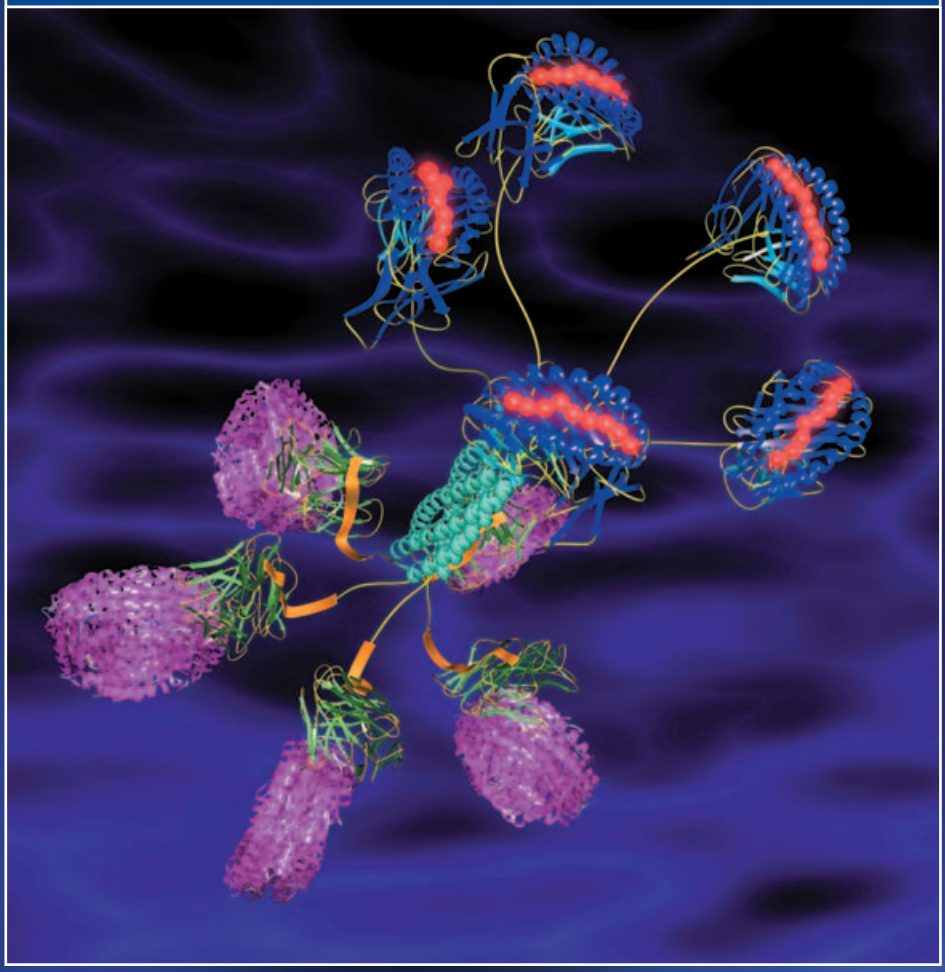
Pro5® MHC Pentamer
Handbook



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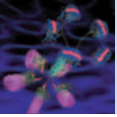
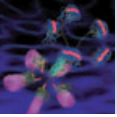
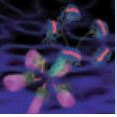


Contents



Section One

Introduction and Assay Principles



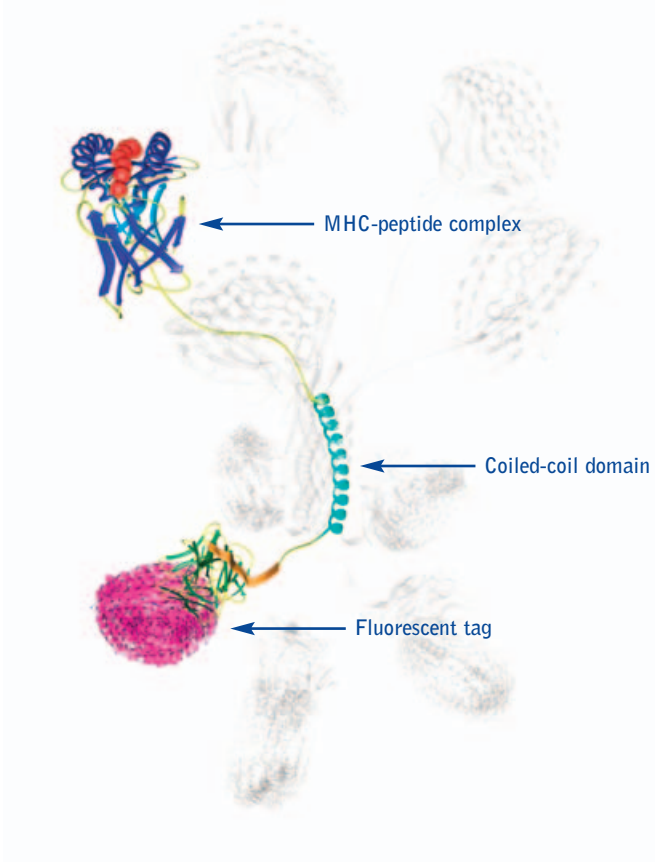
Introduction and Assay Principles

This Pentamer Handbook has been designed to assist all users of ProImmune Pro5® MHC Pentamers and related products. It covers the planning of experiments, preparation of cells, protocols for staining, protocol optimization and troubleshooting. We recommend that even experienced flow cytometry users read this handbook carefully. Follow these guidelines to achieve reliable and consistent results with Pro5® Pentamers and maximize the threshold of detection.

ProImmune has developed Pro5® MHC Class I Pentamers for detecting and enumerating $CD8^+$ single antigen-specific T cells using flow cytometry. Pro5® Pentamers bind to T cell receptors of a particular specificity, as determined by the MHC allele and peptide combination. Pentamers can readily be used to detect and separate antigen-specific T cell populations as rare as 0.02% of lymphocytes. They are also suitable for detailed epitope characterization as well as further immune monitoring. Additional co-staining for intracellular cytokines e.g. IFNγ or IL-2, and/or surface markers e.g. CD69 or CD45RO, can yield functional data for the antigen-specific subset.

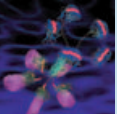
Pro5® Pentamers can also be used in combination with existing technologies such as intracellular cytokine staining and ELISPOT analysis to establish an accurate profile of the functional phenotype of antigen-specific $CD8^+$ T cells. Staining with Pro5® Pentamers provides a quantitative analysis of lymphocytes that express a specific T cell receptor, whereas ELISPOT measures the ability of these cells to produce cytokines. Not all cells that express a particular T cell receptor have an identical ability to produce cytokines. As a consequence the results from these two types of assays will not correspond exactly. Rather they provide complementary information about the profile of an immune response.

Figure 1: Pro5® MHC Class I Pentamer



Pro5® Pentamers comprise five MHC-peptide complexes assembled through a coiled-coil domain. Due to their planar configuration, all five MHC-peptide complexes in the Pentamer are available for binding to complementary T cell receptors (TCRs). Each Pro5® Pentamer also comprises up to five fluorescent or biotin tags for bright and efficient labeling.

Pro5® MHC Pentamers are for research use only, and are not for use in therapeutic or diagnostic procedures.



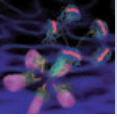
Section Two

Planning Experiments

MHC Alleles

Common Epitope Frequencies

Notes on Planning



MHC Alleles

When planning an experiment it is important to decide which epitopes are to be studied and to know their corresponding Major Histocompatibility Complex (MHC) restriction.

When using a mouse experimental model it is important to know which MHC allele is expressed by the strain of inbred mouse to be used, Table 1.

For example, H-2 Kb MHC Pentamers can only be used with samples from C57BL/6 mice. The T cell receptors of these mice are restricted to H-2 Kb or H-2 Db for class I MHC and therefore will not interact with H-2 Kd Pentamers, for example.

When analyzing human samples it is important to know that the donor is positive for the MHC allele to be investigated. Donor samples therefore either have to be tissue typed or at least serotyped for the allele(s) of interest. Table 2 shows the frequency of the top 30 human class I alleles in four US ethnic groups (White, Black, Hispanic and Asian or Pacific Islander). In certain disease areas and populations the immune response to some epitopes is well characterized, e.g. CMV, EBV. In these cases the published literature will assist experimental planning. The technical support team at ProImmune is happy to offer advice about epitopes and MHC alleles to be used in a given study.

Table 1

MHC alleles expressed by commonly used inbred mouse strains.

			H-2				
			Class I			Class II	
Strain	Appearance	Haplotype	K	D	L	IA	IE
Balb/c	albino	*d*	Kd	Dd	Ld	IAd	IEd
C3H/He	agouti	*k*	Kk	Dk	-	IAk	IEk
C57BL/6	black	*b*	Kb	Db	-	IAb	-
CBA	agouti	*k*	Kk	Dk	-	IAk	IEk

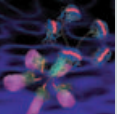
Table 2

Frequency of the top 30 human class I MHC alleles in the North American population

% chance of allele expressed in an individual							
Top 30 expressed alleles							
Allele	White	Allele	Black	Allele	Hispanic	Allele	Asian or Pacific Islander
A*02:01	45.6%	C*04:01	29.0%	A*02:01	37.1%	A*11:01	38.4%
C*07:01	27.7%	C*07:01	25.4%	C*04:01	25.4%	A*24:02	33.7%
A*01:01	27.4%	C*06:02	23.0%	A*24:02	24.9%	C*07:02	33.3%
A*03:01	23.8%	A*02:01	22.3%	C*07:02	24.2%	C*01:02	27.7%
C*07:02	21.5%	A*23:01	20.7%	C*07:01	20.8%	A*33:03	23.3%
C*04:01	21.2%	C*02:02	19.0%	C*03:04	14.4%	C*08:01	21.6%
B*44:02	20.2%	A*03:01	18.7%	A*03:01	14.3%	C*03:04	19.9%
B*07:02	18.1%	C*07:02	18.1%	B*07:02	13.2%	A*02:01	18.1%
B*08:01	18.1%	B*53:01	18.1%	B*35:01	12.8%	B*40:01	15.2%
C*05:01	17.2%	B*07:02	15.8%	C*06:02	12.3%	C*04:01	14.0%
C*03:04	16.8%	C*16:01	15.7%	C*05:01	11.9%	B*58:01	13.3%
C*06:02	15.7%	B*15:03	13.9%	A*01:01	11.4%	B*46:01	12.7%
A*11:01	15.3%	B*58:01	13.5%	A*11:01	11.0%	B*51:01	12.4%
B*40:01	13.6%	A*68:02	12.7%	B*51:01	10.8%	C*03:02	12.0%
A*24:02	12.1%	C*17:01	11.7%	C*16:01	10.6%	B*38:02	11.4%
B*35:01	10.7%	B*45:01	10.8%	B*44:03	9.9%	A*02:07	11.0%
C*03:03	10.6%	B*42:01	10.5%	C*01:02	9.7%	B*15:01	9.4%
B*51:01	10.4%	A*30:01	10.4%	A*29:02	9.7%	A*02:06	9.3%
C*12:03	9.9%	B*35:01	10.1%	C*08:02	9.3%	C*03:03	9.2%
B*15:01	9.6%	A*01:01	10.0%	B*18:01	9.1%	B*15:02	9.1%
A*29:02	8.9%	C*03:04	9.3%	A*31:01	8.9%	A*02:03	8.8%
A*26:01	8.2%	A*30:02	9.2%	B*52:01	8.6%	B*44:03	8.6%
A*32:01	8.2%	B*08:01	8.5%	B*14:02	8.6%	C*14:02	8.4%
C*08:02	7.7%	A*34:02	8.4%	C*02:02	7.6%	B*35:01	7.2%
A*25:01	7.5%	A*74:01	8.4%	C*12:03	7.6%	C*06:02	7.0%
B*57:01	7.1%	A*33:03	8.0%	A*26:01	7.6%	B*54:01	6.9%
B*14:02	6.7%	C*18:01	7.3%	A*68:01	7.1%	B*13:01	6.6%
C*02:02	6.6%	A*29:02	7.2%	B*08:01	7.0%	B*40:02	6.3%
B*18:01	6.4%	B*44:03	6.9%	A*30:02	6.8%	B*55:02	6.3%
B*44:03	6.4%	B*49:01	6.9%	B*44:02	6.5%	A*26:01	6.0%

Data from HLA Matchmaker www.hlamatchmaker.net

Descriptors of ethnic groups are those recommended by the US government.



Common Epitope Frequencies

In certain disease areas and populations, the immune response to some epitopes is well characterized e.g. CMV, EBV. In these cases the published literature will assist experimental planning. The information provided in Table 3 gives an approximate guide to the numbers of healthy donors (expressing the relevant alleles) who would be expected to show a response to these common epitopes that would be detectable by Pentamer staining. The technical support team at ProImmune is happy to offer advice about epitopes and MHC alleles to be used in a given study.

