

GMP Cloudz™ Human Treg Cell Expansion Kit

Catalog Number CLD006-GMP

This package insert must be read in its entirety before using this product.
For Preclinical or *Ex Vivo* Clinical Use Only.
Not for use in Diagnostic Procedures.

TABLE OF CONTENTS

SECTION	PAGE
DESCRIPTION	1
BACKGROUND	1
LIMITATIONS.....	1
PRECAUTIONS.....	1
MATERIALS PROVIDED & STORAGE CONDITIONS	2
OTHER MATERIALS REQUIRED	2
REAGENT PREPARATION.....	3
PROTOCOL FOR HUMAN T CELL EXPANSION	4
DATA EXAMPLES	6
REFERENCES	8
MANUFACTURING SPECIFICATIONS.....	8
END USER TERMS OF USE OF PRODUCT.....	9

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DESCRIPTION

The GMP Cloudz™ Human Treg Cell Expansion Kit is designed for the robust expansion of human regulatory T (Treg) cells from CD4⁺ T cells. The kit contains dissolvable microspheres that are functionalized with CD3 and CD28 antibodies (GMP-grade Cloudz Treg CD3/CD28). The kit also contains GMP-grade 6X Release Buffer which enables easy cell harvesting by quickly dissolving Cloudz Treg CD3/CD28. Each kit contains enough materials to activate and expand Tregs from 1 x 10⁸ CD4⁺ T cells, with greater than 250-fold expansion in 9 days.

BACKGROUND

CD4⁺ T cells differentiate into T helper cells under the influence of various cytokines and cellular interactions that induce expression of specific transcription factors. Naïve CD4⁺ T cells can be induced into Forkhead Box P3 (FoxP3)⁺ Treg cells by activation in the presence of IL-2 and TGF-β *in vitro* (1). Treg cells are a suppressive subset of CD4⁺ T cells that function to antagonize immune responses. Treg cells have the capacity to prevent potentially damaging autoimmune and protective immune responses, so the number of Treg cells is a crucial determinant of the regulatory burden on the immune system (2). Treg cells prevent autoimmune disease, maintain immune homeostasis, and modulate immune responses during infection (3). Continued *in vitro* research on methods of Treg cell expansion and mechanisms of immunoregulation are necessary to elucidate their application in both preclinical and therapeutic settings.

LIMITATIONS

- This reagent should not be used beyond the expiration date indicated on the label.
- Do not use if package is damaged. Use undamaged and sealed bottles only.
- Results may vary due to variations among cells derived from different donors.
- For use in preclinical research or *ex vivo* cell and tissue manufacturing.
- Not intended for direct administration into humans or animals. Not for parenteral use.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

PRECAUTIONS

When handling bio-hazardous materials such as human cells, safe laboratory procedures should be followed and protective clothing should be worn.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF OPENED/RECONSTITUTED MATERIAL
GMP-grade Cloudz Treg CD3/CD28	SPC-4236	16 mL	Store at 2-8 °C*
GMP-grade 6X Release Buffer	SPC-4185	100 mL	

* Provided this is within the expiration date of the kit.

OTHER MATERIALS REQUIRED

- ExCellerate™ Human T Cell Expansion Media, Xeno-free (R&D Systems®, Catalog # CCM030)
- Recombinant Human IL-2 (R&D Systems®, Catalog # PRD202-GMP or 202-GMP)
- Culture Vessel (e.g. plates, culture flasks, culture bags)
- Sterile 1X PBS
- Cell counter

REAGENT PREPARATION

GMP-grade Cloudz Treg CD3/CD28 - GMP-grade Cloudz Treg CD3/CD28 are supplied as a ready-to-use reagent.

Note: *We recommend using the Vialok[®] Vented Vial Access Device (Fresenius Kabi, Catalog # YM020) to access GMP Cloudz Treg CD3/CD28. These needle-free adapters can be attached to the vials allowing the reagent to be removed via a luer-lock syringe.*

GMP-grade 6X Release Buffer - GMP 6X Release Buffer is a ready-to-use concentrate that can be added directly into cell culture media at a 1:6 dilution (*i.e.*, add 100 mL of GMP 6X Release Buffer directly into a cell culture vessel containing 500 mL of culture medium).

1X Release Buffer - If a 1X Release Buffer is desired, add 20 mL of GMP-grade 6X Release Buffer to 100 mL of Sterile 1X PBS in an appropriate reagent vessel.

PROTOCOL FOR HUMAN T CELL EXPANSION

Each vial contains a minimum of 16 mL of GMP-grade Cloudz Treg CD3/CD28, which is sufficient to activate and expand Treg cells from 1×10^8 CD4⁺ T cells. We recommend using 150 μ L of GMP-grade Cloudz Treg CD3/CD28 for every 1 million starting cells.

