Dynabeads® *ClinExVivo*™ CD3/CD28

Sterile Product
For Research Use Only

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1. INTENDED USE

Dynabeads *ClinExVivo* CD3/CD28 are intended for separation (1, 2) and *ex vivo* expansion (1, 5) of human T cells for cell-based clinical research.

2. PRODUCT DESCRIPTION

Dynabeads *ClinExVivo* CD3/CD28 are uniform, superparamagnetic, sterile, non-pyrogenic polystyrene beads with affinity purified mouse anti-human CD3 and CD28 monoclonal antibodies covalently bound to the surface.

Dynabeads *ClinExVivo* CD3/CD28 are produced according to cGMP under aseptic conditions in a fully validated Class 100 clean room using gamma irradiated Dynabeads. All processes are validated. Sterility and endotoxin tests are performed according to United States Pharmacopeia.

2.1 Principle of Isolation and Expansion

Dynabeads *ClinExVivo* CD3/CD28 offer a simple method for isolation (1, 2), activation (3) and expansion (1, 5) of human T cells. Firstly, $CD3^+$ T cells are separated and concentrated from the apheresis product by magnetic cell separation using Dynabeads *ClinExVivo* CD3/CD28 (1, 2). Following separation, the $CD3^+$ T cells are cultured in the presence of the beads. By combining anti-CD3 and anti-CD28 antibodies on Dynabeads, the beads will provide both the primary and co-stimulatory signals that are required for activation and expansion of T cells (4). The activated T cells have been shown to produce IL2, GM-CSF, IFN-γ and TNF-α (1, 4, 5). T cells activated with these Dynabeads can be expanded 100-1000 fold over a 9-14 day culture period (1).

The T cell expansion process can be scaled up using a bioreactor-based process (5). It has been shown that the T cell expansion protocol can be optimized to include expansion of antigen-specific T cells (6).

2.2 Materials Supplied

• 10 ml of Dynabeads *ClinExVivo* CD3/CD28.

Supplied as a sterile suspension containing 4 x 10^8 Dynabeads/ml in phosphate buffered saline (PBS), pH 7.4, w/0.1% human serum albumin (HSA).

2.3 Storage and Stability

When stored in unopened vials at 2-8°C, Dynabeads *ClinExVivo* CD3/CD28 are stable until the expiry date stated on the label.

Keep Dynabeads in liquid suspension during storage and all handling steps, as drying will result in reduced performance.

Resuspend well before use.

2.4 Additional Materials Required

For clinical research procedures, the principal investigator is responsible for ensuring that use of all procedures, reagents, and equipment follow applicable guidelines, standards and regulations. The materials and equipment listed below are recom-

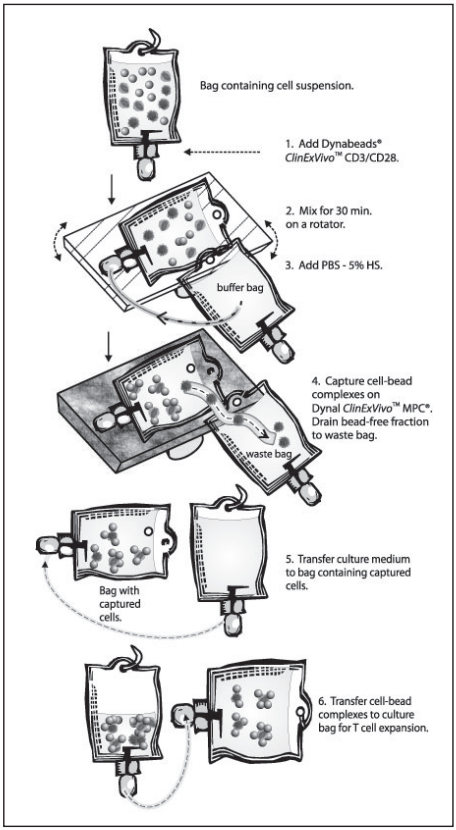

Fig. 1: Schematic flow diagram for magnetic separation of $CD3^+$ T cells with Dynabeads *ClinExVivo* CD3/CD28 and Dynal *ClinExVivo*™ MPC®

mended for use with the Dynabeads *ClinExVivo* CD3/CD28 procedures. Alternative materials and equipment may be used.

