

## An Innovative Standard Operation Procedure for Isolating GMP-Grade CD4<sup>+</sup>CD25<sup>+</sup> T Cells from Non-Mobilized Leukapheresis 2 3 4

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### Abstract 6

This SOP describes a closed system for isolating GMP-grade CD4<sup>+</sup>CD25<sup>+</sup> T cells from non-mobilized 7  
leukapheresis collections (nMLCs), independent of a clean room in a certified GMP premises, by using 8  
CliniMACS format GMP grade reagents (CD25-labeled magnetic beads with/without pre-depletion of 9  
CD8<sup>+</sup> T cells and CD19<sup>+</sup> B cells), a GMP grade-A laminar hood and CliniMACS cell processing system. 10

**Key words** GMP, CD25, Leukapheresis collection, CliniMACS, Magnetic beads 11

### 1 Introduction 12

Good manufacturing practice (GMP) is a production and testing 13  
practice which helps to ensure a quality product [1]. In many 14  
countries pharmaceutical industry and other healthcare providers 15  
follow mandatory legislation and guidelines. The SOP described 16  
here was developed and validated by the National Health Service 17  
Blood and Transplant, Oxford Centre. It complies with the guide- 18  
lines of the Human Tissue Authority in the UK for the procure- 19  
ment and subsequent cell selection to GMP without further 20  
manipulation [2], which is achieved by processing cells within a 21  
closed system, usage of GMP-grade reagents, demonstration of 22  
reproducibility, product safety and traceability during cell 23  
processing. 24

Apheresis is a medical procedure in which the blood of a donor 25  
or patient is passed through an apparatus that separates out one 26  
particular constituent and returns the remainder to the circulation 27  
[3]. Leukapheresis is one type of apheresis which selectively har- 28  
vests mononuclear leucocytes from a donor's circulation using 29  
apheresis machines [4]. Cell type specific gravity, as shown in 30  
Table 1, governs specificity in cell selection. It is worth noting 31

**Table 1**  
**Specific gravity of different blood components**

Blood component	Specific gravity
Plasma	1.025
Platelets	1.040
Lymphocytes	1.050–1.061
Monocytes	1.065–1.070
Granulocytes	1.087–1.092
Red blood cells (RBC)	1.093–1.096

that performance of different apheresis machines can vary in terms of their selectivity. We found when using the setting of Donor Lymphocyte Collection Program for harvesting nMLCs, Cobe Spectra collected significantly higher number of lymphocytes and platelets than Spectra Optia [5]. The setting of apheresis machines targets for hematopoietic stem cells may therefore need to be optimized for lymphocytes [6, 7] and subsequently validated as needed for future works.

Allo-Bone Marrow Transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) is a well-established treatment for a range of malignant and other hematological diseases [8, 9]. Its success depends on graft-versus-leukemia (GvL) or graft-versus-tumor (GvT) effects of donor lymphocytes [10]. However, these lymphocytes may induce graft-versus-host disease (GvHD), a multi-organ acute and chronic disease leading to serious morbidity and mortality in allogeneic HSCT recipients. GvHD contributes to 40% of the immediate transplant related mortality. Furthermore, over half of the patients who survive suffer from chronic GvHD requiring long periods of immunosuppression and reducing their quality of life [8–10]. Elegant studies in mouse models of HSCT have shown that CD4<sup>+</sup>CD25<sup>+</sup> (Tregs) can suppress GvHD while sparing the beneficial effect of GvL [11]. Encouraging results from several clinical trials using Tregs with or without expansion, as prophylaxis or treatment option for acute GvHD have been published [12–17]. A study in Oxford Cancer Centre of 100<sup>+</sup> patients received non manipulated HSCT has shown patients received grafts which contained higher than median Tregs (as percentage of CD4<sup>+</sup> T cells) had a significantly higher 5 years overall survivor [18]. Further clinical trials are required to refine the trial protocols and unravel the mechanisms underline the observed beneficial effect of Tregs in HSCT.

We found that multiple factors, which are likely to be shared by several other published methods, affected the yield of CD4<sup>+</sup>CD25<sup>+</sup> cells [5]. There were no positive correlations between the numbers

of CD4<sup>+</sup> T cells in the CD25<sup>+</sup> products and the total white blood cells (WBC) in the nMLCs. There was a substantial variation in the number of CD4<sup>+</sup> T cells in the nMLCs from different donors despite the fact that these nMLCs have a similar total number of WBC. However, positive correlations between the yield of CD4<sup>+</sup>CD25<sup>+</sup> cells and lymphocyte numbers in the nMLCs and the CD8<sup>+</sup>CD19<sup>+</sup> fractions were observed. In addition, we found complete depletion of CD8<sup>+</sup> T cell and CD19<sup>+</sup> B cell was achieved using the stringent *depletion program 2.1*. However, this CliniMACS negative depletion step was associated with substantial bystander cell loss. For patients receiving non-manipulated grafts, which contained a large number of leukocytes, selection for CD25<sup>+</sup> cells without CD8<sup>+</sup> T cells and CD19<sup>+</sup> B cells depletion maybe a possible option in the future as not only would cell loss be minimized during cell isolation but also a substantial financial saving would be possible.