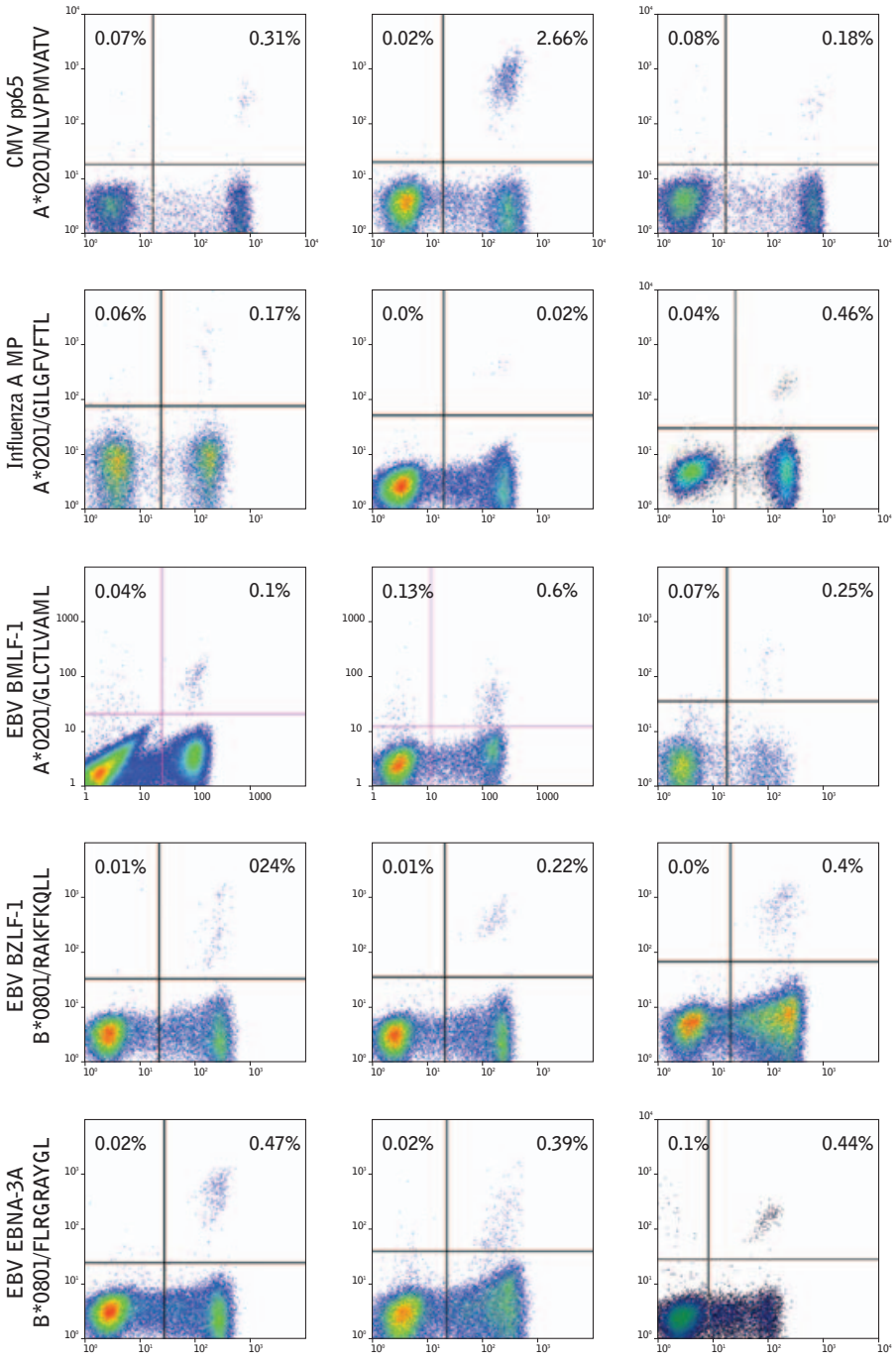
Table 3

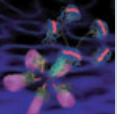
Frequency of healthy donors PBMC giving a detectable Pentamer response

Epitope	Allele	Sequence	Frequency of positive donors
CMV pp65 363-373	A*0201	NLVPMVATV	20%
Influenza A MP 58-66	A*0201	GILGFVFTL	25%
EBV BMLF-1 259-267	A*0201	GLCTLVAML	33%
EBV BZLF-1 190-197	B*0801	RAKFKQLL	50-60%
EBV EBNA-3A 193-201	B*0801	FLRGRAYGL	20-25%

Figure 2 (shown opposite)

Examples of the range of Pentamer positive responses for the commonly expressed epitopes outlined in Table 3. All Pentamer staining was carried out on PBMCs from healthy donors and 3 data plots are shown for each Pentamer specificity.


CMV pp65
A*0201/NLVPMVATV
0.07%
0.31%
0.02%
2.66%
0.08%
0.18%
Influenza A MP
A*0201/GILGFVFTL
0.06%
0.17%
0.0%
0.02%
0.04%
0.46%
EBV BMLF-1
A*0201/GLCTLVAML
0.04%
0.1%
0.13%
0.6%
0.07%
0.25%
EBV BZLF-1
B*0801/RAKFKQLL
0.01%
024%
0.01%
0.22%
0.0%
0.4%
EBV EBNA-3A
B*0801/FLRGRAYGL
0.02%
0.47%
0.02%
0.39%
0.1%
0.44%
CD8



Notes on Planning

The following notes suggest a starting point for planning your experiments.

Protocol worksheet

Prepare a worksheet detailing the staining procedure for each sample. Figure 3 shows an example experimental worksheet.

Experimental controls

The setting up of suitable controls is covered in detail in Section Four.

Equipment

Arrange to have access to the flow cytometer at the appropriate time.

Check the availability of non-standard lab consumables, especially tubes for flow cytometry sample analysis.

Materials

Delivery times for reagents can vary, especially if they are custom manufactured.

Ensure the availability of donors for human blood, frozen samples or the availability of mice for murine samples.

Figure 3

Example experimental worksheet

[Title]

[Date]

Cells: [Description of Cells, e.g. PBMCs from donor #1]

Antibodies & Pentamers:

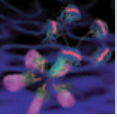
FL1 ctrl: (e.g. anti-CD8 FITC)
FL2 ctrl: (e.g. anti-CD3 R-PE)
FL3 ctrl: (e.g. anti-CD8 Biotin + SA PE Cy5)
FL4 ctrl: (e.g. anti-CD4 APC)

CD8 FITC (1 µl / test) Clone LT8
SA PE Cy5 (optimum µl / test)
Unlabeled Pentamer (2 µl / test)
R-PE-labeled Pentamer (10 µl / test)
APC-labeled Pentamer (10 µl / test)
Biotin-labeled Pentamer (10 µl / test)
Fluorotag (8 µl / test)

Sample#	Cells	Temp.	Sample ID	1st Layer	2nd Layer	Sample#
1	PBMCs donor #1	4°C	unstained	-	-	1
2	PBMCs donor #1	4°C	FL1 ctrl	anti-CD8 FITC	-	2
3	PBMCs donor #1	4°C	FL2 ctrl	anti-CD3 PE	-	3
4	PBMCs donor #1	4°C	FL3 ctrl	anti-CD8 Biotin	SA PE Cy5	4
5	PBMCs donor #1	4°C	FL4 ctrl	anti-CD4 APC	-	5
6	PBMCs donor #1	22°C	Unlabeled Pentamer / R-PE FT	Unlabeled Pentamer	CD8 FITC + R-PE Fluorotag	6
7	PBMCs donor #1	22°C	Unlabeled Pentamer / APC FT	Unlabeled Pentamer	CD8 FITC + APC Fluorotag	7
8	PBMCs donor #1	22°C	R-PE labeled Pentamer	R-PE-labeled Pentamer	CD8 FITC	8
9	PBMCs donor #1	22°C	APC labeled Pentamer	APC-labeled Pentamer	CD8 FITC	9
10	PBMCs donor #1	22°C	Biotin Pentamer + SA PE Cy5	Biotin-labeled Pentamer	CD8 FITC + SA PE Cy5	10

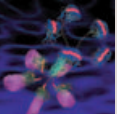
Procedure

1 Prepare cells as appropriate...



Section Three

Cell Preparation Protocols



Cell Preparation Protocols

Care should be taken with preparation of cells to maximize recovery and obtain a sufficient quantity of viable cells for staining. An excessive amount of dead or dying cells will result in poor quality staining and increased background. It is important to take into account the source and storage conditions of the cells when preparing them for flow cytometry and when allocating the number of cells per staining condition.

Red Blood Cell Depletion from Whole Blood

When drawing venous blood use sodium heparin or EDTA as an anti-coagulant. Mix thoroughly and process samples within 24 hours. If storage is necessary prior to processing, store the blood at room temperature (22°C), shielded from light, and on a rocker. DO NOT refrigerate the cells!

Materials and equipment

- Human or murine blood sample
- Universal 30 ml tubes (sterile)
- 50 ml conical tubes (sterile)
- Serological pipettes of appropriate volumes (sterile)
- Ammonium chloride lyzing solution (Appendix I)
- Wash buffer (Appendix I)
- Benchtop centrifuge (NOT refrigerated) with swing-out rotor and appropriate carriers
- Vortex
- Hemocytometer, light microscope

Procedure

Note: this procedure may be scaled up or down according to requirements

1 **Prepare ammonium chloride lyzing solution.**

2 **Take the fresh blood sample and add the appropriate amount of ammonium chloride lyzing solution**

(1 ml per 100 µl blood, or as defined by the manufacturer if using a commercial preparation).

3 **Gently vortex immediately after adding the lyzing solution.**

4 **Incubate at room temperature (22°C) for 15 minutes, shielded from light.**

5 **Centrifuge at 400 x *g* for 5 minutes.**

6 **Carefully aspirate supernatant without disturbing pellet.**

7 **Resuspend cells in 10 ml wash buffer.**

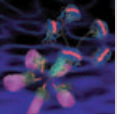
8 **Centrifuge at 400 x *g* for 5 minutes.**

9 **Carefully aspirate supernatant without disturbing pellet.**

10 **Resuspend cells in a final known volume of wash buffer.**

11 **Count live cells using a hemocytometer and light microscope.**

If proceeding straight to staining for flow cytometry, distribute the cells equally between sample tubes.



Density Gradient Centrifugation Separation of Human PBMCs from Whole Blood

Materials and equipment

- Human blood sample
- Disposable Pasteur pipettes (sterile)
- Universal 30 ml tubes (sterile)
- 50 ml conical tubes (sterile)
- Serological pipettes of appropriate volumes (sterile)
- Ficoll-Paque™ PLUS* (GE Healthcare), warmed to room temperature (22°C). *Note: Ficoll-Paque™ PLUS may be substituted by Lymphoprep™ (Axis-Shield), in which case the centrifuge speed in step 4 should be increased to 800 x *g*
- RPMI 1640, warmed to room temperature (22°C)
- Wash buffer (Appendix I)
- Benchtop centrifuge (NOT refrigerated) with swing-out rotor and appropriate carriers
- Hemocytometer, light microscope

Procedure

Note: this procedure may be scaled up or down according to requirements.

1 Take 50 ml fresh blood and split into 2 x 50 ml Falcon tubes. Add 12.5 ml RPMI 1640 to each tube as balanced salt diluent and gently mix by inversion.

2 Add 7.5 ml Ficoll-Paque™ PLUS to each of 4 x 30 ml universal tubes.

3 Carefully layer 18.75 ml diluted blood on top of the Ficoll.

Avoid mixing the two phases.

4 Carefully balance the weight of the tubes then centrifuge at 400 x *g* for 30 minutes at room temperature (22°C).

Accelerate the centrifuge slowly so the gradients do not mix, and ensure the brake is off so that deceleration does not disrupt the density gradient.

5 Immediately remove and discard the top plasma layer using a plastic disposable pipette.

6 Draw off the lymphocyte layer at the Ficoll interface using a serological pipette.

Take care to minimize drawing of Ficoll.

Transfer this fraction to fresh 30 ml tubes (one tube per original tube) and top up tube with RPMI 1640.

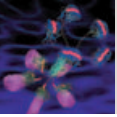
7 Centrifuge at 330 x *g* for 10 minutes (with the brake of the centrifuge on) to wash out the Ficoll.

8 Aspirate the supernatant and resuspend each cell pellet by tapping the tube until no clumps are visible. Vortex gently if necessary. Add room temperature RPMI 1640 then transfer with washings into a single tube.

9 Centrifuge at 330 x *g* for 10 minutes, aspirate supernatant, then resuspend cells in a final known volume of desired medium (e.g. wash buffer or RPMI 1640).

10 Count live cells using a hemocytometer and light microscope.

The cell count should be about 1 million PBMCs for each ml of blood drawn, though this number will vary between individuals. If proceeding straight to staining for flow cytometry, distribute the cells equally between sample tubes.



