STANDARD OPERATING PROCEDURE

Title: Precursor Frequency Determination Using CFSE Dilution Assay and Quantification of Cytokine Secreting Cells Using Intracellular Cytokine Staining	SOP No.: BDR-301 Effective Date: Jan 2013	Version: Page: **1 of 19**
Trial Number : ITN032AD		

Document Approval

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1. PURPOSE AND SCOPE

Described below is the Standard Operating Procedure (SOP) for setting up the assay for determination of precursor frequency of peanut-specific CD4+ T cells (CFSE dilution assay) and quantification of cytokine-producing antigen-specific T cells (intracellular cytokine staining (ICS) assay) in LEAP trial samples.

Each assay/experiment should include peripheral blood mononuclear cell (PBMC) samples from 1 known peanut allergic participant (used as a positive control) and 20 LEAP barcodes

2. RESPONSIBILITY

Dr. Gideon Lack is responsible for ensuring that the individuals executing this procedure are appropriately trained.

3. DEFINITIONS AND ABBREVIATIONS

CFSE	**Carboxyfluorescein succinimidyl ester**
DMSO	**Dimethyl sulfoxide**
PBS	**Phosphate buffered saline**
ICS	**Intracellular cytokine staining**
APC	**Allophycocyanin**
PE	**Phycoerythrin**
PBMC	**Peripheral blood mononuclear cells**

PE-Cy5	**Phycoerythrin-cyanin5**
IL-4	**Interleukin 4**
IL-5	**Interleukin 5**
IL-13	**Interleukin 13**
IFNγ	**Interferon-gamma**
TNFα	**Tumor necrosis factor-alpha**

4. REAGENTS AND MATERIALS

	Type	Supplier	Catalog of Vendor Number
4.1	CFSE	Molecular Probes	C1157
4.2	DMSO	Sigma	D2650
4.3	PMA	Sigma	P8139
4.4	Ionomycin	Sigma	I0634
4.5	Brefeldin A	Sigma	B6542
4.6	Peanut Extract	ALK-Abello	B713 batch# EC-B044
4.7	PBS	Life Technologies	14190-094
4.8	Heat inactivated FBS	Sigma	F4135
4.9	Penicillin/streptomycin	Sigma	P4458

4.10	RPMI1640	Life Technologies	52400-025
4.11	ChemoMetec, Reagent A	Allerod	910-0003
4.12	ChemoMetec, Reagent B	Allerod	910-0002
4.13	ChemoMetec Nucleocassette	Allerod	941-002
4.14	Anti-CD4 APC	BD Pharmingen	555349
4.15	Anti-CD3/anti-CD28 Dynabeads	Invitrogen	111-41D
4.16	Anti-human IFNγ PE	BD Pharmingen	554701
4.17	Anti-human CD3 PECy5	BD Pharmingen	555334
4.18	Anti-human IL-13 PE	BD Pharmingen	554571
4.19	Anti-human IL-5 PE	BD Pharmingen	554395
4.20	Anti-human IL-4 APC	BD Pharmingen	554486
4.21	Anti-human TNFα	BD Pharmingen	554514
4.22	Rat IgG1 κ PE	BD Pharmingen	554885
4.23	Mouse IgG1κ APC	BD Pharmingen	554681
4.24	FACSFlow	BD Pharmingen	342003
4.25	IntraPrep Permeabilization kit	Beckman Coulter	A07803
4.26	NaN_3	Sigma	S8032
4.27	Acrodisc 25 mm syringe filter	Pall Corporation	PN4612
4.28	50 mL polypropylene tubes	Greiner Bio-One	210261
4.29	1.5 mL polypropylene	Fisher Scientific	FB74031

	microcentrifuge polypropylene tubes		
4.30	FACS tubes	BD Falcon	352054
4.31	24-well plate	Greiner Bio-one	662160
4.32	48-well plate	Corning Costar	3548

5. PROCEDURE

5.1 Preparation of the CFSE stock solution:

5.1.1. CFSE is purchased as a 25 mg vial of lyophilized powder and stored desiccated at -20ºC until used.

5.1.2. Add 1 mL DMSO (Hybri-MAX) to the vial and vortex vigorously to dissolve the CFSE.

5.1.3. Transfer 1 mL CFSE into a sterile 50 mL polypropylene tube and add 7.96 mL DMSO (Hybri-MAX) to generate 5 mM CFSE stock solution.