We have made an assumption that all fluid used in this protocol is 1 mL volume equals 1 g weight. This allows us to calculate the volume from a known weight and vice versa. While CD4<sup>+</sup>CD25<sup>+</sup> T cells are selected from nMLCs by following this SOP, the same principles and methods described here are also applicable for other cellular products, which can be enriched from peripheral blood by leukapheresis and express a specific cell surface marker allows for magnetic bead labeling, e.g. magnetic bead labeled with anti-CD34, CD14, CD56 and HLA-tetramer-antigen-complex for hematopoietic stem cells, monocytes, NK cells and pathogen-specific T cells, respectively. Post isolation manipulation is beyond the scope of this SOP. It is worth bearing in mind that complicated post isolation manipulation increases the cost, risk of contamination and complicity in legislation requirement. In vitro suppression assays have been used to demonstrate the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> T cells. They are likely to be applied during validation procedures and as a retrospective measure of in vitro efficacy rather than as a product release criterion, due to the long processing time for these assays and lack of any evidence for a close relationship between these laboratory assays and what happens in the recipient. Much more work is needed to establish the efficacious dose, the timing of administration, the potential side-effects of cellular therapy products to patients and negative impact of GvHD prophylaxis drugs on cellular therapy products such as Tregs.

## 2 Materials

### 2.1 Material for Cell Processing

1. nMLC (*see Note 1*), collected by a qualified Apheresis unit using an Apheresis machine (*see Note 2*). Once the donor is identified and the date of donation is set, sticky labels with a donor/patient-specific barcode are generated for labeling the nMLCs and cellular product along each step of cell processing.