Materials that are not included, but are required to perform the procedures:

- Dynabeads *ClinExVivo* Epoxy – Cat. no. 402.01D
- Dynal *ClinExVivo* MPC (Magnetic Particle concentrator) Cat. no. 121.02
- Dynal MPC™-1 (Magnetic Particle concentrator) Cat. no. 120-01D
- PBS (Gibco) *[Note: PBS must be calcium and magnesium free.]*
- Anti-coagulant solution (e.g. ACD-A, Baxter)
- Pooled Human Serum (HS) (Cambrex or Valley Biomedical) heat inactivated at 56°C for 1 hour or autologous serum *[Note: autologous serum may be used, although significant donor to donor variation should be expected]*
- OpTmizer™ T-Cell Expansion SFM (Gibco) serum free 1x formulation designed to support the culture and expansion of human T cells (or equivalent).
- L-glutamine, (Gibco)
- HEPES buffer, (Gibco)
- IL-2, Proleukin® (Chiron)
- 1-L Bags (Baxter Lifecell® or CellGenix Vuelife™)
- 3-L Culture Bags (Baxter Lifecell® or CellGenix Vuelife™)
- Sampling site coupler with female luer (Charter Medical)
- Terumo TSCD® Sterile Tubing Welder
- 40µm - 80µm In-line Transfusion Filter (Pall)
- 10-lead harness sets (compatible with Terumo SCD 312 welder), (Charter Medical)
- Hemostats tube clamp
- COBE 2991 Cell Washer Disposable Set (COBE/Gambro) or Cytomate Disposable Set (Baxter)
- Cell mixer (e.g. Heidolph Polymax 2040)
- Plasma thawing device (e.g. Thermogenesis MT202)
- Bioreactor (e.g. Wave Bioreactor)

2.5 Media Preparation

Buffer 1:

PBS without Ca^{2+} and Mg^{2+}, with 5% heat inactivated Pooled Human Serum (HS).

Incomplete Medium:

OpTmizer™ T-Cell Expansion SFM (Gibco) serum free 1x formulation designed to support the culture and expansion of human T cells (or equivalent).

Complete Medium:

Prepare fresh Complete Medium every week by adding 200 IU/mL IL-2 to the Incomplete Medium.

Equilibrate Complete Medium to room temperature prior to use.

2.6 Important Information

Follow universal precautions when working with human serum, plasma, or blood products. All human samples must be treated as a potential source of HIV, HBV, and other blood borne pathogens. Gloves and a lab coat must be worn when working with human samples.

Materials contaminated with blood products must be decontaminated by an approved chemical method and disposed of in labeled biohazard containers.

Solution transfers that are not performed in a closed system, such as spike connections and open containers, must be performed under a class 100 biological safety cabinet (BSC) using aseptic techniques.

The protocol below is specifically written to provide guidance for activation (3) and expansion (1, 5) of human T cells from cryopreserved apheresed products. Cultures may also be initiated from non-cryopreserved fresh samples, or samples derived from sources other than apheresis, such as Ficoll separated whole blood (1, 3), cord blood (2) or bone marrow (7). As each sample source and method of T cell or blood collection may vary, procedures may require specific modifications to maximize cell recovery, viability, activation and expansion. Such modifications must be determined empirically. Throughout the protocol below, points to consider will be provided for possible protocol variations that may be appropriate under certain experimental conditions, such as gene modification or working with particular disease tissues.

3. PROTOCOLS

Points to note are listed in brackets (*[…]*) throughout the protocol below. These identify areas where modifications should be considered for specific circumstances.

3.1 Dynabeads Washing Protocol

Wash Dynabeads *ClinExVivo* CD3/CD28 twice before use to avoid potential transfer of residual antibody.

Vials of Dynabeads *ClinExVivo* CD3/CD28 are at negative pressure – liquid must be displaced with air and vice versa.

1. Place one 10 ml vial of Dynabeads *ClinExVivo* CD3/CD28 on the MPC-1 for 1 min.
2. Aseptically remove the supernatant.
3. Remove the vial from the MPC-1.
4. Aseptically add 10 ml Buffer 1 to the vial and mix.
5. Place the vial on the MPC-1 for 1 min.
6. Aseptically remove the supernatant.
7. Repeat steps 4 to 6.
8. Resuspend the beads in 10 ml of Buffer 1 and keep the washed beads in a refrigerator until further use.

3.2 Starting Material

The preferred starting material is cryopreserved human PBMC obtained from leukapheresis product (5). The starting material can also be enriched for specific T cell subsets, such as $CD4^+$ T cells (8) or $CD25^+$ T cells (9, 10, 11).

For optimal T cell activation and expansion it is recommended that freshly collected samples are cryopreserved and thawed prior to use.

[Cryopreservation and subsequent thawing facilitates lysis of granulocytes and other cell types that can suppress T cell activation and expansion. This may be particularly important when working with samples from patients in certain disease states where granulocyte counts are elevated.]