5.1.4. Distribute the 5 mM CFSE stock solution into 10 µL aliquots/tube into sterile 1.5 mL polypropylene microcentrifuge tubes and store the aliquots at -20ºC.

5.1.5. Retrieve one aliquot on the day of the experiment and further dilute the stock solution to a working solution (as described below 5.6.1). **Note**: Fresh 5 mM CFSE stock solutions should be prepared every 3 months and old aliquots should be discarded.

5.2. Preparation of the peanut protein, PMA, ionomycin and brefeldin A stock solutions:

5.2.1. **Peanut protein**:

5.2.1.1. 20 mg lyophilized peanut protein vials are purchased from ALK Abello and stored at -70ºC.

5.2.1.2. Prepare 20 mg/mL stock solution by dissolving the peanut protein in 1 mL PBS per vial and vortexing the vial vigorously to dissolve the peanut protein.

5.2.1.3. Aliquot at 1 mL per tube into sterile 1.5 mL polypropylene microcentrifuge tubes. **Note**: This stock solution can be stored at 4ºC for up to 1 week, stored frozen at -20ºC for up to one month, or stored frozen at -70ºC for up to three months. Fresh stock solutions should be prepared every 3 months and old aliquots should be discarded.

5.2.2. **Phorbol 12-myristate 13-acetate (PMA)**:

5.2.2.1. 1 mg vials of PMA powder are purchased from Sigma and stored in the freezer, protected from light.

5.2.2.2. Prepare 1 mg/mL stock solution by dissolving the powder in 1 mL DMSO (Hybri-MAX) per vial.

5.2.2.3. Aliquot at 5 µL per tube into sterile 1.5 mL polypropylene microcentrifuge tubes and store at -20ºC.

5.2.3. **Ionomycin**:

5.2.3.1. 1 mg vials of Ionomycin powder are purchased from Sigma and stored desiccated at 2-4°C, protected from light.

5.2.3.2. Prepare 1 mg/mL stock solution by dissolving the powder in 1 mL DMSO (Hybri-MAX).

5.2.3.3. Aliquot at 25 µL per tube into sterile 1.5 mL polypropylene microcentrifuge tubes and store at -20ºC.

5.2.4. **Brefeldin A:**

5.2.4.1. 5 mg vials of Brefeldin A powder are purchased from Sigma and stored at 2-8°C.

5.2.4.2. Prepare 5 mg/mL stock solution by dissolving the powder in 1 mL DMSO (Hybri-MAX).

5.2.4.3. Aliquot at 50 µL per tube into sterile 1.5 mL polypropylene microcentrifuge tubes and store at -20ºC.

5.3. Preparation of media:

5.3.1. **Calcium/magnesium-free PBS containing 20% heat inactivated Fetal bovine serum (FBS**)

5.3.1.1. Calculate the volume of media needed for diluting the thawed PBMC. Each PBMC vial will require 10 mL of dilution medium.

5.3.1.2. Prepare the required volume of media by adding 1 part FBS to 4 parts calcium/magnesium-free PBS.

5.3.1.3. For 20 LEAP barcodes (two vials of PBMC per barcode at 5 million PBMC/vial to give a total of 10 million cells) and 1 positive control PBMC aliquots, add 100 mL FBS to

400 mL calcium/magnesium-free PBS. This should be prepared on the day of use and not stored to reduce the risk of contamination.

5.3.2. **Calcium/magnesium-free PBS containing 5% heat inactivated FBS**

5.3.2.1. Add 75 mL of heat inactivated FBS to 1425 mL of calcium/magnesium-free PBS. Store the solution at 4°C.

5.3.3. **Complete culture medium containing autologous serum**:

5.3.3.1. Add 5 mL penicillin/streptomycin solution to a 500 mL bottle of RPMI1640 with L-glutamine and 25 mM HEPES.

5.3.3.2. Store in the fridge at 4ºC for one month after which discard the unused medium.

5.3.3.3.Prepare a container with wet ice to store the autologous plasma samples until ready to prepare the complete culture medium.