2. Peripheral blood samples collected into 4 mL Vacutainer (BD, 367844) pre- and post- leukapheresis collection. 112  
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3. A certified GMP premises (*see* **Note 3**), nMLCs for cell isolation must stay in the defined cell processing space within the premises once received and signed in. 114  
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4. A Grade A laminar Hood, Labcaire, Fisher Scientific, Loughborough, UK. 117  
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5. A Hematology analyzer, Sysmex XE-2100, Sysmex Corp, Kobe, Japan. 119  
120
6. Centrifuge, Heraeus Megafuge 2.0RS, Rotor 2708, Thermo Scientific, Loughborough, UK. 121  
122
7. CliniMACS, Miltenyi Biotec, Surrey, UK. 123
8. Tuber sealer, SEBRA, Haemonetics, Coventry, UK. 124
9. Sterile docking device, Terumo SCD II, Surrey, UK. 125
10. Balance, Excell, UKAS, Middlesex, UK. 126
11.  $-20^{\circ}\text{C}$  freezer, Fisher Scientific, Loughborough, UK. 127
12. Fridge, Fisher Scientific, Loughborough, UK. 128
13. Flat-bed rocker, Grant-Bio, Cambridgeshire, UK. 129
14. Plastic Spencer Wells, Fisher Scientific, Loughborough, UK. 130
15. Plasma press, Fenwal Europe sprl, Mont Saint Guibert, Belgium. 131  
132
16. Cold packs, 216-0196, VWR, Jencons Whitworth, UK. 133
17. Timer. 134
18. One bottle each of CliniMACS format GMP-grade CD8, CD19 and CD25 microbeads for CliniMACS, Miltenyi Biotec, Surrey, UK. 135  
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137
19. 600 mL standard transfer bags, TerumoBCT, Surrey, UK. 138
20. CliniMACS tubing sets, 162-01 and 161-01, Miltenyi Biotec, Surrey, UK. 139  
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21. Pre-system filters, 130-018-101, Miltenyi Biotec, Surrey, UK. 141
22. Zenalb<sup>®</sup> 20, a 200 g/L of human albumin solution for infusion (20% Solution), BPL, Herts, UK. 142  
143
23. CliniMACS buffer (Cat no. 700-25)  $4 \times 1$  L supplemented with 0.5% Zenalb. It must be used at room temperature or  $4^{\circ}\text{C}$  for different parts of the protocol—remove from cold room and allow to come to room temperature (RT) before use for all but the specified  $4^{\circ}\text{C}$  step. 144  
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24. 2.85 mL of Gammaguard (Gammagard, Baxter, Thetford, UK) at 5%. Reconstitute Gammaguard following the manufacturer's instruction. 149  
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	25. 5 mL, 10 mL, 20 mL, and 50 mL Syringes and needles, TerumoBCT, Surrey, UK.	152 153
	26. Forms and bottles for aerobic and anaerobic blood cultures, BD BacTec, Oxford, UK.	154 155
	27. Female sample side couplers, OriGen Biomedical, West Midlands, UK.	156 157
	28. Klerwipe 70/30, Shield Medicare, Farnham, UK.	158
	29. Processing recording sheet (Fig. 1).	159 160
<b>2.2 Materials for Immuno-Fluorescent Staining and Flow Cytometer Analysis</b>		
	1. Centrifuge, Heraeus Megafuge 1.0R, Rotor 2704, Thermo Scientific, Loughborough, UK.	161 162
	2. Tube-Segment-Opener, D-51588, SARSTEDT, Leicester, UK.	163
	3. PBS, Sigma-Aldrich, Dorset, UK.	164
	4. 1.5 mL Eppendorfs, Eppendorf UK Limited, Stevenage, UK.	165
	5. P20, P200, P1000 Gilson Pipettes, Gilson Scientific Ltd., Bedfordshire, UK.	166 167
	6. Filter Tips, Renin, Mettler Toledo Ltd., Leicester, UK.	168
	7. Truecount tubes, Oxford, Becton Dickinson (BD), UK.	169
	8. 4 mL FACS tubes, Oxford, BD, UK.	170
	9. Red cell lyses buffer, Oxford, BD, UK.	171
	10. Antibodies: mouse anti-human CD3-FICT, CD4-PerCPCy5.5, CD8-APC-Cy7, CD20-PE, CD25-PE, BD.	172 173
	11. Pan CD45-APC, R&DSsystem, Abingdon, UK.	174
	12. Live/Dead Fixable dye-blue, Invitrogen, L23105. Optional.	175
	13. FcR blocker, Miltenyi Biotec, Surrey, UK.	176
	14. Anti-FOXP3-APC, Miltenyi Biotec, Surrey, UK.	177
	15. FOXP3 staining kit, Miltenyi Biotec, Surrey, UK.	178
	16. Set of Compbeads, BD.	179
	17. Flow cytometer with UV, blue and red lasers and appropriate long pass and band pass filters (example shown in Table 2).	180 181 182
<b>3 Methods</b>		183
<b>3.1 Preparation for Cell Isolation</b>		
	1. On the day of leukapheresis collection take a pigtail sample ( <i>see Note 4</i> ) from the leukapheresis bag.	184 185
	2. Transfer nMLCs from the leukapheresis bag to a transfer bag by sterile docking of the leukapheresis bag with cells to a transfer bag ( <i>see Note 5</i> ). Release the Spencer Wells forceps to allow the contents of the leukapheresis bag to be completely transferred	186 187 188 189

Donor						
Donor/Patient Name		DOB:		Date of Process:		
Gender:		Hospital Number:		Weight (Kg):	Volume:	
Leukapheresis Sample Analysis by Sysmex						
WBC concentration:	Total WBC	Lymph concentration:	Total lymph			
Leukapheresis Sample Analysis by FACS						
CD8 <sup>+</sup> T cells:		CD19 <sup>+</sup> B Cells:				
CliniMACS Procedure						
CD8 Lot No.	Expiry Date:	CD19 Lot No.	Expiry Date:			
Tubing Set Lot No.	Expiry Date:	Power Filter No.	Expiry Date:			
Process Code:		Process Length:				
Final Volume (ml)						
CD8 <sup>+</sup> CD19 <sup>+</sup> :	CD8 <sup>+</sup> CD19 <sup>-</sup> :		Waste:			
CD25 Lot No.	Expiry Date:		Expiry Date:			
Tubing Set Lot No.	Expiry Date:	Power Filter NO.	Expiry Date:			
Process Code:		Process Length:				
Final Volume (ml)						
CD25 <sup>+</sup> :	CD25 <sup>-</sup> :		Waste:			
Total Cell Counts (by Sysmex)		x10 <sup>6</sup> cells				
	WBC	Mono.	Lymph	Gran.	Platelets	
Leukapheresis*						
CD8 <sup>+</sup> CD19 <sup>+</sup>						
CD8 <sup>+</sup> CD19 <sup>-</sup>						
CD25 <sup>+</sup>						
CD25 <sup>-</sup>						
Total Cell Counts (by FACS)		x10 <sup>6</sup> cells				
	CD45 <sup>+</sup>	CD3 <sup>+</sup> CD20 <sup>+</sup>	CD3 <sup>+</sup> CD8 <sup>+</sup>	CD3 <sup>+</sup> CD4 <sup>+</sup>	CD4 <sup>+</sup> CD25 <sup>+</sup>	CD25 <sup>+</sup> FOXP3 <sup>+</sup>
Leukapheresis*						
CD8 <sup>+</sup> CD19 <sup>+</sup>						
CD8 <sup>+</sup> CD19 <sup>-</sup>						
CD25 <sup>+</sup>						
CD25 <sup>-</sup>						
CD4 <sup>+</sup> CD25 <sup>+</sup> cell product Storage/Issue in Cryobags						
Bags	Vol (ml)	CD4 <sup>+</sup> CD25 <sup>+</sup>	Stored	Date	Fresh/Frozen	
1				Issued		
2						

