate supernatant, re-suspend cells in appropriate volume of complete medium and take a sample for counting.
7. Determine the total viable cell number for the whole preparation and from this determine the number of T cells.

The percentage of T cells in the total mononuclear cell preparation can be determined 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. Alternatively, if this information is not available, then assume that 50% of the total cell number are T cells.

8. Determine the number and volume of Dynabeads® Human T-Expander CD3/CD28 (ThermoFisher Scientific) required for the preparation.

Number of beads = 3 × number of T cells.

$$\text{Vol of beads (ul)} = \frac{\#\text{beads}}{1 \times 10_8} \times 1000$$

9. Vortex mix beads and add the required volume to a 15 mL tube. Prepare the beads following the manufacturer's instructions. Re-suspend the beads in complete medium and add to the cells in flasks.
10. Culture cells undisturbed in static flasks for 3 to 4 days at 37°C with 5% CO_2. From day 3 maintain cells at a cell density of 5×10^5 cells/mL to 1×10^6 cells/mL by adding the required volume of fresh complete media until day 6 or until there is a sufficient number to transfer to a 2L Xuri Cellbag bioreactor.

This basic procedure serves as a general guidance for the initial static phase of T cell cultivation. Depending on the T cell culture application, additional steps can also be carried out at this stage e.g. genetic modification. The use of gas permeable bags, a combination of cytokines or different activation products can also be adapted. Optimal procedures should be defined accordingly to each particular application.

Cultivation in Xuri Cell Expansion Systems

For instructions on how to set up Cellbags for Xuri W5/W25 Cell Expansion System please refer to the protocols (doc 29-1067-93 & 29-1067-90) on the www.gelifesciences.com/xuri webpage

Preparation

Prepare fresh complete medium (either 500 mL or 1 L) bag by adding 5% HI human serum and 350 IU/mL of Xuri IL-2.

Procedure

Following static culture, T cells are transferred into a Xuri Cellbag bioreactor for further cultivation in the Xuri Cell Expansion System W5 or W25.

Prepare the Xuri Cell Expansion system (W5 or W25) using the instructions given in the User Operation Manual. Connect a Xuri Cellbag bioreactor to the Xuri Cell Expansion System, inflate and tare the Xuri Cellbag bioreactor before addition of medium.

1. Prior to transferring cells into the Xuri Cellbag bioreactor, count the cells and determine the total cell number and volume required for transfer.
2. Determine the desired final volume to add to the Xuri Cellbag bioreactor to reach a cell concentration of at least 5×10^5 cells/mL. Take into account that additional volume (approximately 50 mL depending on the length of tubing) will be added after cell inoculum to wash the feed line.

Volume will depend on the application and cell number on the day. The minimal total volume in a 2L Xuri Cellbag bioreactor should be 300 mL at a minimum cell concentration of 5×10^5 cells/mL. If the calculated final volume is >1 L only add enough media to reach a maximum of 1 L for a 2L Xuri Cellbag bioreactor.

3. Before inoculation of cells, calculate the initial volume required to add to the Xuri Cellbag bioreactor for media equilibration:

Initial volume addition to Cellbag =
Final volume in Cellbag - cell inoculum volume - volume to wash line

4. Add complete medium to the Xuri Cellbag bioreactor and let it equilibrate for approximately 2 hours (if using a Xuri Cellbag bioreactor with sensors) or until the temperature of the medium reaches 37°C before inoculating cells. For the 2L Xuri Cellbag bioreactor this should be at least 200 mL and for the 10L Xuri Cellbag this should be at least 300 mL.
5. In a biological safety cabinet, aseptically transfer cell cultures from tissue culture flasks into a small Labtainer™. Attach a piece of sterile weldable extension tubing to the Labtainer containing the cells. Close the clamp on the feed-in line from the media bag.

A small HyClone Labtainer (500 mL or 1 L) is suitable for transferring cells between static flasks and the Xuri Cellbag bioreactor using tube welding.

6. Using a suitable tube welder, connect the bag containing the cells to the weldable tubing onto the feed-in line.
7. Transfer the desired volume of inoculum into the equilibrated Xuri Cellbag bioreactor using the feed pump or gravity flow. Gently agitate the bag to prevent cells settling.
8. Using the tube welder, re-connect the media bag to the weldable tubing on the feed-in line and discard the empty labtainer.
9. Open the media bag clamp and flush residual cells through the line into the Xuri Cellbag bioreactor by pumping complete medium from the feed bag. If required, top up the Xuri Cellbag bioreactor with complete medium to give the desired final density.
10. To sample from a Xuri Cellbag bioreactor, wipe the top of the sampling connector with 70% alcohol (or equivalent). Using aseptic technique, attach a sterile luer lock syringe to the needle-less sampling port. Release the tubing clamp and withdraw a sample into the syringe (1 to 10 mL volume for counting and biochemical analysis).