Alternatively, PBMC from freshly obtained leukapheresis product may be activated and expanded without cryopreservation. If fresh samples are used it is recommended to deplete monocytes with Dynabeads (12, 13). We recommend to use Dynabeads *ClinExVivo* Epoxy for monocyte depletion (see Section 3.4)

For maximum activation and expansion of T cells in PBMC, magnetically capture $CD3^+$ T cells prior to culture initiation (1, 2) (see Section 3.4.2). Magnetic concentration is not required if T cells or T cell subsets have been enriched prior to activation and expansion (e.g. purified $CD4^+$ T cells) (8).

3.3 Separation and Expansion of Cryopreserved Apheresis Cells

This procedure is for separation (1, 2) and expansion (1, 5) of $5x10^8$ $CD3^+$ T cells. Adjust the volumes accordingly when using higher/lower cell numbers.

3.3.1 Thawing and Washing of Cryopreserved Apheresis Cells

1. Remove the required number of bags containing cryopreserved apheresis cells from liquid N_2 vapour storage.
2. Thaw the bag(s) in a plasma thawing device or by equivalent methods.
3. To prevent cell clumping, add anti-coagulant solution aseptically to thawed cells to a final concentration of 10%.
4. Slowly dilute the cell suspension 1:1 in Buffer 1.
5. Wash the cells in Buffer 1 according to the cell washer manufacturer's recommendations.
6. Resuspend the cells in 50-60 ml of Buffer 1. If the volume exceeds 60 ml, perform a centrifugation step to reduce the volume.
7. Incubate the cells for 60 min in Buffer 1 at room temperature to allow dead or dying cells to aggregate and subsequently be removed via a blood filter as described below.
8. Filter the cells through an In-line Transfusion Filter with cut-off between 40-80 µm. The cells are now ready for further processing.
9. Remove 1 ml of the sample from the leukapheresis bag. Calculate the number of $CD3^+$ T cells by flow cytometry, and determine the viability of the cells with trypan blue staining.

3.3.2 Magnetic Separation and Culture of CD3$^+$ T Cells

It has been shown that magnetic pre-selection of CD3$^+$ T cells can improve subsequent T cell expansion (1, 2).

The recommended standard procedure described below uses a ratio of three Dynabeads *ClinExVivo* CD3/CD28 per CD3$^+$ T cell (Fig. 1).

[If the starting sample contains less than 25% CD3$^+$ T cells, replace Buffer 1 with Incomplete Medium in the procedures below.]

1. Dilute the cells to approx. 1 x 10^7 CD3$^+$ T cells/ml in Buffer 1. For the standard procedure of processing 5 x 10^8 CD3$^+$ T cells, dilute the sample in 50-60 ml of Buffer 1.
2. Use a 1L bag for CD3$^+$ T cell separation. Add 100 ml of air to the bag.
3. Add 5 x 10^8 CD3$^+$ T cells to the 1L bag in 50-60 ml Buffer 1.
4. Add 4.0 ml (corresponding to 1.6 x 10^9 Dynabeads) of washed Dynabeads *ClinExVivo* CD3/CD28 per 5 x 10^8 CD3$^+$ T cells and immediately proceed to the next step.
5. Place the bag on a cell mixer and mix for 30 min at room temperature to gently mix the cells and the Dynabeads. *[If the starting sample contains less than 25% CD3$^+$ T cells it may be beneficial to mix the sample for 1-2 hours in Incomplete Medium instead of Buffer 1. Optimise the mixing temperature between 4-25°C for each application.]*
6. Prepare 150 ml of Buffer 1 in a 300 ml bag.
7. After mixing, remove the 1L bag from the mixer and drain 150 ml of Buffer 1 into the bag. *[Note: Handle the 1L bag very gently, to not disrupt the bead/cell complexes.]* Place the 1L bag directly on the Dynal *ClinExVivo* MPC. Adjust the magnet to a 60° angle.
8. Leave the bag on the Dynal *ClinExVivo* MPC for 1 min to capture the bead-bound CD3$^+$ T cells. While on the magnet, open the 1L bag containing the captured cells and drain waste fluid out in waste bag via gravity. Remove the bag containing the captured cells from the magnet immediately after all waste fluid has been drained.
9. Immediately add approximately 300 ml Complete Medium to the 1L bag containing the captured cells and gently resuspend the cell/bead complexes.
10. Obtain a 3L bag
11. Transfer the cell/bead complexes from the 1L bag into a 3L bag. Wash the 1L bag with Complete Medium and transfer the residual cells to the 3L bag.
12. Repeat media wash of the 1L bag and transfer the residual cells to the 3L bag until the volume in the 3L bag has reached 1000 ml.
13. Place the 3L bag in an incubator at 37°C/ 5% CO_2 until Day 3 of culture. *[Note; Using a bioreactor, e.g. Wave Bioreactor, will increase expansion efficiency via perfusion and improved aeration (rocking). Whereas typical cell densities rarely exceed 2-3 x 10^6 T cells/ml in static cultures, bioreactor systems can readily maintain viable T cells at densities of 2-4 x 10^7 T cells/ml.]*
14. Collect a sample of the non-captured cell fraction and manually count the number of non-captured cells.
15. Stain the non-captured cells for CD3 expression and evaluate by flow cytometry to calculate depletion efficacy.