Isolation of Splenocytes from Murine Spleens

Materials and equipment

- Murine spleen
- Disposable Pasteur pipettes
- Polystyrene Petri dish
- Clean scalpel (optional)
- Cell strainer (70 μm Nylon, e.g. Falcon® #2350)
- 2 ml syringe
- Universal 30 ml tubes (sterile)
- 50 ml conical tubes (sterile)
- Serological pipettes of appropriate volumes (sterile)
- Ammonium chloride lyzing solution (Appendix I)
- Phosphate buffered saline (PBS)
- Wash buffer, cooled to 4°C (Appendix I)
- Benchtop centrifuge (NOT refrigerated) with swing-out rotor and appropriate carriers
- Hemocytometer, light microscope

Procedure

1 **Prepare ammonium chloride lyzing solution.**

Leave at room temperature (22°C).

2 **Place a cell strainer in the Petri dish, to serve as a small bowl in which to mash the spleen. Transfer the spleen and 1 ml of PBS directly into the cell strainer.**

If desired, score the outer membrane of the spleen with a clean scalpel before mashing it, but take care to avoid cutting through the strainer mesh.

3 **Remove the plunger from a 2 ml syringe and use the black rubber end to mash the spleen and release the splenocytes into the Petri dish.**

Use grinding circular movements to homogenize the tissue. Periodically, draw up liquid from outside the strainer with a disposable pipette, and wash out the cells from within the strainer. Continue to mash the spleen until all that remains is the white connective tissue of the outer membrane.

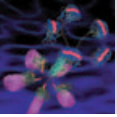
4 **Transfer the homogenized cell suspension into a universal tube. Wash out the Petri dish a few times with fresh PBS to maximize recovery of splenocytes. Make up to the full volume of the tube with PBS.**

5 **Centrifuge cells for 10 minutes at 400 x *g* at room temperature (22°C) and aspirate the supernatant.**

The resulting cell pellet should be red in color. The supernatant may appear cloudy, but this is likely to be due to smaller particles that cannot be centrifuged at this setting.

6 **Resuspend the cell pellet in 2 ml PBS per spleen. Add the appropriate amount of ammonium chloride lyzing solution (1 ml per 100 µl blood, or as defined by the manufacturer if using a commercial preparation) and leave for 15 minutes in the dark at room temperature (22°C).**

Ensure that the cells are fully resuspended before adding the lysis solution in order to avoid excessive clumping.



7 **Centrifuge cells for 5 minutes at 400 x *g* and aspirate the supernatant.**

Take care not to lose cells as the pellet will be loose. The cell pellet should be buff colored with minimal red cell contamination.

8 **Resuspend the cells completely and wash again with a full volume of PBS. Centrifuge for 5 minutes at 400 x *g*, aspirate the supernatant, to then resuspend cells in a final known volume of wash buffer.**

9 **Count live cells using a hemocytometer and light microscope.**

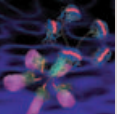
If proceeding straight to staining for flow cytometry distribute the cells equally between sample tubes. Use cells the same day.

Cryopreservation of PBMCs or Ammonium Chloride-lyzed Blood

All reagents should be at room temperature or preferably at 37°C, to maintain the metabolic activity and membrane lipid fluidity of the cells.

Materials and equipment

- Cells from human blood sample (PBMCs or ammonium chloride-lyzed blood)
- Tissue culture grade Dimethyl Sulphoxide (DMSO) (e.g. Sigma #D2650)
- Fetal Calf Serum (FCS)
- Cryogenic vials, 1.8 ml with internal threading (sterile)
- 50 ml conical tubes (sterile)
- Serological pipettes of appropriate volumes (sterile)
- Hemocytometer
- Benchtop centrifuge (NOT refrigerated) with swing-out rotor and appropriate carriers
- Nalgene freezing container (Nalgene #5100 0001), filled with 2-Propanol
- -80°C Freezer
- Liquid nitrogen storage tank, with holders for 1.8 ml cryogenic vials



Preparation

Prepare a Freezing Solution of 10% DMSO in FCS; warm to 37°C.

Label the appropriate number of 1.8 ml cryotubes per sample based on the anticipated cell count (1 - 2 million cells per ml blood drawn). Each tube should contain approximately 5 - 15 million cells. Freezing more cells per tube may lead to cell loss.

Procedure

1 **Centrifuge cells at 400 x *g* for 10 minutes.**

2 **Aspirate supernatant from cell pellet and resuspend the cells by tapping the tube until no clumps are visible. Add the Freezing Solution (warmed to 37°C) to give a cell concentration of 2 x 10^7 per ml.**

3 **Mix the cells gently by tapping the tube without using a pipette.**

Avoid foam formation.

4 **Slowly, over a time period of approximately 2 minutes, add a second, equal volume of warm Freezing Solution to the tube containing the PBMCs while gently swirling the tube to permit complete mixing.**

5 **Aliquot the PBMC suspension into the pre-labeled cryovials; 1 ml into each 1.8 ml vial.**

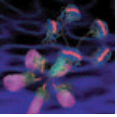
Pipette gently and slowly to minimize shear forces.

6 **Place cryovials into a room temperature Nalgene freezing container filled with 2-propanol.**

7 **Transfer the freezing container to a -80ºC freezer for a minimum of 12 hours.**

Do not open the freezer during this time period. During the cooling process for cryopreservation ice forms first around the cell between -5ºC and -15ºC, leaving the cell super cooled but unfrozen. At this point an osmotic imbalance occurs across the cell membrane, leading to water flux out of the cell. To preserve the cell it must dehydrate slowly at a rate critical to cell survival. Fast cooling leads to intracellular ice formation, which ruptures the cell membrane. Cooling the cells too slowly can lead to electrolyte imbalances or latent heat creation.

8 **After a minimum of 12 hours and maximum of 48 hours, transfer the cryovials into liquid nitrogen tanks for indefinite storage.**



Thawing Cryopreserved Cells

Materials and equipment

- Frozen cell sample
- Benzonase Nuclease (Sigma E1014)
- 50 ml conical tubes (sterile)
- Serological pipettes of appropriate volumes (sterile)
- R10 medium, warmed to 37°C (Appendix I)
- Warm air blower, 37°C glass bead bath, or 37°C water bath
- Benchtop centrifuge (NOT refrigerated), with swing-out rotor and appropriate carriers
- Hemocytometer, light microscope

Procedure

1 Raise the temperature in the cryovial rapidly to between 25°C and 37°C.

If using a 37°C water bath take care to avoid contamination of the cells.

2 Top up the cryovial with warm R10 medium containing 1 U/ml Benzonase Nuclease.

3 Transfer the cells from the cryovial into a 50 ml conical tube.

At this point, the contents of up to 3 cryovials from the same sample can be pooled.

4 Very slowly, over 1 to 2 minutes, add R10 medium containing 1 U/ml Benzonase Nuclease (warmed to 37°C), diluting the total to 5x the original volume (e.g. add 4 ml buffer to 1 ml thawed cells). Subsequently, add somewhat faster (over approximately 30 seconds) an equal amount of warm R10 medium containing 1 U/ml Benzonase Nuclease (e.g. an additional 4 ml).

The gradual dilution of DMSO avoids osmotic shock and the warm temperature ensures that the cells can actively compensate the osmotic pressure.

The use of Benzonase Nuclease prevents cells clumping during the thawing process.

5 Centrifuge cells at 330 x *g* for 10 minutes with rapid acceleration and brake on.

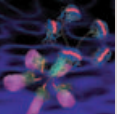
6 Aspirate supernatant and resuspend the cell pellet by tapping (avoid shear forces) and add 10 ml warm R10 medium (plus Benzonase Nuclease if desired).

7 Centrifuge cells at 330 x *g* for 10 minutes with rapid acceleration and brake on.

8 Aspirate supernatant, then resuspend cells in desired medium (e.g. R10 medium) to a final known volume.

9 Count live cells using a hemocytometer and light microscope if desired.

10 Proceed to allocation of cells for staining for flow cytometry or bead sorting.



Allocation of Cells Prior to Staining

The number of cells required per staining condition will depend on the frequency of antigen-specific T cells expected. If starting with a population that is likely to have a high proportion of dead and dying cells, the number of starting cells allocated should be increased to ensure that a sufficient number of live cells can be gated. See Section Five: Procedure for data acquisition.

Fresh PBMCs

These are often obtained by density gradient centrifugation (protocol page 16) or leukapheresis. Allocate 1-2 x 10^6 lymphoid cells per staining condition.

Previously frozen PBMCs

Allocate 1-2 x 10^6 lymphoid cells per staining condition (protocol for thawing cells, page 24).

Whole blood

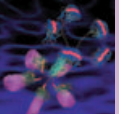
Obtain a whole blood sample treated with anti-coagulant such as sodium heparin. Allocate 1 ml of whole blood per staining condition (1 x 10^6 cells). Lyze red blood cells with ammonium chloride lyzing solution (protocol page 14).

T cell line

Allocate 2-5 x 10^5 cells per staining condition. Fewer cells are required due to the high frequency of antigen-specific T cells.

Section Four

How to Stain Cells

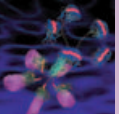


Introduction

Labeling cells for flow cytometry involves using reagents that are specific to cellular markers, such as fluorescently labeled antibodies for detecting phenotypic cell surface molecules, and MHC Pentamers for detecting single antigen-specific T cell receptors. The markers are typically cell surface proteins, although it is also possible to label intracellular proteins by first permeabilizing cells.

Many antibodies used in flow cytometry are directly conjugated to a fluorochrome. However, unlabeled and biotinylated primary antibodies can also be used in combination with a fluorescent-labeled secondary antibody. A single cell sample can be simultaneously analyzed for multiple markers using antibodies conjugated to different fluorescent labels. Ongoing advances in fluorochrome chemistry and cytometry instrumentation provide many flexible options for multi-parameter cell labeling and analysis.

During preparation cells should be at room temperature (22°C) to maintain the metabolic activity and membrane lipid fluidity of the cells. Subsequently, unfixed cells should be kept cold, on ice (4°C) during staining and analysis. This will help to ensure that cell surface markers are not internalized following the interaction of the binding reagent (antibody or Pentamer) with the cell surface receptor. The cells must be in single cell suspension for the antibody labeling to work successfully.



Setting Up Appropriate Controls

Instrument controls

1 Unstained sample: a sample of unstained cells is required to enable correct set up of the flow cytometer forward scatter and side scatter voltage settings (Section Five).

2 Single color staining: set up a single color stain of cells for each fluorochrome channel of the flow cytometer to be used in the experiment. This is to enable the correct compensation settings to be made (Section Five). For example, for two-color staining with FITC and R-PE use one set of cells stained with only anti-CD8 FITC for FL1 compensation and a second set of cells stained with only anti-CD3 R-PE for FL2 compensation. Any antibody may be used that stains a distinct population in the cell sample, but it must also leave a negative unstained population. Anti-CD8 and anti-CD3 are particularly suitable for cytotoxic T cell staining. Refer to Appendix III for information about which fluorescent labels may be used alongside each other.

3 Multicolor staining: a sample stained with two colors may be prepared using the two antibodies that were used in the single color staining; this sample may be used to check that the compensation settings are correct.

Other controls

Positive control

Pro5® Pentamers should be tested against a specific T cell line or clone. Use T cells that have not been stimulated recently as this has been shown to cause down-regulation of T cell receptors. Cells should not be used for a minimum of 3-4 days after stimulation. If possible wait 10 days after stimulation for best results. If a T cell line is not available it is possible to use PBMCs from a known positive donor. In this situation the frequency of positive cells will be much lower and more cells will be required per stain (at least 1 x 10^6). Ideally, collect functional data using a technique such as ELISPOT, to indicate the frequency of positive cells that should be expected.

Negative control

Cells obtained from an unexposed (seronegative) individual may be used. To control for non-specific staining it is also advisable to stain the cell sample with either the A*0201 Human Negative Control Pro5® Pentamer, or a mismatched Pro5® Pentamer (irrelevant MHC allele and/or irrelevant peptide).

ProImmune offers a negative control Pentamer (peptide code N01), which consists of a multimeric HLA-peptide complex assembled with an irrelevant peptide antigen; it is unable to bind T cell receptors on $CD8^+$ cells. Any staining achieved by using the negative control Pentamer is genuinely non-specific, so this reagent is ideal for use when studying a low frequency of antigen-specific T cells.

Markers for cells to be excluded

In order to exclude certain cells from the analysis, cell markers may be stained and 'gated out' during the acquisition or analysis of results. For example, the exclusion of B cells and/or NK cells is likely to reduce most of the non-specific background (see next page for an example of murine cell staining). These markers could all be conjugated with the same fluorochromes in order to maximize the number of other channels available for analysis of the desired cell markers.