5.3.3.4. Filter two 0.25 mL aliquots of autologous plasma from the pediatric PBMC barcodes through an Acrodisc 25 mm syringe filter with 0.2 μm Supor membrane.

5.3.3.5. Store on wet ice till used to prepare complete culture medium by mixing 5.7 mL RPMI with 0.3 mL autologous plasma.

5.3.4. **Calcium/magnesium-free PBS containing 1% heat inactivated FBS and 0.1% NaN_3**

5.3.4.1.Add 5 mL of heat inactivated FBS and 500 mg of NaN_3 to 455 mL of calcium/magnesium-free PBS. Store the solution at 4°C.

5.3.5. **4% paraformaldehyde solution**

5.3.5.1. Add 4 g of paraformaldehyde to 90 mL distilled water and 100 uL 2N NaOH.

5.3.5.2. Heat to 60°C to dissolve.

5.3.5.3. Adjust pH to 7.4 with 1N HCl.

5.3.5.4. Store at 4°C for 1 week or dispense in 5 mL aliquots and store at -20°C for long term storage.

5.4. Peanut allergic PBMC positive control

5.4.1. Include 1 positive control in each experiment. The positive control to be used with this assay is PBMC from a peanut allergic pediatric donor outside of the LEAP study. This positive control serves the purpose of validating the stimulatory potential of the peanut extract and must be run prior to assaying LEAP study barcodes whenever a new batch of peanut extract is prepared (once every three months if stored at -70°C).

5.4.2. If the positive control shows proliferation in response to the peanut extract, then the LEAP study barcodes can be assayed using this batch of peanut extract.

5.4.3. The same positive control used to validate a batch of peanut extract must also be run with every LEAP study barcode run (20 barcodes/week). If no more PBMC aliquots from the same positive control donor are available, then use PBMC from another confirmed peanut allergic pediatric donor.

5.5 PBMC thawing

5.5.1 Prepare a tray with dry ice (at least 1.5 cm deep).

5.5.2 Identify the barcodes of the positive control and twenty LEAP PBMC barcodes that will be placed in culture. Each vial contains 5 million PBMCs, so two vials of each barcode will need to be thawed.

5.5.3 Identify the location of the PBMC cryovials in the boxes from the liquid nitrogen storage tank.

5.5.4 Remove the PBMC vials from the liquid nitrogen storage boxes and keep them on dry ice until ready to thaw.

5.5.5 Prewarm the water bath (GLS Aqua 12 Plus from Grant) to 37ºC.

5.5.6 Inset the cryovials into a foam float so that the base of the cryovial is submerged in the water but the lid is placed above the waterline.

5.5.7 Place the float with the cryovials in the water bath and leave them in until there is only a small granule of frozen PBMC remaining in the cryovials.

5.5.8 Remove the cryovials from the foam float and wipe the water from the walls with lab wipes/tissue paper and spray with 70% ethanol.

5.5.9 Working rapidly, open the cryovials in the Class 2 laminar air flow safety cabinet, mix the contents of the cryovial once with a pipette P1000 (1000 Pipet-Lite SL from Rainin Instrument, Oakland CA or an equivalent Gilson or Eppendorf P1000) and transfer the contents into a sterile 50 mL polypropylene tube .

5.5.10 Dilute the PBMC slowly with calcium/magnesium-free PBS containing 20% heat inactivated FBS by adding the PBS drop-by-drop in order to dilute very slowly the DMSO that was used as cryoprotective agent. **Note:** as a general rule, the first milliliter should be added during the first

minute, the next 2 mL over the next minute, 3 mL over the following minute and 4 mL over the next minute in order to dilute the thawed PBMC 1:10.

5.5.11 Centrifuge for 5 minutes at 300g, at room temperature (20ºC). Discard the supernatant, resuspend and pool the 10 million PBMC from each LEAP barcode in 1 mL PBS containing 5% FBS.

