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Dynabeads® CD3/CD28

(Research version of Dynabeads® *ClinExVivo*™ CD3/CD28)

For research use only.

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1. INTENDED USE

Dynabeads CD3/CD28 are intended for separation and *in vitro* expansion of human T cells. This product is the research version of Dynabeads *ClinExVivo* CD3/CD28.

2. PRODUCT DESCRIPTION

Dynabeads CD3/CD28 are uniform 4.5 µm, superparamagnetic polystyrene beads coated with a mixture of monoclonal antibodies against the CD3 and CD28 cell surface molecules of human T cells.

2.1 Principle of Isolation and Expansion

Dynabeads CD3/CD28 offer a simple method for isolation and expansion of human T cells. Firstly, $CD3^+$ T cells are separated and concentrated from the apheresis product by magnetic cell separation using Dynabeads CD3/CD28. 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. Activated T cells produce IL2, GM-CSF, IFN-g and TNF-α. T cells activated with Dynabeads CD3/CD28 can be expanded 100-1000 fold over a 9-14 day culture period.

2.2 Materials Supplied

• 10 ml of Dynabeads CD3/CD28

Supplied as a suspension containing $1x10^8$ 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 CD3/CD28 are stable until the expiry date stated on the label.

Store opened vials at 2-8°C and avoid bacterial contamination.

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

Materials that are not included, but are recommended to perform the entire protocol:

- Dynabeads®M-450 Epoxy –(Cat. No. 140.11)
- Magnet: See www.invitrogen.com/magnets-selection for magnet recommendations
- PBS: Gibco, Cat. No. 20012-027 (Ca^{2+} and Mg^{2+} free)
- Pooled Human Serum (HS) heat inactivated at 56°C for 1 hour
- Human Serum Albumin (HSA)
- OpTmizer™ T-Cell Expansion SFM (Gibco) serum free 1x formulation designed to support the culture and expansion of human T cells (or equivalent).
- RPMI-1640, Gibco, Cat. No. 12633-012
- L-glutamine, Gibco, Cat. No. 25030-081
- HEPES buffer, Gibco, Cat. No. 15630-080
- Proleukin® (IL-2), Chiron, or Biosource IL-2 Cat. No. PHC 0023.
- Appropriate sterile culture vessels.

2.5 Important Information

Follow standard 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.

The protocol below is specifically written for the activation and expansion of human T cells from cryopreserved human PBMC obtained from leukopheresis products or Ficoll® separated whole blood, cord blood or bone marrow. Cultures may also be initiated from non-cryopreserved fresh samples. As sample sources and T cell or blood collection methods may vary, procedures may require specific modifications to maximize cell recovery, viability, activation and expansion. Such modifications must be determined empirically. Possible protocol variations that may be appropriate under certain experimental conditions are highlighted throughout the protocol, such as gene modification or working with particular disease tissues.

3. PROTOCOLS

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

3.1 Dynabeads Washing Protocol

Dynabeads should be washed before use with the aid of a magnet.

1. Resuspend the Dynabeads in the vial.
2. Transfer the desired volume of Dynabeads to a tube.
3. Add the same volume of Buffer 1 as the initial volume of Dynabeads, or at least 1 ml, and mix.
4. Place the tube in a magnet for 1 minute until the Dynabeads are separated; discard the supernatant. Remove the tube from the magnet.
5. Resuspend the washed Dynabeads in the same volume of Buffer 1 as the initial volume of Dynabeads.

3.2 Media Preparation

Buffer 1: PBS, pH 7.4, with 5% heat-inactivated HS or 1% HSA.

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

Complete Medium: Add 200 IU/ml IL-2 to the incomplete medium.

Store at 2-8°C for a maximum of 7 days.

[Note: Autologous serum may be used, but expect donor to donor variation, IL-2 concentrations can be increased to enhance cell growth, although in static culture there is no significant increase in expansion for normal T cells when IL-2 levels are increased beyond 200-500 IU. Regulatory T cells may benefit from use of IL-2 at ≥500IU/ml. For IL-2 products where activity is not available in IU, typically 6-10ng/ml bioactive IL-2 is sufficient for optimal T cell expansion.]

3.3 Starting Material

The preferred starting material is cryopreserved human PBMC obtained from leukapheresis product or Ficoll® separated whole blood. The starting material can also be enriched for specific T cell subsets, such as $CD4^+$ T cells or $CD25^+$ T cells.

[The cryopreservation and subsequent thawing process 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 with elevated granulocyte counts.]

Alternatively, PBMC from freshly obtained leukapheresis products or Ficoll separated whole blood may be activated and expanded without cryopreservation. If fresh samples are used, deplete monocytes with Dynabeads M-450 Epoxy (see Section 3.7)

For maximum activation and expansion of T cells in PBMC magnetically capture $CD3^+$ T cells prior to culture initiation (see Section 3.4). 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).