Table 4

Markers to exclude unwanted cell types from Pentamer analysis

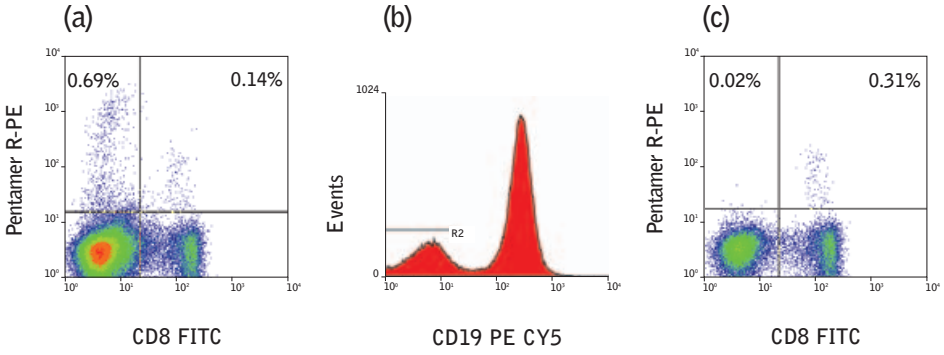
Antibody	Cell Marker	Species
CD19	B cells	Mouse / Human
CD49b	NK cells	Mouse
CD56	NK cells	Human
CD4	Helper T cells	Mouse / Human
CD11c	Macrophages	Mouse / Human
CD13	Monocytes / Neutrophils	Mouse / Human

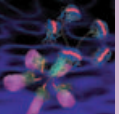
Improvement of Data by Exclusion

It may be helpful to exclude certain cell types from the analysis. For example, B cells (CD19-positive), may contribute to non-specific staining. If there is more than one set of cells to exclude they may all be stained with antibodies labeled with the same color. They can then be eliminated from analysis by setting a single 'unwanted cell' gate.

Figure 4

The figure shows the effect of gating out B cells from C57BL/6 splenocytes that were previously immunized against a model ovalbumin antigen. 1 x 10^6 splenocytes were stained with 1 test (10 µl) R-PE labeled Pentamer, followed by 1 test anti-CD8 FITC (clone KT15) plus anti-CD19 PECy5 (clone 6D5) using the standard protocol for labeled Pentamer staining. (a) shows the live splenocyte population in which there is considerable non-specific staining (upper left quadrant) making it difficult to verify that the specific staining (upper right quadrant) is truly antigen-specific. (b) R2 is set upon the CD19-negative cells. (c) The density plot is gated upon live cells and R2 to exclude B cells. The non-specific staining is removed, illustrating that in this case it was attributable to B cells, and the Pentamer-positive staining can now be clearly identified.





Fluorescent-labeled Pentamer Cellular Staining Protocol

Materials and equipment

- Cell sample, eg. blood sample (RBC depleted), PBMCs or T cell line
- Pro5® MHC Class I Pentamer labeled with R-PE or APC, specific for cells of interest
- Fluorescent labeled anti-CD8 antibody
- Additional fluorescent labeled antibodies, as required
- Plastic disposable Pasteur pipettes
- Universal 30 ml tubes
- 50 ml conical tubes
- Serological pipettes of appropriate volume
- Wash buffer, chilled to 4°C (Appendix I)
- Fix buffer, chilled to 4°C (Appendix I)
- 12 x 75 mm polystyrene tubes for flow cytometry (e.g. BD Biosciences #352052)
- Benchtop refrigerated centrifuge with swing-out rotor and appropriate tube carriers
- Microcentrifuge
- Vortex

Procedure for washing cells

Dispense 1 ml wash buffer into each sample tube and spin at 400 x *g* for 5 minutes in a chilled centrifuge at 4°C. Check for presence of a cell pellet before discarding the supernatant. Resuspend cell pellets in residual liquid (~50 µl).

Figure 5: Labeled Pentamer staining protocol

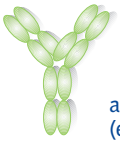
Figure 5

+

1 x 10^6 cells in
50 µl per tube

1 test labeled Pro5®
MHC Pentamer
(e.g. R-PE)

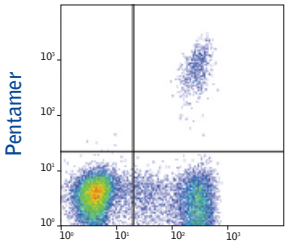
10 minutes room
temperature
then wash



anti-CD8 mAb
(e.g. FITC labeled)

20 minutes on ice

2 washes



CD8 Analyze by flow cytometry

Procedure

1 Centrifuge Pro5® Pentamer in a chilled microcentrifuge at 14,000 x *g* for 3 minutes.

This will help to remove protein aggregates from the solution that may contribute to non-specific staining. Maintain reagents on ice (4°C), shielded from light, until required. Ensure that you do not aspirate any part of the pelleted aggregates when taking test volumes for staining.

2 Allocate the correct number of cells (page 26) into individual sample tubes for each staining condition. Wash the cells with ice-cold wash buffer and resuspend in the residual volume (~50 µl).

Keep tubes chilled on ice for all subsequent steps except where otherwise indicated.

3 Add one test (10 µl) of labeled Pentamer to the cells and mix by pipetting up and down.

4 Incubate the Pentamer-stained samples at room temperature (22°C) for 10 minutes, shielded from light.

5 Wash cells with 2 ml wash buffer per tube and resuspend in residual liquid (~50 µl).

6 Add an optimal amount of each secondary antibody and mix by pipetting up and down.

e.g. anti-CD8 and other co-staining antibodies such as anti-CD3.

Control samples should also be stained at this stage.

7 **Incubate samples on ice (4ºC) for 20 minutes, shielded from light.**

8 **Wash cells twice with 2 ml wash buffer per tube. Resuspend cells thoroughly after each wash.**

9 **Add 200 µl of fix buffer. Vortex samples thoroughly.**

It is important to vortex well when adding fixative so that cells do not clump. Store tubes in the dark in the refrigerator until ready for data acquisition. The cells may sediment at this stage and must be vortexed again before analysis. The morphology of the cell changes after fixing, so it is advisable to leave the samples for three to four hours before proceeding with data acquisition. Samples can be stored in the dark in a refrigerator for up to 2 days. Cells MUST NOT BE FIXED and must be used immediately if Fluorescent Activated Cell Sorting (FACS) is to be performed.

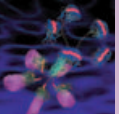
Notes

1 In order to optimize the protocol it is advisable to vary incubation time and temperature for the Pentamer. Optimal conditions can be antigen dependent. A ten minute incubation at room temperature commonly works best. See Protocol Optimization - Changing temperature and time (page 46).

2 A single test quantity of labeled Pentamer is equivalent to 10 µl of reagent. Ideally, the amount of Pentamer added should be titrated in order to find the optimum working dilution to use for each individual Pentamer and cell type. See Protocol Optimization - Titrating the amount of Pentamer (page 48).

3 Ideally, the amount of antibody added should be titrated in order to find the optimum working dilution. See Appendix II: Optimal titration of antibodies.

4 Some anti-CD8 antibodies can cause blocking of the MHC-T cell receptor interaction. Clones LT8 for human cells and KT15 for murine cells have been tested by ProImmune and shown not to interfere with the interaction. Both of these antibody clones are available from ProImmune. See Section Eight: Troubleshooting Guide for more about selecting the correct anti-CD8 antibody.



Unlabeled Pentamer Cellular Staining Protocol

Materials and equipment

- Cell sample, eg. blood sample (RBC depleted), PBMCs or T cell line
- Pro5® MHC Class I Pentamer, specific for cells of interest
- Pro5® Fluorotag (R-PE or APC labeled)
- Fluorescent labeled anti-CD8 antibody
- Additional fluorescent labeled antibodies, as required
- Plastic disposable Pasteur pipettes
- Universal 30 ml tubes
- 50 ml conical tubes
- Serological pipettes of appropriate volume
- Wash buffer, chilled to 4°C (Appendix I)
- Fix buffer, chilled to 4°C (Appendix I)
- 12 x 75 mm polystyrene tubes for flow cytometry (e.g. BD Biosciences #352052)
- Benchtop refrigerated centrifuge with swing-out rotor and appropriate tube carriers
- Microcentrifuge
- Vortex

Procedure for washing cells

Dispense 1 ml wash buffer into each sample tube and spin at 400 x *g* for 5 minutes in a chilled centrifuge at 4°C. Check for presence of a cell pellet before discarding the supernatant. Resuspend cell pellets in residual liquid (~50 µl).

Figure 6: Unlabeled Pentamer cellular staining protocol

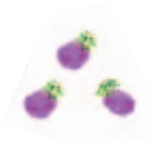
Figure 6

1 x 10^6 cells in
50 µl per tube

+

1 test unlabeled
Pro5® MHC Pentamer

10 minutes room
temperature
then wash



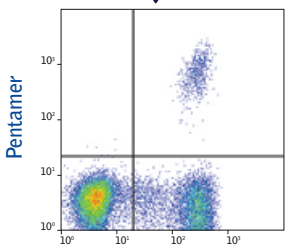
Pro5® Fluorotag
(e.g. R-PE)

+

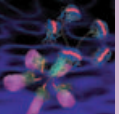
anti-CD8 mAb
(e.g. FITC labeled)

20 minutes on ice

2 washes



CD8 Analyze by flow cytometry



Procedure

1 **Centrifuge Pro5® Pentamer and Pro5® Fluorotag reagents in a chilled microcentrifuge, at 14,000 x *g* for 3 minutes.**

This will help to remove protein aggregates from the solution that may contribute to non-specific staining. Maintain reagents on ice (4°C), shielded from light, until required. Ensure that you do not aspirate any part of the pelleted aggregates when taking test volumes for staining.

2 **Allocate the correct number of cells (page 26) into individual sample tubes for each staining condition. Wash the cells with ice-cold wash buffer and resuspend in the residual volume (~50 µl).**

Keep tubes chilled on ice for all subsequent steps except where otherwise indicated.

3 **Add one test (2 µl) of unlabeled Pentamer to the cells and mix by pipetting up and down.**

4 **Incubate the Pentamer-stained samples at room temperature (22°C) for 10 minutes.**

5 **Wash cells with 2 ml wash buffer per tube and resuspend in residual liquid (~ 50 µl).**

6 **Add 8 µl of Fluorotag and an optimal amount of each secondary antibody and mix by pipetting up and down.**

e.g. anti-CD8 and other co-staining antibodies such as anti-CD3.

Control samples should also be stained at this stage.

7 **Incubate samples on ice (4°C) for 20 minutes, shielded from light.**

8 **Wash cells twice with 2 ml wash buffer per tube. Resuspend cells thoroughly after each wash.**

9 **Add 200 µl of fix buffer. Vortex samples thoroughly.**

It is important to vortex well when adding fixative so that cells do not clump. Store tubes in the dark in the refrigerator until ready for data acquisition. The cells may sediment at this stage and must be vortexed again before analysis. The morphology of the cell changes after fixing, so it is advisable to leave the samples for three to four hours before proceeding with data acquisition. Samples can be stored in the dark in a refrigerator for up to 2 days. Cells MUST NOT BE FIXED and must be used immediately if Fluorescent Activated Cell Sorting (FACS) is to be performed.

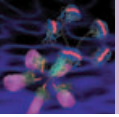
Notes

1 In order to optimize the protocol it is advisable to vary incubation time and temperature for the Pentamer. Optimal conditions can be antigen dependent. A ten minute incubation at room temperature commonly works best. See Protocol Optimization - Changing temperature and time (page 46).

2 A single test quantity of unlabeled Pentamer is equivalent to 2 µl of reagent. Ideally, the amount of Pentamer added should be titrated in order to find the optimum working dilution to use for each individual Pentamer and cell type. See Protocol Optimization - Titrating the amount of Pentamer (page 48).

3 Ideally, the amount of antibody added should be titrated in order to find the optimum working dilution. See Appendix II: Optimal titration of antibodies.

4 Some anti-CD8 antibodies can cause blocking of the MHC-T cell receptor interaction. Clones LT8 for human cells and KT15 for murine cells have been tested by ProImmune and shown not to interfere with the interaction. Both of these antibody clones are available from ProImmune. See Section Eight: Troubleshooting Guide for more about selecting the correct anti-CD8 antibody.



Biotin-labeled Pentamer Cellular Staining Protocol

Materials and equipment

- Cell sample, eg. blood sample (RBC depleted), PBMCs or T cell line
- Pro5® MHC Class I Pentamer, specific for cells of interest
- Fluorescent-labeled streptavidin conjugate
- Fluorescent-labeled anti-CD8 antibody (different fluorescence to the streptavidin conjugate)
- Additional fluorescent labeled antibodies, as required
- Plastic disposable Pasteur pipettes
- Universal 30 ml tubes
- 50 ml conical tubes
- Serological pipettes of appropriate volume
- Wash buffer, chilled to 4°C (Appendix I)
- Fix buffer, chilled to 4°C (Appendix I)
- 12 x 75 mm polystyrene tubes for flow cytometry (e.g. BD Biosciences #352052)
- Benchtop refrigerated centrifuge with swing-out rotor and appropriate tube carriers
- Microcentrifuge
- Vortex

Procedure for washing cells

Dispense 1 ml wash buffer into each sample tube and spin at 400 x *g* for 5 minutes in a chilled centrifuge at 4°C. Check for presence of a cell pellet before discarding the supernatant. Resuspend cell pellets in residual liquid (~50 µl).