CELL CULTURE SETUP AND ACTIVATION

1. Prepare starting cells by thawing/washing according to desired protocol.
2. Count cells.
3. Resuspend starting cells in ExCellerate Human T Cell Expansion Media containing 400 IU/mL of IL-2 and transfer into desired culture vessel.
4. Mix GMP-grade Cloudz Treg CD3/CD28 by vortexing the vial for 5-10 seconds immediately before use.
5. Add 150 μ L of GMP-grade Cloudz Treg CD3/CD28 for every 1 million starting cells.
6. Gently mix cells with GMP-grade Cloudz Treg CD3/CD28 by shaking or rocking for approximately 30 seconds.
7. Culture cells in a humidified incubator (37 °C, 5% CO₂) for 9 days.

Note: *Monitoring Treg cell expansion may be desired. Every 2-3 days retrieve a sample of your cell culture suspension and count the cells. For accurate cell counts, it is highly recommended to dilute the cells in 1X Release Buffer prior to counting. Based on cell counts, add additional complete cell culture medium to each culture vessel to maintain your preferred cell density of choice (~ 5×10^5 cells/mL is recommended). If required, transfer cell suspensions to larger culture vessels. Culture cells in humidified incubator (37 °C, 5% CO₂) and continue to monitor cell density until the desired fold-expansion is achieved.*

DISSOLUTION OF GMP-GRADE CLOUDZ TREG CD3/CD28 AND CELL COLLECTION

Protocol 1 - Direct addition of GMP-grade 6X Release Buffer into cell culture medium.

GMP-grade 6X Release Buffer is a ready-to-use concentrate that can be added directly into cell culture media at a 1:6 dilution (*i.e.*, add 100 mL of GMP-grade 6X Release Buffer directly into a cell culture vessel containing 500 mL of culture medium). After addition of the buffer, agitate the sample to ensure GMP-grade Cloudz Treg CD3/CD28 is completely dissolved (approximately 5 minutes). Collect Treg cells by centrifugation for downstream applications.

Protocol 2 – Dissolution Using 1X Release Buffer

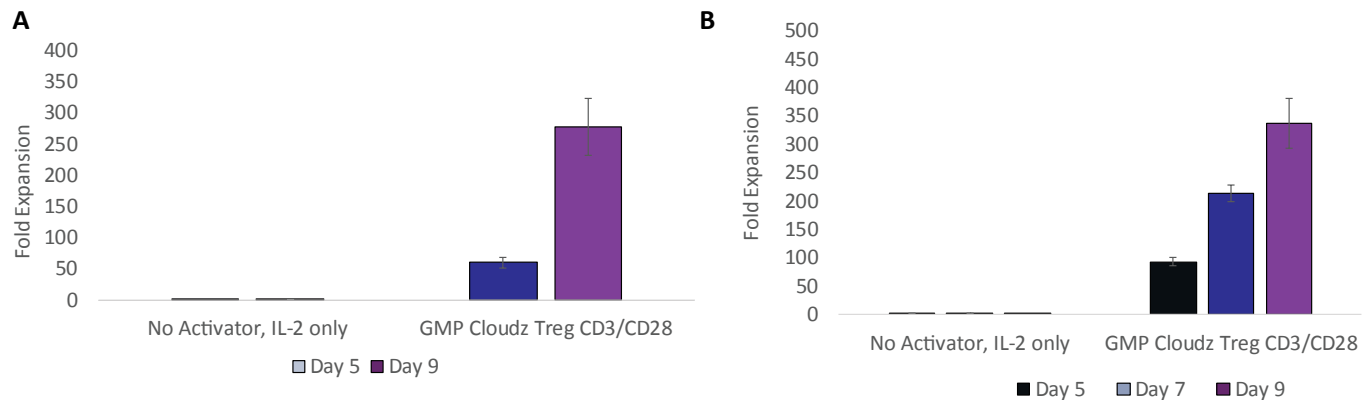
1. Prepare 1X Release Buffer as indicated in Reagent Preparation Section.
2. Transfer Treg cell suspension (still containing GMP-grade Cloudz Treg CD3/CD28) into sterile centrifuge tubes. Centrifuge at 300 x g for 5 minutes at room temperature.
3. Remove the supernatant. Re-suspend the cell pellet based on the following recommendations:

AMOUNT OF GMP-GRADE CLOUDZ TREG CD3/CD28 PARTICLES ADDED TO EACH SAMPLE ON DAY 0	VOLUME OF 1X RELEASE BUFFER
75 µL - 300 µL	2 mL
300 µL - 1200 µL	4 mL
1.2 mL - 6 mL	8 mL

Note: For larger volumes of GMP-grade Cloudz Treg CD3/CD28 particles in each sample, scale up volume of 1X Release Buffer as needed.