3.4 Magnetic Separation and Expansion of Cryopreserved $CD3^+$ T cells

For fresh samples, see Section 3.7

[Note: Magnetic separation serves two purposes. Firstly, the process tends to synchronize the T cell activation process, accelerating contact between Dynabeads and T cells, as well as promoting early T cell aggregation. Secondly, during the magnetic selection process, T cells are enriched, leaving behind monocytes in the unselected fraction with other cells that might inhibit T cell activation and expansion. Most notably, monocytes can rapidly phagocytose beads at 37°C, thereby reducing the absolute number of beads capable of contacting T cells, in turn reducing the level of T cell activation and expansion.]

1. Determine the percentage of $CD3^+$ T cells in the sample by flow cytometry or other suitable methods.
2. For PBMC, resuspend cells in a tube at roughly 2-5 x 10^7 $CD3^+$ cells/ml in Buffer 1, but not exceeding a maximum of 2 x 10^8 total nucleated cells/ml. For previously enriched/purified T cells (e.g. $CD4^+$), resuspend the cells at 5-10 x 10^7 cells/ml.
3. Add washed Dynabeads CD3/CD28 at a 3:1 bead: $CD3^+$ T cell ratio

[Note: Bead:$CD3^+$ T cell ratio can be dropped as low as 1:1 while still achieving nearly equivalent T cell capture. Lower capture/activation initiation ratios, such as 1:1, may be preferred when the starting T cell pool is more prone to activation induced cell death or where lower levels of T cell expansion are desired.]

4. Rotate the sample at 1 rpm for 30 min at between 4-25°C. Optimise the mixing temperature between 4-25°C for each application.

[If starting PBMC have relatively low CD3 content, e.g. less than 25%, it may be beneficial to rotate for 1-2 hours at 4-25°C in Incomplete Medium or Buffer 1.]

5. Increase the volume in the tube to sufficient levels for magnetic selection by adding Incomplete Medium or Buffer 1 before placing the tube in a Dynal MPC for 1-2 min.
6. Remove supernatant and stain with anti-CD3 antibody for flow cytometric analysis to calculate depletion efficiency.
7. Gently resuspend bead:cell complexes to an estimated 0.5 x 10^6 -1 x10^6 $CD3^+$ T cells/ml in Complete Medium (assuming 100% capture of $CD3^+$ T cells from PBMC). Typically, 0.5 x 10^6 $CD3^+$ T cells/ml are optimal for culture initiation. Depending upon the particular application (e.g. gene modification), cultures can be initiated with seeding densities up to 1 x 10^6 $CD3^+$ T cells/ml.
8. Plate cells at 2.5-5 x 10^6 $CD3^+$ cells in a total volume of 5 ml/well (0.5-1.0 x 10^6 cells/ml) of a 6 well culture plate in a humidified incubator at 37°C/ 5% CO_2 for 3 days.

[Note: smaller or larger culture vessels/wells can be used - adjust volumes accordingly. For example, in a 24 well format, total volume should be approximately 1 ml/well; in a 12 well format, total volume should be approximately 2 ml/well. For flasks, culture volumes may vary, however it is important that static cultures do not exceed ~1.2 cm in volume depth, otherwise expansion potential and viability may be compromised due to reduced gas exchange.]

3.5 Counting and Splitting of Cultures

Evaluate cell concentration daily, beginning on day 3 of culture. It may be useful to conduct regular phenotypic analyses by flow cytometry (e.g. days 3, 5, 8, and end of culture) to track T cell purity, phenotype and activation state. Similarly, supernatants may be collected to measure cytokine secretion patterns to further characterize T cell activity.

1. Gently mix contents of wells to dissociate beads and cells. Mixing should be sufficient to disrupt all visible bead:cell complexes.
2. Remove 50 µl sample and mix in 50 µl trypan blue (do not remove beads before counting).
3. Count cells manually using a hemocytometer.

[Caution: Insufficient mixing of bead:cell complexes may result in cell count underestimates as they will not migrate efficiently under hemocytometer coverslips.]

4. When $CD3^+$ T cell density is >1 x 10^6 cells/ml, dilute cells to approximately 0.5 x 10^6 $CD3^+$ T cells/ml in culture medium.