5.5.12 Repeat for all 20 LEAP barcodes and 1 peanut allergic positive control individually or in batches of three barcodes (six PBMC vials).

5.6 CFSE labeling

5.6.1 Prior to labeling the PBMC with CFSE, remove a 10 µL CFSE 5 mM stock solution aliquot from the -20ºC freezer and dilute it by adding 990 µL PBS.

5.6.2 Mix well and transfer 20 µL of this CFSE working solution to the 1 mL PBMC resuspended in PBS. This will result in a 1 µM final concentration for CFSE.

5.6.3 Incubate the tube for 10 minutes at 37ºC.

5.6.4 Dilute the PBMC by adding approximately 49 mL PBS containing 5% heat inactivated FBS to facilitate the removal of unbound CFSE.

5.6.5 Recover the labeled PBMC by centrifugation (10 minutes, 300g) at room temperature.

5.6.6 Resuspend the CFSE-labeled PBMC in 1 mL complete culture medium supplemented with 5% autologous plasma.

5.7 PBMC count and viability assessment

5.7.1 Assess the viability of the CFSE-labeled PBMC from step 5.6.6 using the NucleoCounter™ (ChemoMetec, Allerod, Denmark) as described below.

5.7.2 Collect 10 µL PBMC into a sterile 1.5 mL polypropylene microcentrifuge tube for the total count sample and 10 µL PBMC into a sterile 1.5 mL polypropylene microcentrifuge tube for the viability count sample.

5.7.3 **For the total count sample:**

5.7.3.1 Add 90 µL PBS and 100 µL ChemoMetec Reagent A to the cells and vortex 1 second on medium speed on a Whirlimixer™ (Fisher).

5.7.3.2 Add 100 µL ChemoMetec Reagent B, vortex 1 second, and then collect the sample into a counting ChemoMetec Nucleocassette.

5.7.3.3 Place the Nucleocassette into the Nucleocounter™ and run.

5.7.3.4 Calculate the total number of PBMC taking into consideration the 30x dilution factor.

5.7.4 **For the viability count sample:**

5.7.4.1 Add 190 µL PBS to the cells, vortex 1 second and collect the sample into a counting ChemoMetec Nucleocassette.

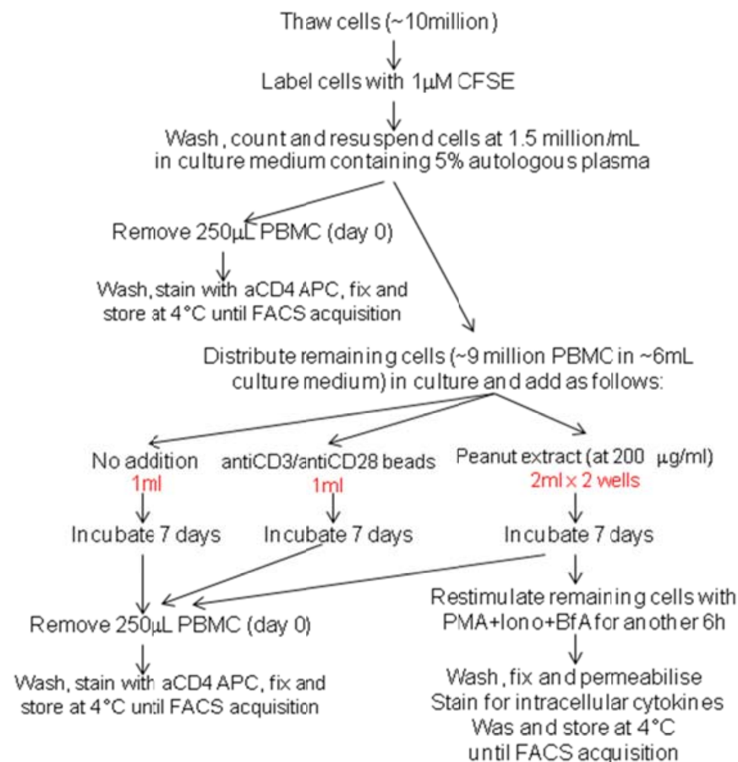
5.7.4.2 Place the microcassette into the cell counter and run.