Operator's signature: \_\_\_\_\_

\*1 in 10 dilutions of these samples is used for Sysmex and FACS analysis.

**Fig. 1** Processing recording sheet for CliniMACS selection of CD4<sup>+</sup>CD25<sup>+</sup> cells from non-mobilized leukapheresis

Table 2  
Cytometer set ups

Laser	Antigen	Conjugate	Filter setting
UV (355 nm, 20 mW)	Viability	Live/dead fixable dye	Non, 450/50
Blue (488 nm, 100 mW)	CD3	FITC	505LP, 530/30
	CD20 or CD25	PE	550LP, 575/26
	CD4	PerCP-Cy5.5	685LP, 695/40
Red (633 nm, 40 mW)	CD8	APC-Cy7	755LP, 780/60
	CD45 or FOXP3	APC	Non, 660/20

- to the transfer bag (*see Note 6*). Seal the tube with a tube sealer. Detach both bags.
3. Weigh the bag. Record the net volume of nMLC onto recording sheet. Store the bag at 4 °C overnight.
4. Label a 1.5 mL Eppendorf as NEAT nMLC. Transfer the contents of the pigtail sample to this vial (*see Note 7*).
5. Make a master dilution of nMCL for Sysmex and FACS cell quantitation. Label a 1.5 mL Eppendorf as 1:10 nMLC. Transfer 450 µL of PBS and 50 µL of NEAT nMLC to this vial (*see Note 8*).
6. Perform cell count by Sysmex (*see Note 9*) and FACS analysis (*see Subheading 3.3, step 7*) to determine the absolute number of cells of interest in the nMLC.
7. Record cell number onto the recording sheet after Sysmex and FACS analysis. The total number of WBC and the total percentage of CD8<sup>+</sup> T cells and CD19<sup>+</sup> B cells should be entered into the CliniMACS for the depletion step.

3.2 Reagent Preparation

- Perform the following preparations in a grade A GMP tissue culture hood.
1. Draw the thawed Gammaguard into a 5 mL syringe.
2. Draw the contents of CD25 bead bottle into a 10 mL syringe.
3. Draw the contents of CD8 and CD19 bead into a 20 mL syringe.
4. Each time replace the needles with female sample side couplers. Store these syringes in individual sealed bags in a GMP-grade fridge.
5. Prepare 4 × 1 L CliniMACS buffer supplemented with 0.5% Zenalb (*see Note 10*).
6. Record the Lot number and expiry date onto the recording sheet at the end of reagent preparation.

3.3 Immuno-  
Fluorescent Staining  
for Lymphocyte  
Phenotyping

Sample preparation:	222
1. Label Eppendorfs correspondent to each pigtail sample. Transfer samples from pigtail tubes to Eppendorfs following <b>Note 7</b> .	223
2. A one in ten master dilution is made from nMLC and CD25 <sup>-</sup> fractions due to high cell concentration follow <b>Note 8</b> .	225
3. Label one set of Trucount tubes according to the name and number ( <i>n</i> ) of samples for lymphocyte phenotyping.	227
Antibody preparation:	229
See Table 3 for details.	230
Staining procedure:	231
4. Transfer 100 μL of each sample to the correspondent Trucount tubes. Accurate sample volume is important for calculation. Apply the two steps pipetting method or use an electronic pipette for obtaining accurate sample volume.	232
5. Transfer 40 μL of the antibody mix from panel one to each Trucount tube; mix well; and leave the tubes to incubate in the dark for 15 min at room temperature.	233
6. At the end of incubation, add 1 mL of red cell lyses buffer to each tube; check the tubes frequently. Once the solution turned from cloudy to clear (take about 4–6 min at RT), the samples are ready for analysis on FAC machine.	234
7. CST beads must be run on the day of experiment for instrument set up. Compbeads staining with single color is used for setup compensation. Instrument setup by CST beads is recommended for achieving maximal detection sensitivity.	235
Lymphocyte concentration (#/μL) = # of CD45 <sup>+</sup> events / # of events in absolute count beads region × # of beads per test* /test volume (100 μL).	236
* This number is Trucount tube batch specific; it is on the bag of Trucount tube. Make sure to use correct number.	237
Absolute number of lymphocyte in the sample = Lymphocyte concentration (#/μL) × volume of the sample × dilution factor.	238