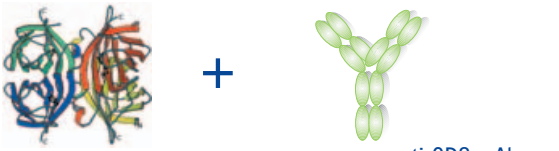
Figure 7: Biotin-labeled Pentamer cellular staining protocol

Figure 7

1 x 10^6 cells in
50 µl per tube

1 test biotin-labeled
Pro5® MHC Pentamer

10 minutes room
temperature
then wash

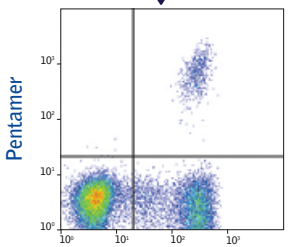


Fluorescent -
labeled
Streptavidin
(e.g. R-PE)

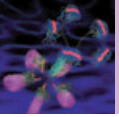
anti-CD8 mAb
(e.g. FITC labeled)

20 minutes on ice

2 washes



CD8 Analyze by flow cytometry



Procedure

1 **Centrifuge Pro5® Pentamer in a chilled microcentrifuge, at 14,000 x *g* for 3 minutes.**

This will help to remove protein aggregates from the solution that may contribute to non-specific staining. Maintain reagents on ice (4°C) until required. Ensure that you do not aspirate any part of the pelleted aggregates when taking test volumes for staining.

2 **Allocate the correct number of cells (page 26) into individual sample tubes for each staining condition. Wash the cells with ice-cold wash buffer and resuspend in the residual volume (~50 µl).**

Keep tubes chilled on ice for all subsequent steps except where otherwise indicated.

3 **Add one test (10 µl) of biotin-labeled Pentamer to the cells and mix by pipetting up and down.**

4 **Incubate the Pentamer-stained samples at room temperature (22°C) for 10 minutes.**

5 **Wash cells with 2 ml wash buffer per tube and resuspend in residual liquid (~50 µl).**

6 **Add an optimal amount of fluorescent-labeled streptavidin and each secondary antibody and mix by pipetting up and down.**

e.g. anti-CD8 and other co-staining antibodies such as CD3.

Control samples should also be stained at this stage.

7 **Incubate samples on ice (4°C) for 20 minutes, shielded from light.**

8 **Wash cells twice with 2 ml wash buffer per tube. Resuspend cells thoroughly after each wash.**

9 **Add 200 µl of fix buffer. Vortex samples thoroughly.**

It is important to vortex well when adding fixative so that cells do not clump. Store tubes in the dark in the refrigerator until ready for data acquisition. The cells may sediment at this stage and must be vortexed again before analysis. The morphology of the cell changes after fixing, so it is advisable to leave the samples for three to four hours before proceeding with data acquisition. Samples can be stored in the dark in a refrigerator for up to 2 days. Cells MUST NOT BE FIXED and must be used immediately if Fluorescent Activated Cell Sorting (FACS) is to be performed.

Notes

1 In order to optimize the protocol it is advisable to vary incubation time and temperature for the Pentamer. Optimal conditions can be antigen dependent. A ten minute incubation at room temperature commonly works best. See Protocol Optimization - Changing temperature and time (page 46).

2 A single test quantity of biotin-labeled Pentamer is equivalent to 10 µl of reagent. Ideally, the amount of Pentamer added should be titrated in order to find the optimum working dilution to use for each individual Pentamer and cell type. See Protocol Optimization - Titrating the amount of Pentamer (page 48).

3 Ideally, the amount of antibody added should be titrated in order to find the optimum working dilution. See Appendix II: Optimal titration of antibodies.

4 Some anti-CD8 antibodies can cause blocking of the MHC-T cell receptor interaction. Clones LT8 for human cells and KT15 for murine cells have been tested by ProImmune and shown not to interfere with the interaction. Both of these antibody clones are available from ProImmune. See Section Eight: Troubleshooting Guide for more about selecting the correct anti-CD8 antibody.

Protocol Optimization

Changing Temperature and Time

The incubation temperature and time are variable conditions where the protocols for Pro5® MHC Pentamers and conventional MHC tetramers differ. It is recommended to set up staining conditions for a number of different temperatures and times. Pentamer (as well as tetramer) complexes have different optimal staining parameters, dependent on the peptide affinity and MHC-allele used.

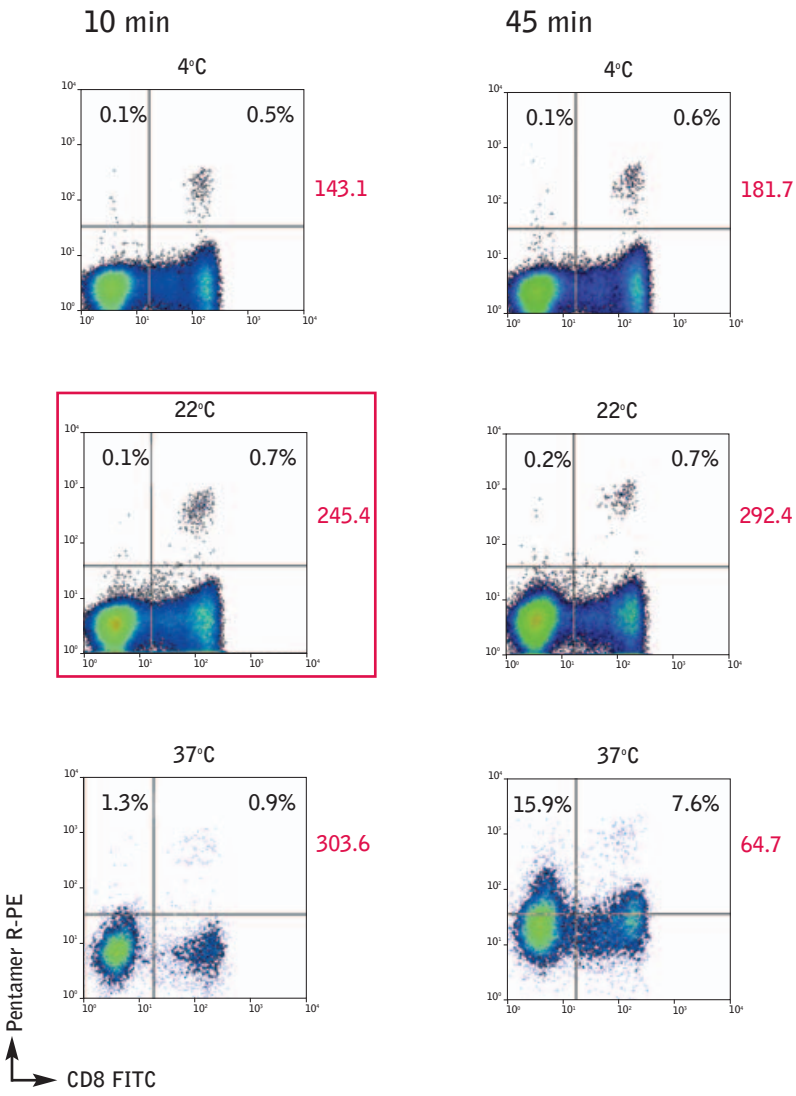
Staining at room temperature (22°C) for 10 minutes is recommended in the first instance. Incubation at 4°C for 40 minutes or at 37°C for 5 to 10 minutes may be tested in order to optimize the signal to noise ratio. The higher the incubation temperature, the shorter the incubation time required.

Figure 8

Anti-CD8 staining is shown on the x-axis; Pentamer staining is shown on the y-axis. The mean fluorescence intensity is shown in red.

The density plot in the red box (temperature 22°C) shows a higher mean fluorescence intensity compared with the first plot at 4°C. When the temperature is increased to 37°C a higher level of non-specific staining is seen (1.3%) and base line negative staining is raised. These events interfere with the positive events under investigation and affect the data significantly. Although the percentage of CD8-positive and Pentamer-positive cells has increased (0.9%) some of these events are likely to be Pentamer-negative. Fewer cells can be visualized due to clumping that occurs at the higher temperature. Similar characteristics are seen in the plots when a longer staining time (45 minutes) is tried. The conclusion from this experiment is that staining conditions of 10 minutes at 22°C should be used for this particular Pentamer and cell sample.

Figure 8


10 min
45 min
4°C
0.1%
0.5%
143.1
4°C
0.1%
0.6%
181.7
22°C
0.1%
0.7%
245.4
22°C
0.2%
0.7%
292.4
37°C
1.3%
0.9%
303.6
37°C
15.9%
7.6%
64.7
Pentamer R-PE
CD8 FITC

Titrating the Amount of Pentamer

The amount of Pentamer added to each staining condition should be titrated in order to find the optimum working dilution to use for each individual Pentamer and cell type. A suggested titration range would be doubling dilutions from 1 test quantity down to $^{1}/_{16}$ test quantities.

Figure 9

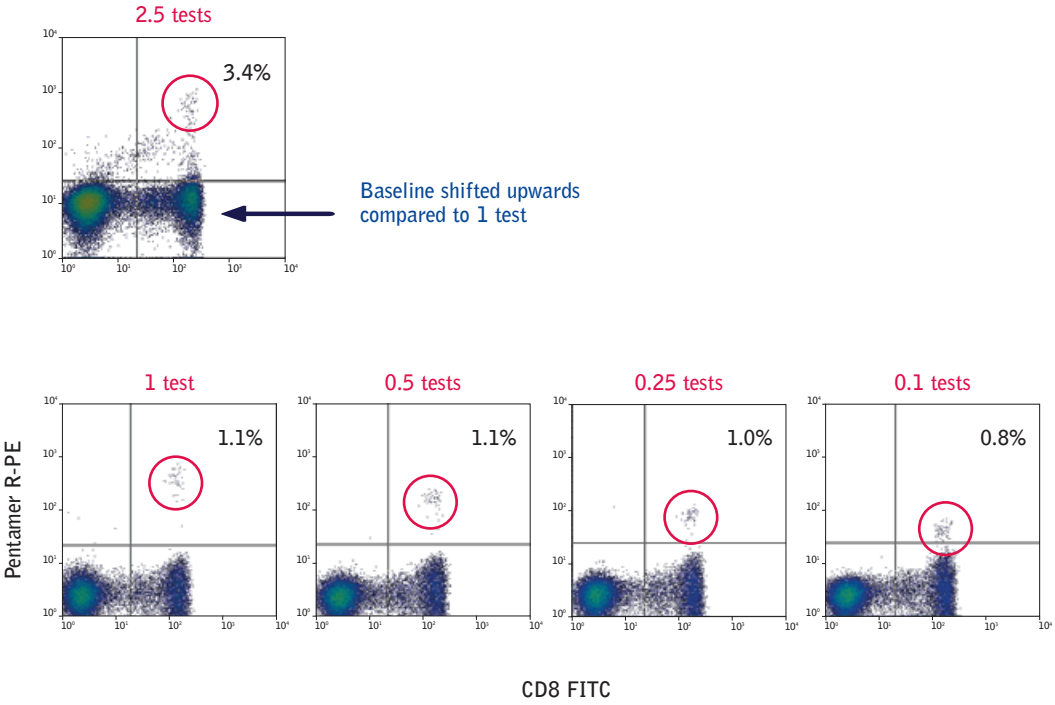
The figure illustrates the importance of titrating the Pentamer. 1 x 10^{6} peripheral blood mononuclear cells (PBMCs) were stained with either 2.5, 1.0, 0.5, 0.25 or 0.1 test equivalents of Pentamer for 10 minutes at 22°C. Following 2 washes, cells were incubated with an optimal dilution of anti-CD8-FITC for 20 minutes at 4°C. 100,000 live events were collected.

When excess Pentamer is used (2.5 tests) the baseline (negative cells) is shifted upwards out of the first log decade of the density plot when compared with the results seen with 1 test. This shift is due to excess protein present in the staining reaction and leads to false positive results as the negative population is seen in the same quadrant as the CD8-positive, antigen-specific events.

When 1 test of Pentamer is used a clear population of antigen-specific T cells is seen (1.1% of total cells gated). The negative cells and the CD8-positive Pentamer-negative cells are well within the lower quadrant. The stain of the Pentamer is bright. Comparing 0.5 test of Pentamer with 1 test shows that the mean fluorescence intensity has reduced. With 0.25 test of Pentamer the frequency of cells detected is lower (1.0%). This is due to some events appearing in the lower quadrants of the plot and being indistinguishable from the negative population. When 0.1 test of Pentamer is used the fluorescence intensity has significantly decreased, affecting the frequency of cells detected.