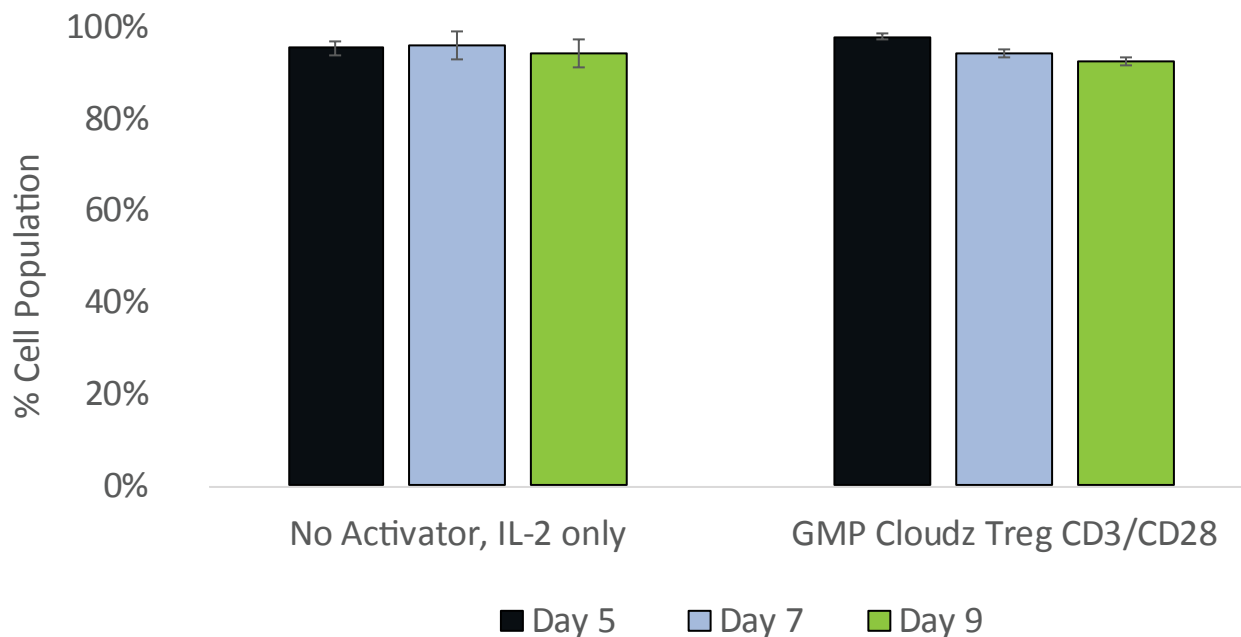
4. Agitate this suspension to dissolve GMP-grade Cloudz Treg CD3/CD28 by pipetting (up and down 5 times), rocking, or shaking for no more than 5 minutes.
5. Centrifuge the cell suspension at 300 x g for 5 minutes at room temperature.
6. Remove the supernatant. Re-suspend the cell pellet in appropriate media or buffer for downstream analysis or applications.