**Table 3**  
**Antibody mix for lymphocyte typing**

CD45	CD3	CD4	CD8	CD20
APC	FITC	PERCPCy5.5	APC-Cy7	PE
SAMPLE × <i>n</i>	5 μL	10 μL	10 μL	5 μL
× <i>n</i>	× <i>n</i>	× <i>n</i>	× <i>n</i>	× <i>n</i>

*N* = number of samples to be stained



Total number of lymphocyte subsets (e.g.  $CD3^+CD4^+$  T cells) = Total number of lymphocyte in the sample  $\times$  % of  $CD3^+CD4^+$  cells in the lymphocyte gate.

Collect Sysmex and FACS report, Bead CE certificate and the recording sheet ready for the next day process.

### 3.4 Leukapheresis Preparation

Wash to reduce the number of platelets and the volume of the nMLC for the bead labeling.

1. Sterile dock the bag with cells to the MACS buffer bag. Increase the volume of nMLC to 550 mL (*see Note 11*). Seal the tube with a tube sealer and detach the MACS buffer bag.
2. Sterile dock the bag with cells to a new empty transfer bag (*see Note 12*).
3. Place the bags and a balance bag (*see Note 13*) in the buckets of a centrifuge (*see Note 14*). Centrifuge at  $200 \times g$  for 10 min with brake off. Total time required for this step is about 20 min.
4. At the end of centrifugation, carefully remove the bag with cells from the bucket. To remove the supernatant, hang the bag onto the plasma presser or extruder, push the hook forward. Release the presser from the stationary or fixed position to allow it to come into contact with the bag. Release the Spencer Wells to allow supernatant to flow from the nMLC bag to the empty transfer bag. Try to remove as much supernatant as possible without disturbing the pellet. Avoid moving the position of the bag during releasing supernatant.
5. Clamp the tube with two Spencer Wells when the cell pellet starts to be disturbed. Seal the tube with a tube sealer. Detach the bags.
6. Resuspend the pellet (*see Note 15*).
7. Sterile dock the syringe containing Gammaguard to the bag containing cells. Release Spencer Wells and push the plunge to transfer Gammaguard to the cell bag. Seal the tube and detach the bags. Mix Gammaguard and cells by inverting the bag a few times.
8. Increase the volume of nMLC for bead labeling. Sterile dock MACS buffer bag to the bag with cells. Transfer buffer to nMLC bag as described in **Note 11**. The volume of cell suspension for the negative depletion is 90 mL.

### 3.5 CD8 and CD19 Depletion

1. Transfer CD8 and CD19 beads to the nMLCs bag. Connect the syringe containing CD8 and CD19 beads and the bag containing cells by sterile docking. Seal the tube with tube sealer after the contents of the syringe has been completely transferred to the bag. Detach the syringe. Mix well and