The conclusion from this experiment is that 1.0 test or 0.5 test of Pentamer should be used. Any non-specific staining seen with high concentrations of Pentamer can be reduced to an ideal level. Further titration below this level will reduce the signal intensity. The level of fluorescence intensity required for the application should also be taken into account.

Figure 9



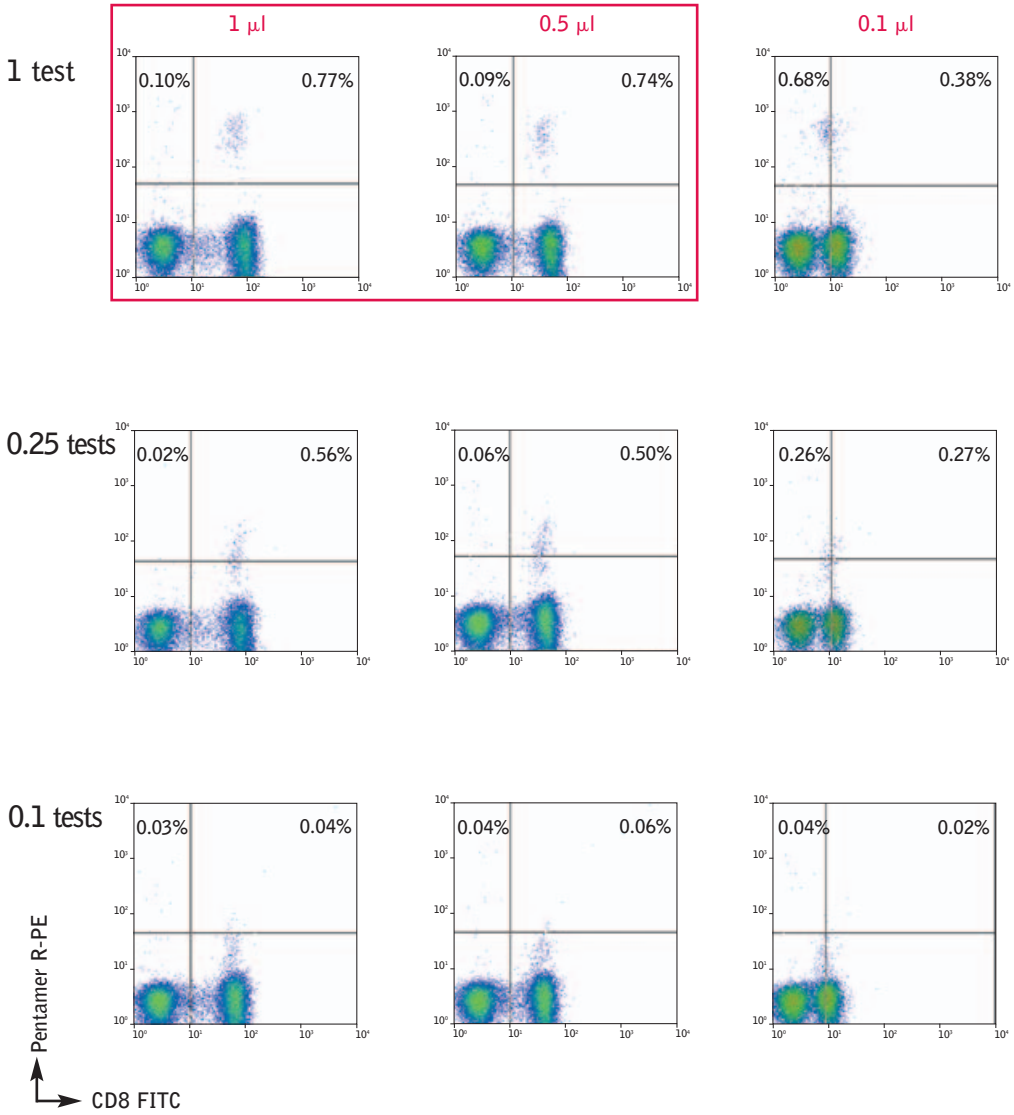
Cross-titration of Pentamer with Anti-CD8

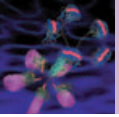
It is important to cross-titrate the Pentamer against the anti-CD8 antibody. If the concentration of both reagents is too high, background staining will interfere with data analysis. If the quantity of reagent used is too little, the fluorescence intensity signal may be too low to analyze results accurately. Cross-titration of the reagents enables the reduction of background staining, whilst maintaining an optimum fluorescence intensity level. Figure 10 illustrates the importance of cross-titration of Pentamer against anti-CD8 monoclonal antibody.

Figure 10

1 x 10^6 PBMCs were stained with either 1.0, 0.25 or 0.1 test equivalents of R-PE-labeled Pentamer for 10 minutes at 22°C. Following 2 washes, cells were incubated with either 1.0, 0.5 or 0.1 µl of FITC-labeled anti-CD8 (clone LT8) for 20 minutes at 4°C. These results show that the Pentamer should be used at 1 test quantity (10 µl) and the anti-CD8 antibody at either 1.0 or 0.5 µl per sample, depending on the desired degree of separation of positive from negative cells.

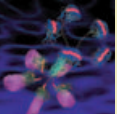
Figure 10


1 µl
0.5 µl
0.1 µl
1 test
0.10%
0.77%
0.09%
0.74%
0.68%
0.38%
0.25 tests
0.02%
0.56%
0.06%
0.50%
0.26%
0.27%
0.1 tests
0.03%
0.04%
0.04%
0.06%
0.04%
0.02%
Pentamer R-PE
CD8 FITC



Section Five

Data Acquisition



Procedure

1 Set up the flow cytometer:

turn on cytometer and carry out any specific start-up procedures according to your laboratory protocol.

2 Select instrument settings:

before analysis of cells the instrument must be correctly set up using unstained cells. Forward and side scatter voltage settings are determined using cells that have no fluorescent staining (page 57).

3 Adjust the compensation settings:

correct compensation is vital when making simultaneous immunofluorescent measurements from several labeled, cell bound antibodies or Pentamers (page 60).

4 Collect data from sample tubes:

collect as many events as possible so that the population of interest can be clearly defined and distinguished above background.

For controls, it is normally sufficient to collect between 10,000 - 15,000 gated events (live lymphocytes). For Pentamer-stained samples it is important to collect a larger number of events, eg. 50,000 - 200,000 **gated live lymphocytes** as a starting recommendation. This will ensure that low-frequency responses can be detected. For example, a 0.1% population will yield 100 dots for every 100,000 events collected.

5 After data acquisition is complete carry out cytometer shutdown procedures according to your laboratory protocol.

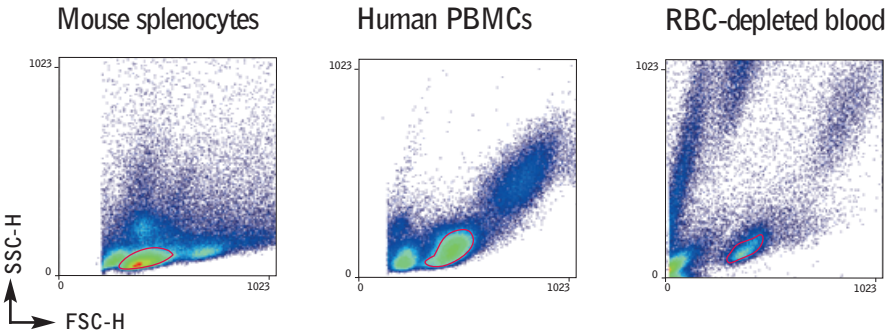
Identification of the Cells of Interest

Light scatter is used to analyze specific subsets of cells, such as lymphocytes, within a heterogeneous population and is commonly used to exclude dead cells, cell aggregates and cell debris from the fluorescence data. As cells pass through the laser beam of the flow cytometer the amount of light scattered varies according to the size (forward scatter) and granularity (side scatter) of each cell. This allows large and very granular cells to be differentiated from the smaller less granular lymphocytes in the blood. It can be difficult to identify the correct live lymphocyte population, but it is very important to do this correctly in order to minimize non-specific staining events seen. For example, dead or dying cells take up proteins from solution easily and therefore contribute disproptionately to non-specific background.

Forward scatter is strongly influenced by cell size; the larger the cells, the further along the FSC-axis they appear. Each dot represents one event. Side scatter reflects granularity or internal cell structure; the larger and more granular a cell the further up the SSC-axis it appears. Cell populations that are close together may be most easily distinguished using a density plot. It is important to ensure that the forward-scatter (FSC) and side-scatter (SSC) gates are set correctly on the population of interest.

Figure 11 shows the correct live lymphocyte gating for three different cell preparations.

Figure 11



Examples of density plots for three different cell preparations.

The populations seen in the lower left corner are made up of dead or dying cells, red blood cells and cell debris - these cells are not of interest. A tight gate has been set around the lymphocytes (outlined in red). Cells seen on the right are likely to be granulocytes and monocytes.

Instrument Set Up

Use instrument settings suitable for analysis of lymphoid cells. These should be determined empirically for each different cytometer. Pass a sample of unstained cells through the cytometer and view these on a 2D density plot showing forward scatter (FSC) on the x-axis and side scatter (SSC) on the y-axis.

The FSC and SSC voltages should be set so that the live lymphocyte gate is centered at coordinates 400/200, figure 12a; use the 'voltage' and 'amp gain' adjusters in the detectors/amps window of the flow cytometer controls. If the FSC voltage is set too high the cells will appear too far to the right on the x-axis and consequently the cells of interest may be off the scale. Ensure that the FSC and SSC mode is set to 'linear' or 'lin' in the detectors/amps window of the flow cytometer.

Setting the Live Lymphocyte Gate

It is essential to set a tight gate on the lymphocyte population of interest. This reduces the appearance of non-specific staining in the density plot. This is especially important when looking at small T cell populations. The flow cytometer software gives a name to the population of gated cells, e.g. R1 (Region 1).

Figure 12b shows only the cells gated in R1 on a FL1/FL2 density plot. In this figure FITC is in the FL1 channel on the x-axis; R-PE is in the FL2 channel on the y-axis. When cells are unstained the R1 population should be set to lie within the first log decade (lower left corner) of the density plot on both axes.

Figure 12a

Set FSC/SSC voltages so that the live lymphocyte gate is centred at co-ordinates 400/200

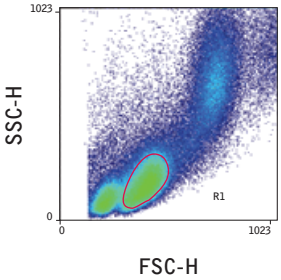
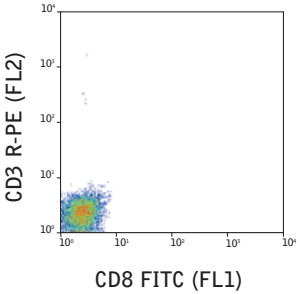


Figure 12b

Set FL1/FL2 voltages so that the cells in the live lymphocyte gate (R1) are contained within 1st log decade of FL1 and FL2

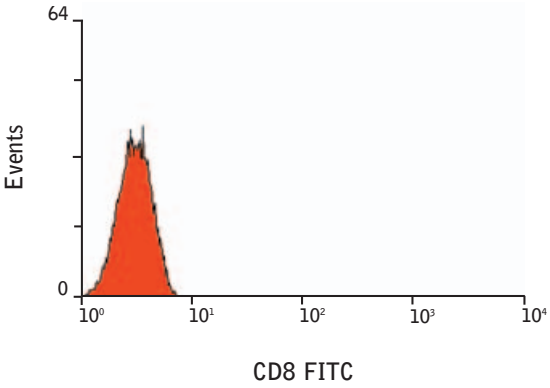


Voltage Settings for Individual Colors

Following live lymphocyte gating, set the voltages for each individual fluorescent channel to be used. Ensure that the 'FL' channels are set to 'log' mode (Detectors/Amps window of flow cytometer controls). Adjust the voltage for FL1 until the cells are contained within the first log decade, figure 13. Repeat the procedure for all the channels to be used.

These instrument settings can be used as a starting point for future experiments with the same cell type.

Figure 13



Compensation

A variety of different fluorochromes may be used for multi-color, immunofluorescence analysis of several cell bound antibodies or entities. Figure 14 shows the significant spectral overlap of the emission wavelengths of a number of fluorochromes that are commonly used for flow-cytometric analysis. The gray bands represent the wavelength range detected by the flow cytometer channels (the filter path bands, FL1, FL2 etc.). Fluorescence from FITC (green) is measured in the FL1 channel (525 nm) and R-PE fluorescence (orange) is detected in the FL2 channel (575 nm). Due to the overlap of the emission spectra, some green fluorescence from the FITC molecule will be detected by the FL2 channel and some orange fluorescence from R-PE will be detected by the FL1 channel. If the overlap is not corrected by compensation, the data will include the signal from each of the fluorochromes that has been detected by the inappropriate detector. This could lead to incorrect analysis of results due to the presence of false-positive cell populations, figure 15.