DATA EXAMPLES



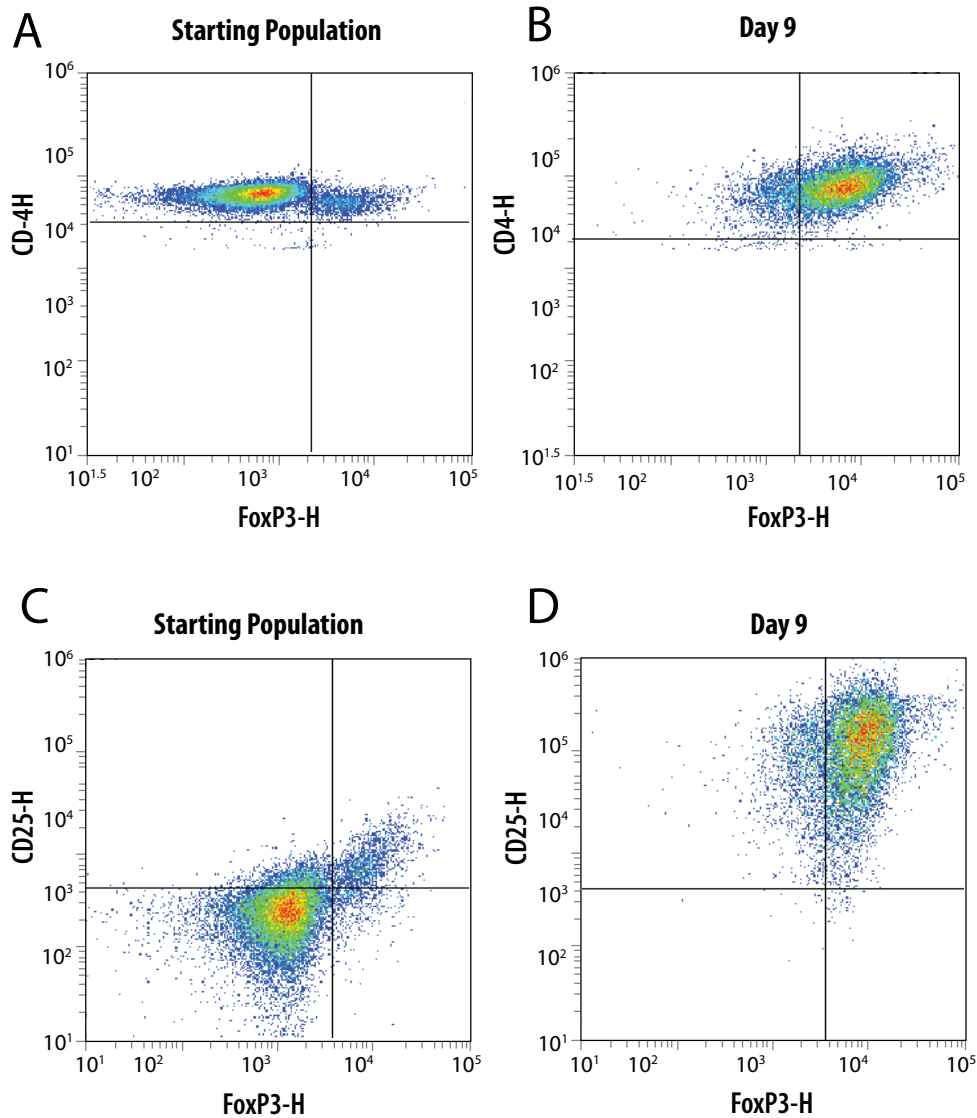
Fold Expansion of Treg Cells using the GMP Cloudz Human Treg Cell Expansion Kit.

A. Primary human CD4⁺ cells were activated with GMP Cloudz Treg CD3/CD28 and cultured for 9 days in ExCellerate T Cell Expansion Media (R&D Systems®, Catalog # CCM030) and 400 IU/mL IL-2 (R&D Systems®, Catalog # 202-GMP). Expansion of FoxP3⁺ Treg cells was evaluated using flow cytometry on Day 5 and Day 9 and fold expansion of was calculated relative to Day 0. **B.** Similarly, expansion of CD127⁻ Treg cells were calculated using flow cytometry on Days 5, 7 and 9, and fold expansion was calculated relative to Day 0. Error bars reflect standard deviation across 3 donors.



Viability of Human Treg after expansion with GMP Cloudz Human Treg Cell Expansion Kit.

Primary human CD4⁺ cells were activated with Cloudz CD3/CD28 and cultured for 9 days in ExCellerate T Cell Expansion Media (R&D Systems®, Catalog # CCM030) and 400 IU/mL IL-2 (R&D Systems®, Catalog # 202-GMP). Cell viability 9 days after expansion was greater than 90%. Error bars reflect standard deviation across 3 donors.



Increased FoxP3-positive CD4 Cells following Treg Expansion. Flow cytometry data showing expression of the Treg cell marker, FoxP3, in CD4⁺ T cells on Day 0 (**A, C**) and Day 9 (**B, D**) following expansion using the GMP Cloudz Human Treg Expansion Kit. Nine days after expansion, the cells were fixed, permeabilized, and stained with CD4-FITC, CD25-APC, and FoxP3-PE antibodies. Cells in panels A and B were stained with FoxP3 and CD4. Cells in C and D were stained with FoxP3 and CD25. Flow gates were determined based on isotype controls.

REFERENCES

1. Feuerer, M. *et al.* (2009) *Nat. Immunol.* **10**:689.
2. Liston, A. and D.H. Gray (2014) *Nat. Rev. Immunol.* **14**:154.
3. Campbell, D.J. and M.A. Koch (2011) *Nat. Rev. Immunol.* **11**:119.

MANUFACTURING SPECIFICATIONS

R&D Systems® GMP-grade products are produced according to relevant sections of the following documents: WHO TRS, No. 822,1992 Annex 1, Good Manufacturing Practices for Biological Products; USP Chapter 1043, Ancillary Materials for Cell, Gene and Tissue Engineered Products and USP Chapter 92, Growth Factors and Cytokines Used in Cell Therapy Manufacturing.

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