- incubate for 30 min at controlled RT, no higher than 25 °C. 299  
 Rotate gently on the flat bed rocker mixer. 300
2. Wash cells to remove excessive beads. At the end of incubation, 301  
 sterile dock the bag with MACS buffer bag. Transfer buffer to 302  
 the bag with cells (*see Note 11*). Clamp and seal the tube then 303  
 sterile dock the cell bag to a new transfer bag (*see Note 12*) 304  
 before centrifugation. 305
  3. Repeat **steps 4–6** under Subheading **3.3**. At the end of centri- 306  
 fugation, change the temperature setting of the centrifuge to 307  
 4 °C for all the rest of centrifugation steps. 308
  4. Increase the volume of nMLCs to 100 mL for CliniMACS run. 309  
 Sterile dock the cell bag and MACS buffer. Transfer MACS 310  
 buffer to the now completely resuspended cells (*see Note 11*). 311  
 Seal and detach the bags. 312
  5. To connect a 162-01 tubing set with cell bag, buffer bag and 313  
 cell collection bag. In a GMP grade A hood, check to make sure 314  
 that all connections in the tubing set are properly sealed; con- 315  
 nect a bag of **cold** MACS buffer and a pre-system filter to the 316  
 tubing set. **Clamp** tubes connecting CliniMACS tubing set to 317  
 the MACS buffer bag and the pre-system filter with Spencer 318  
 Wells (*see Note 16*). Sterile dock a new transfer bag to the 319  
 output tube of the 162-01 tubing set for collecting cells. 320
  6. Hook up the MACS buffer bag, cell bag and the waste wash 321  
 bag onto the CliniMACS. Install the tubing set following 322  
 CliniMACS instructions. Select *depletion program 2.1* from 323  
 the manual. Input the total number of WBC and the total 324  
 percentage of CD8 and CD19 labeled cells. Set machine to 325  
 RUN and release Spencer Wells. Once the cell suspension starts 326  
 to fill the power filter, gently tap the filter till there are no air 327  
 bubbles going up; this minimizes air bubbles trapped on the 328  
 filter and increases efficiency. While the negative depletion is in 329  
 progress, sandwich the bag for the negative fraction (contain- 330  
 ing CD8<sup>+</sup>CD19<sup>+</sup> cells) between two cold blocks. The deple- 331  
 tion takes about 80 min and uses  $\leq 1$  L of buffer for an average 332  
 size of nMLC. 333
  7. While the depletion is in progress record Lot number and 334  
 expiry date of the 162-01 tubing set used and processing 335  
 time (this will show on CliniMACS machine) onto the record- 336  
 ing sheet. Label the bags using sticky labels. 337
  8. At the end of CliniMACS depletion step, record the Clini- 338  
 MACS protocol number onto the recording sheet; clamp or 339  
 seal all the tubes in and out of the CliniMACS tubing set and 340  
 remove the bags from hooks. 341
  9. Detach the bag containing CD8<sup>+</sup>CD19<sup>+</sup> fraction and 342  
 promptly proceed to the CD25 positive isolation. 343

10. Take a pigtail sample (*see Note 4*) from each of the bags containing CD8<sup>+</sup>CD19<sup>+</sup> and CD8<sup>-</sup>CD19<sup>-</sup> cells. Label the samples.
11. Weigh the bags and record the net volume onto the recording sheet.

### 3.6 CD25 Isolation

1. For the CD25 beads labeling, the incubation volume is 380 mL ( $\pm 10\%$ ). Increase or decrease the volume of the CD8<sup>-</sup>CD19<sup>-</sup> fraction depending on its volume. If the weight of the cell bag is in the range of 380–440 g go straight into CD25 bead selection.
2. Transfer CD25 beads to the bag of cells via a sample side coupler using a sterile docking system. Mix well. Sandwich the bag in between one cold block stored in a fridge at 4 °C and one stored in a freezer at -20 °C. Wrap in cling film and place the bundle on a rocker and incubate for 15 min.
3. Wash the cells to remove excessive beads. At the end of incubation, sterile dock the bag with cells with a bag containing cold MACS buffer. Transfer MACS buffer to cell bag (*see Note 11*).
4. Seal the bag then sterile dock the bag with cells to a new transfer bag (*see Note 12*) before centrifugation.
5. Repeat steps 4–6 under Subheading 3.3 above.
6. Resuspend cell pellet (*see Note 15*).
7. Increase the volume of CD8<sup>-</sup>CD19<sup>-</sup> to 100 mL for CliniMACS run (*see Note 11*).
8. In a GMP grade A hood, connect the bag with cells, the pre-system filter and buffer bag to a CliniMACS tubing set 161-01 by repeating Subheading 3.4, step 5 (*see Note 16*).
9. Hook up buffer bag, bag of cells, and waste collection bag. Install the tubing set to the CliniMACS. Select program *enrichment 3.1*. Press RUN and release the Spencer Wells.
10. While the positive selection is in progress, record Lot number and expiry date of the 161-01 tubing set used, and processing time onto the recording sheet. Label the bags using sticky labels generated earlier.
11. At the end of the enrichment program, record the CliniMACS protocol number onto the recording sheet; seal all the tubes in and out of the CliniMACS tubing set. Remove all bags and the tubing set and then shut down CliniMACS.
12. Weigh the bags containing CD25<sup>+</sup> and CD25<sup>-</sup> fractions and record the net volume of the content (*see Note 17*). Take a pigtail sample from both CD25<sup>-</sup> and CD25<sup>+</sup> fractions for Sysmex and FACS analysis (*see Note 4*). Label the pigtail samples.