Figure 14

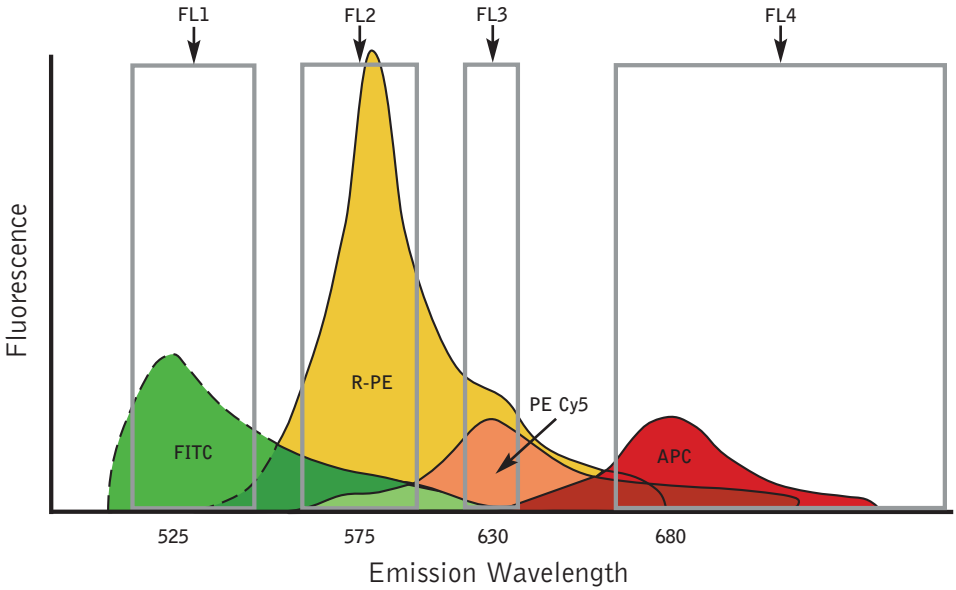
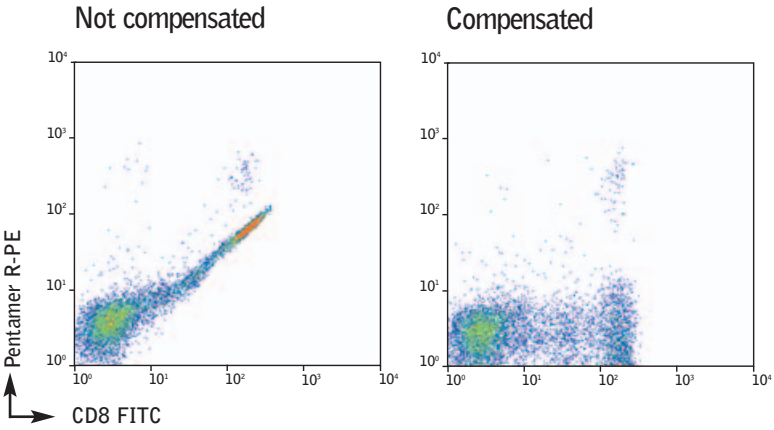


Figure 15a

Figure 15b



15a: The antigen-specific population stained with labeled Pentamer is fairly clear to see in the upper half of the plot, but there is interference from the CD8-positive, Pentamer-negative population.

15b: After compensation the antigen-specific population is much clearer and is seen as a set of well defined events separate from the negative population.

How to set compensation

Use single-color stained controls to adjust compensation settings for all the fluorescent channels required for the experiment. For example, for two-color analysis, use one sample with FITC labeled anti-CD8 and one with R-PE labeled anti-CD3.

Select a marker that will stain many cells (~50-70%) in order to facilitate setting the compensation for that channel. A marker that occurs at low frequency (<1% of cells) will only stain a few cells, making it difficult to set the compensation. It is also important to choose a stain that creates both a positive and negative population of cells.

Double-stained control samples are helpful for fine-tuning the compensation settings. Choose control antibodies that stain mutually exclusive cell populations. Alternatively, use a tube of cells derived by mixing aliquots of the FITC and R-PE single-color controls.

Following the initial instrument set up proceed to set the compensation using single-stained control samples. Set the compensation so that the center of the positively stained cell population lies in line with the center of the negative cell population and parallel with the appropriate axis, figure 16.

Figure 16

16a: Cells are stained with FITC-labeled anti-CD8 only. Events are seen in the second log-decade of the y-axis, due to detection of FITC fluorescence emission in the FL2 channel. Compensation is set for the FL1 channel to correct this.

16b: Cells have been compensated and a theoretical horizontal line can be drawn parallel to the x-axis, through the center of the population of CD8-negative cells and that of the CD8-positive cells.

16c: Cells are stained with R-PE-labeled anti-CD3 only. Events are seen in the FL1 FITC channel.

16d: Once the compensation in the FL2 channel has been set correctly the R-PE stained cells are aligned vertically above the negative cell population.

Figure 16a

Before compensation

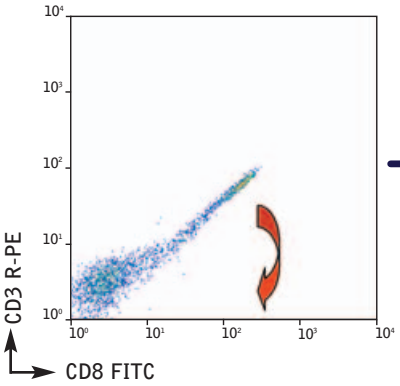


Figure 16b

After compensation

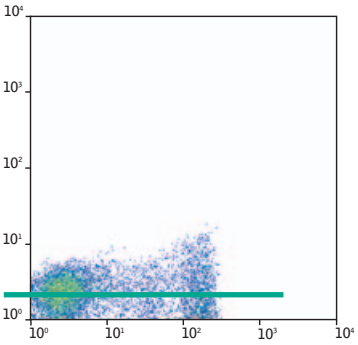


Figure 16c

Before compensation

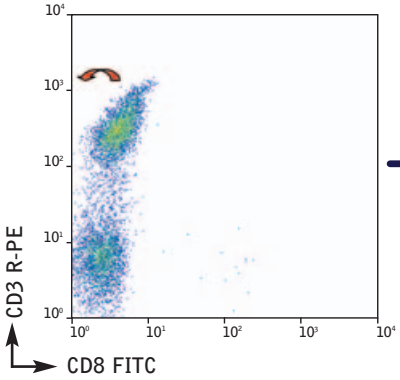
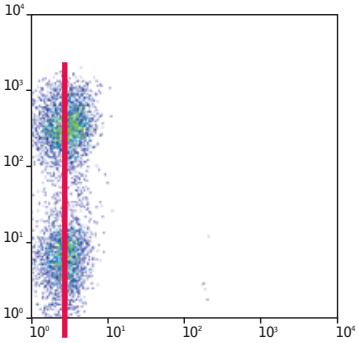
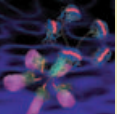


Figure 16d

After compensation





Section Six

Data Analysis and Interpretation

Choice of Plot

Setting Gates and Quadrants

Examples

Choice of Plot

The acquired data must be exported to a specialized software program for data analysis and interpretation. The data should be viewed using the most appropriate choice of plot. Most software analysis programs for flow cytometry have a choice of different types of plot for data analysis, e.g. 1-Dimensional (1D) histogram plot, 2-Dimensional (2D) dot plot, 2D density plot. The 2D density plot is most commonly used as it highlights discrete populations most effectively. A small population of cells would be hard to identify using a histogram plot. For example, the low frequency of Pentamer positive events expected in a PBMC or mouse splenocyte sample.

Setting Gates and Quadrants

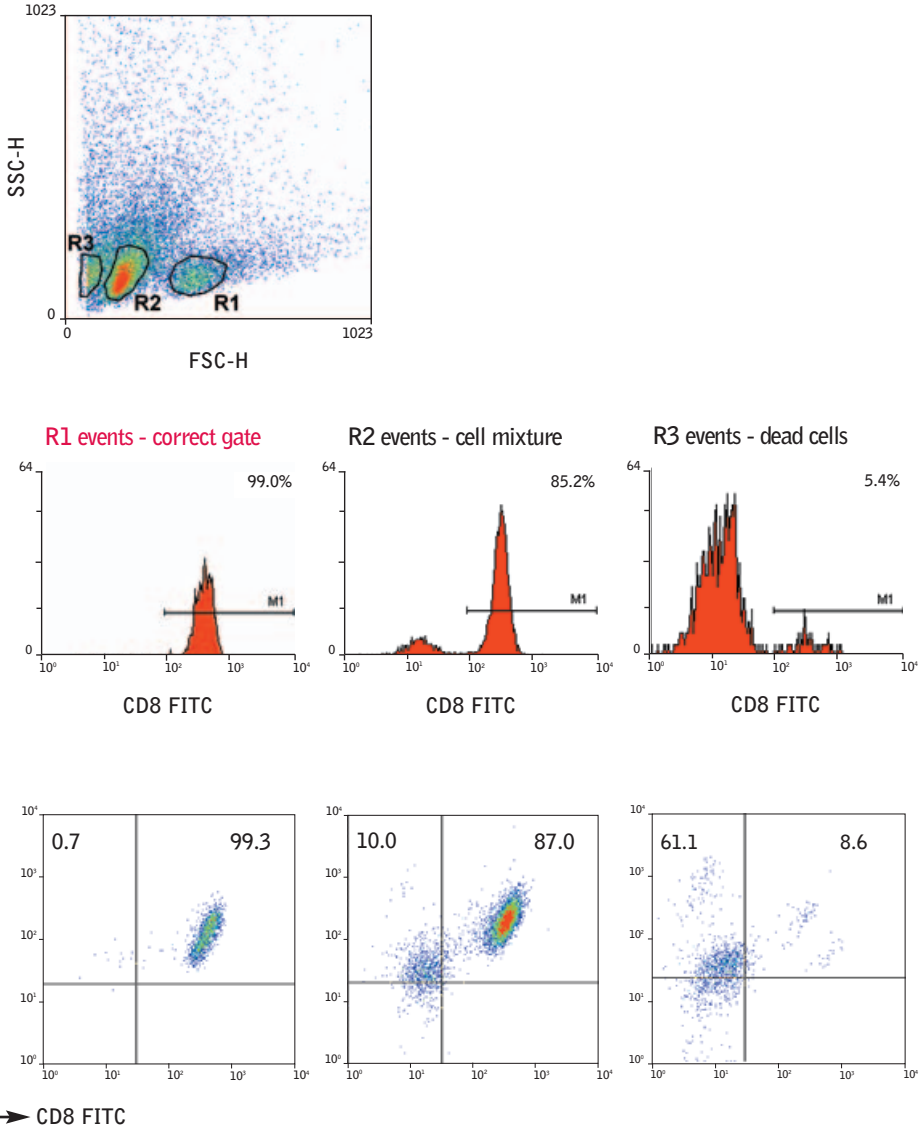
Using the control sample data, set the live lymphocyte gate and add quadrants to each 2D plot between the positive and negative cell populations. Correct gating distinguishes the cells of interest from those that are unwanted and irrelevant.

Figure 17

The figure illustrates the different profiles of three cell populations (R1, R2, R3) in a T cell clone sample. In this particular T cell clone, 100% of cells should be CD8 positive and specific for a single Pentamer. The sample was stored frozen before use, therefore there is a significant proportion of dead and dying cells. The three populations were gated and their CD8-positive and Pentamer-positive cells are shown.

R1, R2 and R3 events may be distinguished by their forward and side scatter profiles. R1 defines cells that are almost exclusively CD8-positive (mature T cells) and Pentamer-positive (i.e. the correct population). Two-thirds of the R2 population are CD8-positive and not all stain for Pentamer. The FSC vs. SSC profile indicates that these cells are likely to be mature T cells approaching apoptosis. The population of cells defined by R3 has no CD8-positive cells within it, though a significant number of cells stain for the antigen-specific Pentamer. Their FSC properties define them as very small, therefore they are most probably dead cells and debris.

Figure 17


1023
SSC-H
R3
R2
R1
0
0
1023
FSC-H
R1 events - correct gate
R2 events - cell mixture
R3 events - dead cells
99.0%
85.2%
5.4%
M1
CD8 FITC
0.7
99.3
10.0
87.0
61.1
8.6
Pentamer R-PE
CD8 FITC

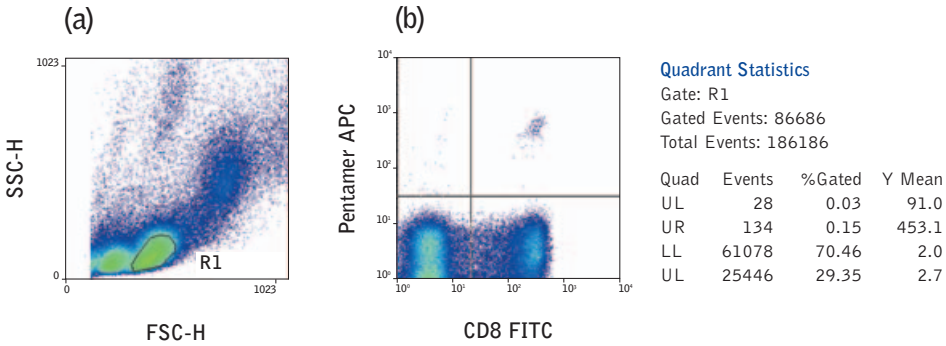
Examples

Typical analysis of PBMCs stained with Pentamer (APC-labeled) vs. CD8 (FITC)

1 Create a 2D density plot showing FSC vs. SSC. Draw a tight region around the lymphocyte population (R1), figure 18a.

2 Create a second density plot, gated only on events encircled within R1, showing Pentamer-stained cells on the y-axis and CD8-stained cells on the x-axis. Draw a quadrant that clearly separates positive cells from negative cells on both axes, and use quadrant statistics to obtain quantitative information about the frequency of antigen-specific CD8-positive T lymphocytes, figure 18b.