5.7.4.3 Calculate the number of dead PBMC taking into consideration the 20x dilution factor.

5.7.5 Using the two counts, determine PBMC count and % viability. Record the viability into the experiment datasheet.

5.8 Setting the CFSE labeled PBMC in culture

5.8.1 Based upon the counts, resuspend the PBMC in complete culture medium at 1.5 million cells /mL. Should the 6 mL complete medium prepared as described above (point 5.3.3) be insufficient, prepare additional medium. Then, setup the experiment using the outline below:



5.8.2 Remove 250 μL PBMC for assessing day 0 CFSE labeling.

5.8.3 Transfer the day 0 PBMC to a FACS tube and wash with 4 mL PBS by centrifuging at 300g for 5 minutes.

5.8.4 Resuspend the PBMC in 45 uL PBS and stain with 5 uL of anti-CD4 APC for 1 hour.

5.8.5 After the incubation, wash the PBMC with 4 mL PBS by centrifuging at 300g for 5 minutes, resuspend the pellet in 200 μL PBS + 200 μL paraformaldehyde (4% dissolved in PBS, pH=7.4) and fix the cells at room temperature for 20 minutes.

5.8.6 Add 3 mL PBS containing 1% heat inactivated FBS and 0.1% NaN_3, centrifuge the tubes for 10 minutes at 600g and resuspend the fixed cells in 1 mL PBS containing 1% heat inactivated FBS and 0.1% NaN_3.

5.8.7 Store at 4ºC until ready to be acquired by FACS, together with the 7-day cultured PBMC.

5.8.8 The tubes should be labeled as Barcode.001 (for LEAP PBMC) and PC-DDMONYYYY.001 (for peanut allergic positive control PBMC).

5.8.9 Distribute the remaining PBMC in cultures as follows:

5.8.10 For the peanut-stimulated cultures, distribute 3 million live cells in 2 mL per well in duplicate on a 24-well plate.

5.8.11 Add 20 μL of peanut extract (20 mg/mL) to give a final concentration of 200 μg peanut extract/mL.

5.8.12 For the control cultures, distribute 1.5 million live cells in 1 mL per well in duplicate, on a 48-well plate.

5.8.13 One well is used as a negative (unstimulated) control and nothing is added to it.

5.8.14 To the other positive control well, add anti-CD3/anti-CD28 Dynabeads at 3:1 bead:PBMC ratio. Note: If more PBMC are available, additional peanut-stimulated wells should be set using the same PBMC and peanut concentrations

5.8.15 Fill the outside wells with sterile PBS to prevent evaporation and incubate the plates for 7 days in a dry incubator.

5.8.16 Harvest the LEAP barcodes and anti-CD3/anti-CD28-stimulated positive control run in parallel on day 7.

5.9 PBMC collection, re-stimulation and fixation:

5.9.1 After 7 days, mix the PBMC cultures well and collect 250 μL of the cell suspension from the peanut-stimulated, no antigen and the anti-CD3/anti-CD28-stimulated wells for precursor frequency determination for every LEAP barcode and peanut allergic positive control sample.

5.9.2 Transfer the cells to FACS tubes labeled as follows:

(i) Barcode.002 (LEAP Day 7 without antigen PF)

(ii) Barcode.003 (LEAP Day 7 with peanut antigen PF)

(iii) Barcode.004 (LEAP Day 7 anti-CD3/CD28 beads PF)

(iv) PC-DDMONYYYY.002 (Positive control Day 7 without antigen PF)

(v) PC-DDMONYYYY.003 (Positive control Day 7 with peanut antigen PF)

(vi) PC-DDMONYYYY.004 (Positive control Day 7 anti-CD3/CD28 beads PF)

5.9.3 Wash cells with 4 mL FACS buffer by centrifuging at 300g for 5 minutes.

5.9.4 Resuspend in 45 uL PBS and stain with 5 uL anti-CD4 APC for 1 hour.

5.9.5 Wash the PBMC with 4 mL FACS buffer by centrifuging at 300g for 5 minutes.

5.9.6 Resuspend the pellet in 200 µL PBS + 200 µL 4% paraformaldehyde pH 7.4 and fix the cells for 20 minutes at room temperature.