3.3.3 Counting and Splitting of Cultures

The cell concentration should be evaluated daily beginning on day 3 of culture.

1. Gently mix the bag to help dissociate cell/bead complexes and to resuspend the cells before removing 1-2 ml cell suspension for counting.
2. Again, mix the sample well to resuspend cells so as to ensure maximum dissociation of beads from cells. This will improve cell count accuracy.
3. Take an aliquot of cells and mix 1:1 with trypan blue staining solution and manually count on a hematocytometer (do not remove the Dynabeads before counting).
   Determine cell density and viability. *[Caution: insufficient mixing of bead/cell complexes may result in cell count underestimates as they will not migrate efficiently under hemocytometer coverslips.]*
4. Stain cells for CD3 expression and evaluate by flow cytometry to calculate the number of CD3$^+$ T cells in the bag.
5. Determine the total cell volume by weighing the bag.
6. When CD3$^+$ T cell density exceeds 1 x 10^6 cells/ml, dilute the cells to approximately 0.5 x 10^6 CD3$^+$ T cells/ml in Complete Medium.
7. Split the cultures to new 3L bags when needed. *[Note: T cell growth typically slows as T cell concentrations increase above 1-2 x 10^6 T cells/ml, so adjust T cell numbers to ~0.5 x 10^6/ml to help maintain the cells in log phase growth.]*
8. Repeat counting of cells daily and dilute cells in fresh Complete Medium to 0.5 x 10^6/ml.

3.3.4 Harvesting of Expanded CD3$^+$ T Cells

1. Harvest cells on an optimal day for your application (usually day 8-12).

2. Remove the 3L bags from the incubator. Remove a sample from a representative number of bags for cell count and FACS analysis. Perform the cell counts as described above.
3. Remove the beads by passing the cell culture over the Dynal *ClinExVivo* MPC using gravity-driven flow. Determine the angle of the MPC empirically between 0-60°. To achieve a flow rate of up to 150 ml/min, the bags containing cells and beads must be suspended from the pole so that the fluid level is 85-90 cm above the cell collection bags.
4. Concentrate the cells and washed using a cell washer.
5. Perform a final bead removal twice by placing the bag on the Dynal *ClinExVivo* MPC. Adjust the magnet to a 60° angle. After 1 min, drain the cells into a new bag via gravity.
6. Determination of residual beads can be performed as described in Reference 13.

3.3.5 Cryopreservation of Expanded $CD3^+$ T Cells

1. Prepare cryopreservation medium and cryopreserve the expanded T Cells.
2. Store the final product of expanded T cells in the vapour phase of a liquid N_2 storage unit.

3.4 Separation and Expansion of Fresh (non-cryopreserved) Apheresis Cells

[Note: During thawing of cryopreserved PBMC, contaminating granulocytes tend to lyse, thereby diminishing inhibitory effects they may have upon T cell expansion (5). If working with fresh, non-cryopreserved samples, allow for contaminating granulocytes.

It is recommended that for initiation of cultures with non-cryopreserved samples, the Magnetic Concentration method described above is employed (See 3.3.2).

Overall T cell activation and expansion can be further improved by specifically depleting phagocytic cells, such as monocytes, using Dynabeads ClinExVivo Epoxy prior to magnetic concentration and culture initiation (12, 13). This is particularly useful when monocyte levels are >15% of total PBMC.]

1. Dilute PBMC to 5-10 x 10^6 cells/ml in Incomplete Medium (pre-warmed to 37°C).
2. Wash Dynabeads *ClinExVivo* Epoxy as described above for Dynabeads *ClinExVivo* CD3/CD28 (see 3.1).
3. Add Dynabeads *ClinExVivo* Epoxy to the PBMC at a 1-2 beads per total nucleated cells in a sterile culture bag. It may be preferred to use 2 beads per nucleated cell when monocyte levels are >15% of total PBMC.
4. Place the bag into a humidified incubator at 37°C/ 5% CO_2 for one hour.