[Note: The rate of cell growth tends to diminish as cell concentrations exceed 1-2 x 10^6 $CD3^+$/ml]

5. At the end of culture (day 9-14) count cells and remove beads with a magnet.

3.6 Culture Initiation Without Magnetic Concentration of CD3+ T Cells

1. Add 5 x 10^6 enriched or purified T cells (e.g. purified $CD4^+$ T cells) to 1.5 x 10^7 washed Dynabeads CD3/CD28 in a final volume of 10 ml culture media, placing 5 ml/well in a 6 well tissue culture plate to give a final concentration of 0.5 x 10^6 $CD3^+$ T cells/ml. Mix gently.
2. Culture cells in a humidified incubator at 37°C/5% CO_2 for 3 days
3. Cell concentration should be evaluated daily beginning on day 3 of culture.
4. Counting of cells and splitting of culture; see Section 3.5.

[As decribed earlier, cells can be cultured in smaller or larger culture vessels by adjusting cell numbers and culture volumes accordingly.]

3.7 Separation and Expansion of Fresh (non-cryopreserved) Samples

[Note: During the thawing process of cryopreserved PBMC, contaminating granulocytes tend to lyse, thereby diminishing any inhibitory effects upon subsequent T cell expansion. If working with fresh, non-cryopreserved samples, take into consideration the level of contaminating granulocytes when designing a culture initiation protocol.

For initiation of cultures with non-cryopreserved samples, use the Magnetic Concentration method described above (see Section 3.4).

Overall T cell activation and expansion can be further improved by specifically depleting phagocytic cells, such as monocytes, using Dynabeads M-450 Epoxy prior to magnetic concentration and culture initiation. 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 M450 Epoxy as described above for Dynabeads CD3/CD28 (see Section 3.1).
3. Add Dynabeads M-450 Epoxy to the PBMC at 1-2 beads per total nucleated cells in a sterile culture vessel. 2 beads per nucleated cell can be used when monocyte levels are >15% of total PBMC. Use any sterile culture vessel, including bags, tissue culture flasks, tissue culture plates or sterile tubes.
4. Place the culture vessel containing beads and cells into a humidified incubator at 37°C/ 5% CO_2 for one hour. If using a culture flask, lay the flask on its side to maximize culture surface area.
5. After 1 hour, remove Dynabeads M-450 Epoxy and any cells that have ingested beads by applying an appropriate Dynal MPC for 1-2 min.
6. Collect non-selected cells and initiate culture as described in Section 3.4 or 3.6 above, depending upon whether you want to incorporate the magnetic concentration step. For maximal activation and expansion, the magnetic concentration method is recommended. Note: If magnetic concentration is planned after the monocyte/phagocytic cell removal step, keep cells in Incomplete Medium throughout magnetic concentration instead of using Buffer 1.
7. For larger volumes, where PBMC and beads are in bags, beads and bead:cell complexes can be removed on the Dynal *ClinExVivo*™ MPC™ (Cat. No. 121-02D). The magnet angle can vary between 0° and 60° and should be determined empirically.
8. Transfer the non-captured cell fraction containing monocyte-depleted T cells to an appropriate culture vessel (plate, flask, or bag).
9. Count cells and measure $CD3^+$ T cell content of sample by flow cytometry.
10. Adjust the concentration of cells to 1-5 x 10^7 $CD3^+$ T cells/ml in Incomplete Medium.
11. Capture $CD3^+$ T cells as described above in Section 3.4.
12. Resuspend captured T cells and Dynabeads CD3/CD28 to approximately 0.5-1 x 10^6 $CD3^+$ T cells/ml in Complete Medium containing IL-2 and transfer to an appropriate culture vessel. Incubate at 37°C/5% CO_2 until Day 3 of culture.
13. Count and split cell culture as described above in Section 3.5.

3.8 Procedures Incorporating Gene Transduction.

Typically, for all culture conditions described earlier, T cells from normal donor tissues begin cycling and start to divide between day 2 and 3 of culture. Days 2, 3 and/or 4 of culture are recommended as optimal days for transduction using moloney-based vectors, whereas day 1, 2 and/or 3 are recommended for lentivirus based vectors. 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 to enter cell cycle and cell division may not commence until 1, 2 or even 3 days later than typically observed for tissues from healthy donors. T cells from patients with HIV infection, tissues from patients undergoing chemotherapy, or patients with certain kinds of cancer (e.g. chronic lymphocytic leukemia) may be slower to start cell cycling. 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 Certifications

Invitrogen Dynal AS complies with the Quality System Standards ISO 9001:2000 and ISO 13485: 2003.

4.2 Technical Service

Contact details for further technical information can be found at http://www.invitrogen.com/contact.

Certificate of Analysis/Compliance is available upon request.

4.3 Precautions

Material Safety Data Sheet is available upon request.

5. WARNINGS AND LIMITATIONS

This product is for research use only. Not intended for any animal or human therapeutic or diagnostic use unless otherwise stated.

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.

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