**Table 4**  
**Antibody mix for Treg semi-quantification**

	CD3	CD4	CD25	FOXP3 APC
	FITC	PERCPCy5.5	PE	Fix&Perm
FMO FITC		10 $\mu$ L	20 $\mu$ L	20 $\mu$ L
FMO PERCPCy5.5	10 $\mu$ L		20 $\mu$ L	20 $\mu$ L
FMO PE	10 $\mu$ L	10 $\mu$ L		20 $\mu$ L
FMO APC	10 $\mu$ L	10 $\mu$ L	20 $\mu$ L	
SAMPLE $\times n$	10 $\mu$ L	10 $\mu$ L	20 $\mu$ L	20 $\mu$ L
	$\times n$	$\times n$	$\times n$	$\times n$

**3.7 Immuno-  
Fluorescent Staining  
for Semi-  
Quantification of  
Regulatory T Cells  
Number in the Donor  
Peripheral Blood and  
the nMLCs Before and  
Throughout Each Step  
of the GMP Cell  
Isolation Process**

13. In the GMP grade A hood, take a sample from the negative fraction (*see* **Note 18**); inoculate 3 mL of the negative fraction to each of the aerobic and anaerobic blood cultures. Fill in the form and send the bottles to local microbiology department.
14. Operators must complete and sign the production form.
1. Label another set of FACS tubes according to the name and number ( $n$ ) of samples and FMOs (Table 4) for Treg semi quantification.
2. Transfer 100  $\mu$ L of each (diluted or neat) sample as used for the lymphocyte quantification to the correctly-labeled FACS tubes including FMO-1 controls for Treg quantification.
3. Add 40  $\mu$ L of Treg antibody mix or FMO mixes to the cell suspension. **Mix well** and leave to incubate at RT for 20 min.
4. At the end of incubation, top up the tubes with 3 mL of PBS and centrifuge at  $300 \times g$  for 5 min.
5. Discard the supernatant. Vortex tubes to resuspend cells (*see* **Note 19**).
6. Lyse red cells as described in the lymphocyte staining protocol and wash cells once with 3 mL of PBS and centrifuge at  $300 \times g$  for 5 min.
7. Discard the supernatant and blot the tube on a piece of tissue paper. This will reduce the residual buffer to around 50  $\mu$ L. Vortex tubes to resuspend cells.
8. Live/Dead stain is optional. Make the Live/Dead stain working solution by diluting the stock of Live/Dead solution 1/1000 in PBS (*see* **Note 20**). Add 1 mL of this **Live/Dead stain** working solution to each FACS tube. Leave the sample to incubate at RT for 10 min.

9. At the end of incubation, top up the tubes with 3 mL of PBS and centrifuge at  $300 \times g$  for 5 min.
  10. Discard the supernatant. Vortex tubes to resuspend cells.
  11. Add 0.5 mL of freshly prepared diluted **fix buffer** from the FOXP3 staining buffer kit to each tube; incubate the tubes in a fridge @ 4 °C for at least 30 min.
  12. At the end of incubation, top up the tubes with 3 mL of PBS and centrifuge at  $300 \times g$  4 °C for 5 min.
  13. Discard the supernatant. Vortex tubes to resuspend cells.
  14. Wash the cell one more time in 1 mL of **perm buffer** per sample.
  15. Discard the supernatant and blot the tube on tissue paper. Vortex tubes to resuspend cells.
  16. Add 5 µL of FcR blocker to the cell suspension and incubate for 5–10 min at RT.
  17. Add 20 µL of anti-FOXP3 to each tube and incubate the tubes at 4 °C in the dark for 30 min.
  18. At the end of incubation, top up the tubes with 1 mL of perm buffer, leave it to stand for 2 min before centrifuge at  $300 \times g$  4 °C for 5 min.
  19. Discard the supernatant and resuspend the pellet in PBS.
  20. Repeat **steps 18** and **19**. Cells are ready for FACS analysis.
- (Assume cells lost to different subsets in the sample occurred in equal proportion.)
- Number of Treg = Absolute number of CD3<sup>+</sup>CD4<sup>+</sup> T cell in a sample × % of CD25<sup>+</sup>FOXP3<sup>+</sup> cells in the CD3<sup>+</sup>CD4<sup>+</sup> T cell gate of the sample.