    Close the tubing clamp and remove the syringe. Wipe the top of the sampling port again and replace the cap. Pinch the sampling port tubing a few times to ensure that any liquid in the tubing drains back into Xuri Cellbag bioreactor.

At low rocking rates (<15 rpm) it is sometimes difficult to get a representative sample due to cells settling. In this situation, it is recommended to raise the rocking rate to 15 rpm for a few minutes prior to sampling. The rocking is stopped during sampling. Before taking a sample, make sure that the sampling connector is firmly attached to the luer-lock coupling of the Xuri Cellbag bioreactor by screwing it clock-wise.

11. If culture volume is lower than 1L, add fresh complete medium to the Xuri Cellbag bioreactor as the cells grow, being careful to maintain cell number at least at $5x10^5$/mL. The minimal culture volume recommended to start in the Xuri Cellbag bioreactor is 300 mL.

12. During a perfusion culture, waste media is withdrawn and fresh complete medium is added. This can be done using Xuri Cellbag bioreactors with an internal perfusion filter and harvest/waste and feed pumps.
    When the culture has reached 1L volume and the cell concentration >2 × 10^6 cells/mL, perfusion can be started (dependent also on the metabolite levels). The standard perfusion rate should ensure that lactate levels remain below 25 mmol/L and ammonium levels remain below 2 mmol/L. This broadly equates to the following:

Cell concentration	Perfusion rate
2 × 10^6 cells/mL	500 mL/day
10 × to 15 × 10^6 cells/mL	750 mL/day
>15 × 10^6 cells/mL	1000 mL/day

    Fresh complete media and waste bags may need to be attached throughout the culture, depending on the perfusion rate. Feed and waste bags can be changed using the weldable extension tubing.

13. The culture can also be continuously monitored for pH and dissolved oxygen (DO) when using the Xuri Cellbag bioreactor with DO and pH sensors, following the instructions described in the Xuri Cell Expansion System W25 operator manual.

14. To end cultivation, prepare a harvest container. Stop aeration and rocking, clamp off all connections and disconnect the air inlet tube and filter heater. Cells can be harvested through the harvest line in the Xuri Cellbag bioreactor.

    For end of cultivation, do not use the waste line that is connected through the filter to harvest the cells as this will damage the cells.

PBMC isolation process requirements

Item	Supplier	Catalogue code
Ficoll-Paque PREMIUM	GE Healthcare	17544202
Dulbecco's PBS without calcium and magnesium	GE Healthcare	SH30028.03
For cryopreservation (optional): e.g. HyCryo Stem Cryopreservation Media	GE Healthcare	SR30002.02
Human Serum*	-	-

Static culture process requirements

Item	Supplier	Catalogue code
Xuri T Cell Expansion Medium, 500 mL	GE Healthcare	29185230
Xuri T Cell Expansion Medium, 1L	GE Healthcare	29185231
Xuri IL-2	GE Healthcare	29062790
Dynabeads Human T-Expander CD3/CD28 Or Dynabeads CD3/CD28 CTS™	Thermo Fisher Scientific	111.41D 40203D
Human Serum*	-	-

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Cultivation in Xuri Cell Expansion Systems process requirements

Item	Supplier	Catalogue code
Xuri T Cell Expansion Medium, 500 mL	GE Healthcare	29185230
Xuri T Cell Expansion Medium, 1L	GE Healthcare	29185231
Xuri IL-2	GE Healthcare	29062790
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

For your local office contact information, visit
www.gelifesciences.com/contact

GE Healthcare UK Limited
Amersham Place
Little Chalfont, Buckinghamshire,
HP7 9NA, UK

http://www.gelifesciences.com

GE Healthcare offices:

GE Healthcare Bio-Sciences AB
Björkgatan 30, 751 84 Uppsala,
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5, D-79111 Freiburg,
Germany

GE Healthcare Bio-Sciences Corp.
100 Results Way, Marlborough,
MA 01752
USA

GE Healthcare Dharmacon.
2650 Cresent Drive, Lafayette,
CO 80026
USA

Hyclone Laboratories, Inc,
925 W 1800 S, Logan
UT 84321
USA

GE Healthcare Japan Corporation
Sanken Bldg. 3-25-1, Hyakunincho,
Shinjuku-ku, Tokyo 169-0073,
Japan

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