5.9.7 Add 3 mL PBS containing 1% heat inactivated FBS and 0.1% NaN_3, centrifuge the tubes for 10 minutes at 600g and resuspend the fixed cells in 1 mL PBS containing 1% heat inactivated FBS and 0.1% NaN_3.

5.9.8 Store tubes at 4ºC until acquisition.

5.9.9 After collecting the 250 µL as described above, restimulate the peanut-stimulated cultures with PMA and ionomycin, in the presence of brefeldin A as follows:

(i) PMA (final concentration 50 ng/mL): add 0.5 mL PBS per 5 µL PMA aliquot and add 20 µL of the diluted PMA per well.

(ii) Ionomycin (final concentration 1 ug/mL): add 0.225 mL PBS per 25 µL aliquot then add 20 µL of the diluted ionomycin per well.

(iii) Brefeldin A (final concentration 10 ug/mL): add 450 µL PBS per aliquot and add 20 µL of the diluted solution per well.

Incubate the plates at 37°C for 6 hours.

5.9.10 After the 6 hour incubation, resuspend the cells from the wells by pipetting and then collect in FACS tubes labeled as follows:

(i) Barcode.005 (LEAP Day 7 with peanut antigen IFNγ+IL-4)

(ii) Barcode.006 (LEAP Day 7 with peanut antigen IL-13+TNFα)

(iii) Barcode.007 (LEAP Day 7 with peanut antigen IL-5)

(iv) Barcode.008 (LEAP Day 7 with peanut antigen Isotype control)

(v) PC-DDMONYYYY.005 (Positive control Day 7 with peanut antigen IFNγ+IL-4)

(vi) PC-DDMONYYYY.006 (Positive control Day 7 with peanut antigen IL-13+TNFα)

(vii) PC-DDMONYYYY.007 (Positive control Day 7 with peanut antigen IL-5)

(viii) PC-DDMONYYYY.008 (Positive control Day 7 with peanut antigen Isotype control)

5.9.11 Resuspend cells in 50 µL PBS, add 0.1 mL formaldehyde (Fixative reagent 1 from the IntraPrep Permeabilization) and fix cells for 15 minutes at room temperature.

5.9.12 Add 3 mL PBS containing 1% heat inactivated FBS and 0.1% NaN_3 and centrifuge the tubes for 10 minutes at 600g.

5.9.13 Resuspend cells in 1 mL PBS containing 1% heat inactivated FBS and 0.1% NaN_3.

5.10 PBMC intracellular staining:

5.10.1 Pellet the fixed cells at 600g for 5 minutes and permeabilise with 0.2 mL permeabilisation buffer (Reagent 2 from the IntraPrep Permeabilization kit) for 5 minutes at room temperature.

5.10.2 Distribute 50 µL of the cells in FACS tubes for staining with the different antibody cocktails. Mix the antibodies (5 µL of each one per sample) and add to the cells (final staining volume is 65 µL).

The following combinations of BD antibodies are used:

Tube 1: PE mouse anti-human IFNγ (clone B27) PE-Cy5 mouse anti-human CD3 (clone UCHT1), APC rat anti-human IL-4 (clone MP4-25D2)

Tube 2: PE rat anti-human IL-13 (clone JES10-5A2), PE-Cy5 mouse anti-human CD3 (clone UCHT1), APC mouse anti-human TNF (clone MAb11)

Tube 3: PE rat anti-human IL-5 (clone TRFK5), PE-Cy5 mouse anti-human CD3 (clone UCHT1)

Tube 4: PE rat IgG1 κ isotype control, PE-Cy5 mouse anti-human CD3 (clone UCHT1), APC mouse IgG1 κ isotype control (clone R3-34)

5.10.3 Incubate for 40 minutes at room temperature in the dark.

5.10.4 Wash cells with 4 mL PBS by centrifuging at 600g for 5 minutes, resuspend in FACS buffer (BD FACSFlow) and acquire using a BD FACSCalibur instrument according to ITN SOP BDR-302.

Appendix:

SOP BRD-301 checklist 021213.docx