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## 4 Notes

1. Use of nMLCs for research and development requires ethical permission from local Ethics Committee.
2. Spectra Optia (TerumoBCT, Surrey, UK) is the one currently provided on contract to NHS National Blood and Transplant in the UK.
3. Certified GMP premises is regulated, maintained, and inspected according to national guidelines. For details of guidelines in UK consult the MHRA at <http://www.mhra.gov.uk/Howweregulate/Medicines/Inspectionandstandards/GoodManufacturingPractice/index.htm>

4. To take a pigtail line sample, the line is stripped back six times while mixing the bag, before being sealed off and removed using the RF tube sealer. The length of the line to be taken is dependent on the cell concentration of the solution. Samples such as nMLCs, and CD25<sup>-</sup> fraction have a high cell concentration. 1 cm of line containing cell solution is sufficient. Master dilutions must be prepared from the neat solutions for cell quantitation by Sysmex counter (takes about 200  $\mu$ L per run) and FACS (take 100  $\mu$ L per FACS panel). 1.5 cm of line or approximately 500  $\mu$ L of cell solution to be taken for other samples (CD8<sup>+</sup>CD19<sup>+</sup>, CD8<sup>-</sup>CD19<sup>-</sup> and CD25<sup>+</sup>).
 

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5. Sterile dock the bags (or sample side coupler). Make sure there is sufficient length of tube to allow for the sterile docking. First, clamp the tubes from both the bags with Spencer Wells, then perform the sterile docking using the sterile docking device. Check the sterile docking is intact. The Spencer Wells provide an additional guarantee of an enclosed system, but can still allow for liquid transfer to take place when they are released at the time indicated in the protocol.
 

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6. The size of a leukapheresis bag is too big for the height of hanger of the CliniMACS. The weight of a leukapheresis and a 600 mL transfer bag is 40 g and 30 g, respectively.
 

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7. To empty the contents of a pigtail line, cut one end of the tube and insert the open end to a vial or Eppendorf tube. Insert a Tube-Segment-Opener to the other end of the tube. The contents of the pigtail line will drain freely into the vial. Check the vial labeling is correct and discard the pigtail line and the Tube-Segment-Opener.
 

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8. Apply the two-step pipetting technique or use an electronic pipette to minimize pipetting error. Use a master dilution to avoid inconsistency.
 

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9. Operate the Sysmex counter following the instrument manual.
 

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10. Wipe the detachable metal lid with an alcohol wipe before lifting it from a Zenalb bottle. Draw 2.5 mL of Zenalb<sup>®</sup> 20 from the bottle. Replace the needle with a sample side coupler. Connect the syringe to the tube of a buffer bag using a sterile docking device. Inject the contents of the syringe into the buffer bag. Seal the tube using a tube sealer and detach the syringe from the bag. Repeat this for the remaining three bags of buffer.
 

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11. Once the sterile docking is completed, place the bag with nMLC on a balance and top up the bag with MACS buffer by releasing the two Spencer Wells and monitor the weight. The volume for all washing steps and CliniMACS runs is 550 mL and 100 mL, so clamp the tube with Spencer Wells when the weight of the bag is 580 g or 130 g, respectively. The volume
 

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- for CD8 and CD19 beads incubation is 90 mL, so clamp the bag when the weight of bag reaches 120 g.
12. The empty bag is for discarded supernatant after centrifugation. Docking before centrifugation minimizes the risk of disturbing the cell pellet.
13. Wrap the empty bag and tube around the Spencer Wells so the empty bag and the Spencer Wells can be tucked down tightly and neatly in the centrifugation bucket in the next step. Have the non-covered side of the bag facing outside of the bucket. Tuck down with tissue and make sure the bag stands upright without folds and bends, so that a compact pellet will form at the end of centrifugation step.
14. A balance bag is prepared in advance by transferring 550 mL of distilled water into a transfer bag. The tube is sealed and clamped with a Spencer Well for balancing purposes.
15. To disperse the cell pellet, hold the two ends of the bag and roll it against the edge of a bench. Hold the bag against a light source to check for any visible cell clumps. Repeat this until no visible clumps remain before proceeding to the next step. It is important to completely resuspend the cell pellet to minimize cell loss in the pre-system filters. Make sure the cell pellet is well resuspended before increasing the volume as better cell resuspension is achieved in a smaller volume.
16. If the tubes leading to the pre-system filter are not clamped, cells and buffer will flood the CliniMACS set. These cells will be flushed to the waste bag during the subsequent CliniMACS tubing set preparation. It is therefore important to clamp all the tubes that lead to the pre-system filter before hanging up CliniMACS tubing set.
17. The content of CliniMACS positive isolation is 50 mL by default. We found the real volume is between 45 and 50 mL. We recommend recording the real volume to avoid over-estimation of cell number.
18. Use the negative fraction to inoculate each of the aerobic and anaerobic blood cultures to minimize the waste of CD25<sup>+</sup> cells.
19. Make sure cell pellets are well resuspended after each centrifugation.
20. Make sure cells are washed in PBS to remove proteins before staining with Live/Dead dye as this dye is amine reactive.

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