[During the one hour incubation, beads and cells come into contact via gravity at the bottom of the bag and monocytes will ingest beads.]

5. Remove the bag of cells and beads from the incubator after 1 hour. Gently mix the cells and beads before removing Dynabeads *ClinExVivo* Epoxy and any cells that have ingested beads using a Dynal *ClinExVivo* MPC. Adjust the primary magnet to a 60° angle and transfer non-captured cells to a new 1L bag using gravity.
6. Allow ~50mL of Incomplete Medium to flow into the bag containing the bead:cell complexes. Mix well and transfer the medium containing residual cells to the bag containing unbound cells.
7. Count the cells in the bag, stain for CD3 and evaluate by flow cytometry to calculate the number of $CD3^+$ T cells in the sample.
8. Adjust the cell concentration to 1 x 10^7 $CD3^+$ T cells/ml in Incomplete Medium.
9. Perform capture of $CD3^+$ T cells as described above (Section 3.3.2). *[Note: magnetic concentration should be done in Incomplete Medium in place of Buffer 1 after monocyte/phagocytic cell depletion using Dynabeads ClinExVivo Epoxy.]*
10. Following capture of $CD3^+$ T cells, prepare cells for culture initiation as described in Section 3.3.2.
11. Place the bag with cells and Dynabeads *ClinExVivo* CD3/CD28 in an incubator at 37°C/ 5% CO_2 until Day 3 of culture.
12. Count and split cell culture as described above (Section 3.3.3).

3.5 Procedures Incorporating Gene Transduction

Typically, for all culture conditions described earlier, T cells from normal donor samples begin cycling and start to divide between day 2 and 3 of culture (4, 8). Day 1, 2 and/or 3 are recommended as optimal days for transduction using lentivirus based vectors (14). Magnetic removal of beads prior to transduction will diminish overall cell expansion, but should not affect viability. Leaving beads in during the retroviral transduction process should be acceptable for most transduction applications.

[Note: T cells obtained from patients with various diseases and/or undergoing various treatments may be slower at entering cell cycle and cell division may not commence until 1, 2 or even 3 days later than typically observed for samples from healthy donors. For example, T cells from patients with HIV infection

may be slower to start cell cycling, as may be samples from patients undergoing chemotherapy, or patients with certain kinds of cancer (e.g. chronic lymphocytic leukemia) (1, 8, 15). Thus it is important to monitor T cell activation markers, such as CD25, as well as cell division to determine optimal splitting schedules and timing for gene modification.]

4. GENERAL INFORMATION

4.1 Certification

Invitrogen Dynal AS conforms to the Quality Systems Standard ISO 9001:2000 and ISO 13485:2003 with the following scope:

"Development, manufacturing, marketing and sales of Dynospheres®, Dynabeads® and associated products to customers that work within immunology, biological and clinical research, cell based therapy and *in vitro* diagnostics."

In the United States, Dynabeads *ClinExVivo* CD3/CD28 is available for use in clinical trials under an approved IND or IDE.

4.2 USA (Device Master File)

A Device Master File is held with the United States Food & Drug Administration (FDA), which will assist users with their application for FDA approvals on their clinical trials. If cross-referencing the Device Master File is of interest to an Investigational New Drug (IND) Application or other applications, please contact Invitrogen Dynal with the sponsor's and/or investigator's full name and address, along with project name and aim. This information is required by Invitrogen Dynal to issue a Letter of Authorisation, informing the FDA who has been authorised to cross-reference the Master File for their IND application.

4.3 Technical Service

Please contact Invitrogen Dynal AS for further technical information at www.invitrogen.com

Certificate of Analysis (CoA) is available upon request.

4.4 Precautions

Material Safety Data Sheet (MSDS) is available at http://www.invitrogen.com.

5. WARNINGS AND LIMITATIONS

For research use only/not involving the generation of T cells for use in therapy.

If mammalian cells are used in this procedure, appropriate laboratory guidelines must be followed. Detailed information on such guidelines can be obtained from: Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body fluids and Tissue: Approved Guideline: M29-A; ISBN 1-56238-339-6; http//:www.nccls.org.

Handle all samples as if capable of transmitting disease. All work should be performed wearing gloves and appropriate protection.

5.1 Trademarks and Patents

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5.2 Intellectual Property Disclaimer

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5.3 Warranty

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Claims for merchandise damaged in transit must be submitted to the carrier.

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5.4 Limited Use Label License

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