Figure 18



Quadrant Statistics
Gate: R1
Gated Events: 86686
Total Events: 186186

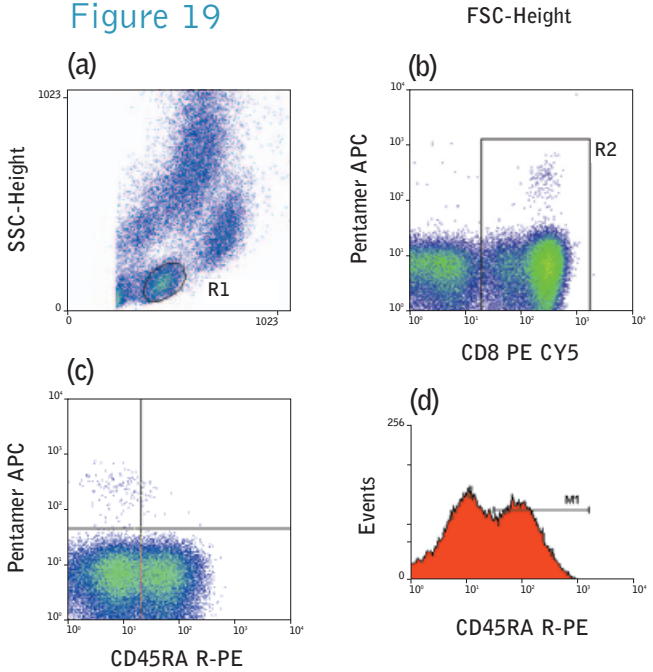
Quad	Events	%Gated	Y Mean
UL	28	0.03	91.0
UR	134	0.15	453.1
LL	61078	70.46	2.0
UL	25446	29.35	2.7

Typical analysis of PBMCs triple-stained with Pentamer (APC) vs. CD8 (PE-Cy5) vs. CD45RA (PE)

This three-color analysis enables the phenotype of the antigen-specific cells to be analyzed further using antibodies specific for activation markers or cytokines.

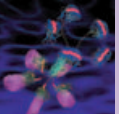
1. Create a 2D density plot showing FSC vs. SSC. Draw a tight region around the lymphocyte population (R1), figure 19a.
2. Create a second density plot, gated only on events encircled within the R1 region. Draw a second gate (R2) to select only CD8-positive cells, figure 19b.
3. Create a third density plot, gated on events within regions R1 and R2, and draw a quadrant to separate positive and negative cells, figure 19c. Draw a histogram to aid in accurate setting of quadrant lines, figure 19d. Use quadrant statistics to evaluate the proportion of Pentamer-positive cells that co-express a phenotypic marker.

Figure 19



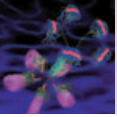
Quadrant Statistics
Gate: R1 and R2
Gated Events: 54358
Total Events: 175588

Quad	Events	%Gated	Y Mean
UL	128	0.24	233.0
UR	22	0.04	185.3
LL	28786	52.96	6.3
UL	25422	46.77	5.9



Section Seven

Applications of Pro5® Pentamers



Use of Pro5® Pentamers for Cell Separation

Separation of Antigen-Specific T Cells

In addition to detecting antigen-specific T cells, Pro5® MHC Pentamers may be used in conjunction with magnetic beads to enrich or deplete for the T cell population of interest.

Magnetic bead sorting is a simple solution for applications requiring enrichment or depletion of antigen-specific T cells; for example, in the development of therapeutic applications, generation of T cell lines, PCR cloning, gene profiling, and cell culture experiments. Alternatively, if magnetic bead sorting is used as a pre-enrichment step before Fluorescence Activated Cell Sorting (FACS) it will reduce both the number of cells that will have to be sorted and the required sorting time.

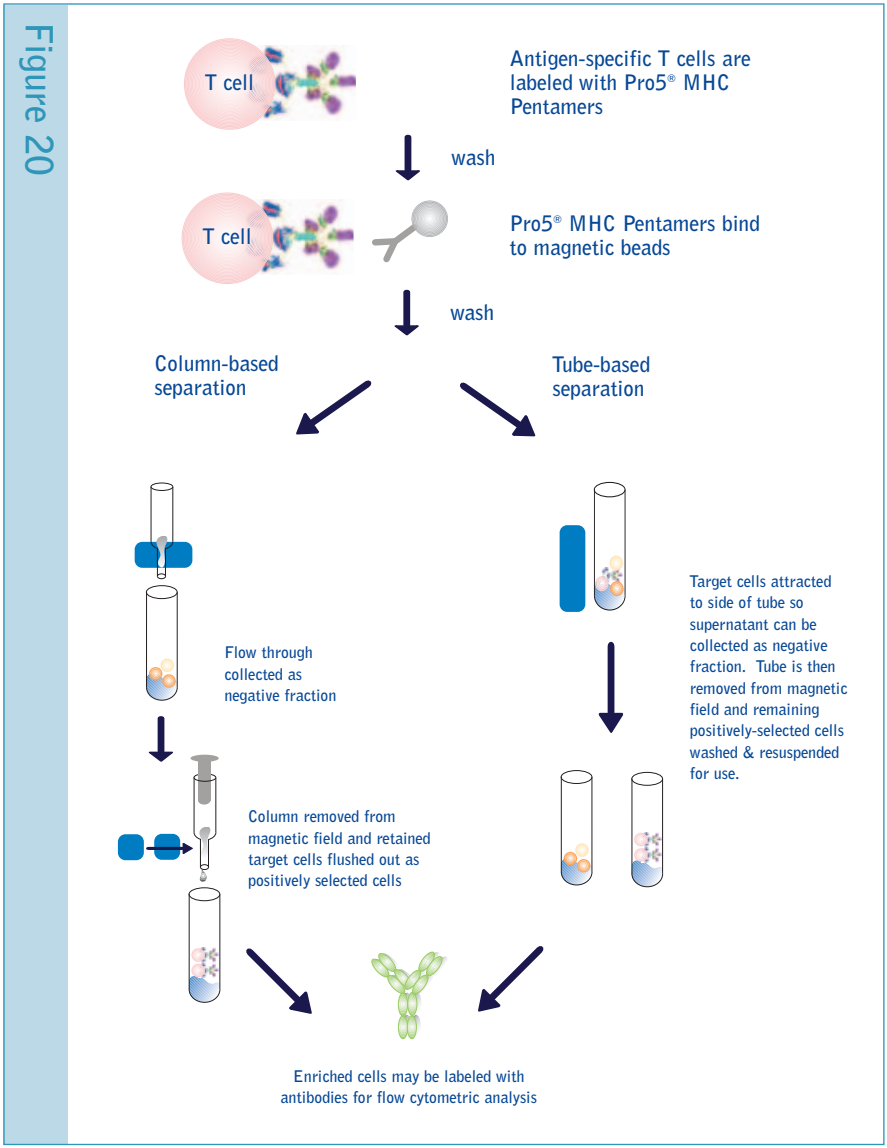
There are currently two main technologies for magnetic bead separation, column- and tube-based methods, illustrated in figure 20.

In the column-based method, cell-bead complexes pass through a separation column, which is placed in a strong, permanent magnet. The column matrix retains bead-bound cells while non-labeled cells flow through. Following removal of the column from the magnetic field, the retained cells may be eluted.

In the tube-based method, cell-bead complexes are removed from the cell suspension using an external magnet that draws the complexes to the inner edge of the tube allowing supernatant to be removed. Removing the tube from the magnetic field enables resuspension of the cell-bead complexes.

Figure 20

General protocols for bead-enrichment



Column-Based Isolation of Antigen-Specific Cells Using Anti-Fluorochrome Beads

Materials and equipment

- Pro5® MHC Class I Pentamer, labeled with R-PE or APC, specific for cells of interest
- Anti-fluorochrome magnetic beads e.g. anti-R-PE or anti-APC, (Miltenyi Biotec / BD Biosciences) to detect the fluorescence of the Pentamer, suitable for use with magnetic columns
- Anti-CD8 antibody, conjugated with a different fluorescent label to the Pentamer
- Wash buffer, de-gassed (remove air prior to use) (Appendix I)
- Fix solution (Appendix I)
- Magnetic stand e.g. MACS Multistand, Miltenyi Biotec #130-042-303
- Separation unit e.g. MiniMACS separation unit, Miltenyi Biotec #130-042-102
- Magnetic columns e.g. MS Columns, Miltenyi Biotec #130-042-201
- 12 x 75 mm polystyrene tubes for flow cytometry (e.g. BD Biosciences #352052)
- Benchtop refrigerated centrifuge with swing-out rotor and appropriate tube carriers

Procedure for washing cells

Dispense 1 ml wash buffer into each sample tube and spin at 400 x *g* for 5 minutes in a chilled centrifuge at 4ºC. Check for presence of a cell pellet before discarding the supernatant. Resuspend cell pellets in residual liquid (~50 µl).

Procedure

Note: procedure may need adjustment depending on the particular system used

1 Stain 1 x 10^7 cells with 50 µl (5 tests) fluorescent-labeled Pentamer for 10 minutes at room temperature (22°C).

2 Wash samples, centrifuge and discard the supernatant.

3 Resuspend cells in 80 µl wash buffer and add 20 µl anti-fluorochrome magnetic beads per tube (or as directed by the manufacturer).

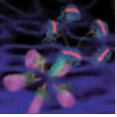
4 Incubate for 15 minutes at manufacturer's recommended temperature (Miltenyi Biotech, 4-8°C, BD Biosciences, 6-12°C).

5 Wash cell-bead complexes, centrifuge and resuspend in 500 µl wash buffer (de-gassed).

6 Wash a column suitable for positive-selection with 500 µl wash buffer (de-gassed) and place on magnetic stand.

7 Load cell-bead complexes onto the column.

Antigen-specific T cells labeled with Pentamer-bead complexes will be retained on the column (positive fraction).



8 Collect the negative fraction that elutes from the column, including 3 column washes of 500 µl each.

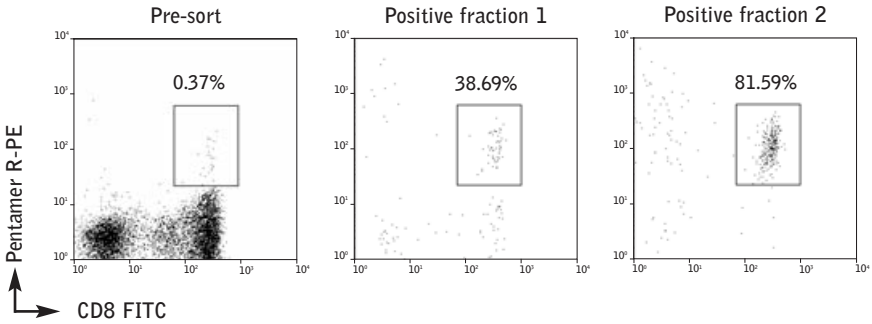
9 Remove column from magnet and flush out the positive fraction by adding 1 ml wash buffer onto the column and applying a plunger (provided with the column).

10 To obtain a purer antigen-specific cell population, the positive cell fraction may be passed over a second column.

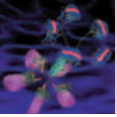
11 Stain cells from pre-isolation, positive and negative fractions with anti-CD8 antibody for flow cytometric analysis.

Figure 21

The figure shows an experiment in which the column-based method of magnetic separation was used to enrich antigen-specific T cells from peripheral blood cells (PBMCs). 1 x 10^7 PBMCs were incubated with 5 tests (50 µl) R-PE-labeled Pentamer for 10 minutes at room temperature (22°C), followed by 20 µl anti-R-PE microbeads (Miltenyi Biotec) for 15 minutes at 4°C. Cells were passed over an 'MS' column using the MidiMACS system and the flow-through fraction discarded. The column was then removed from the magnetic field and the retained target cells flushed out as positively selected cells (positive fraction 1). These cells were then passed over a second 'MS' column in order to enrich the antigen-specific T cells of interest further (positive fraction 2). Resultant cells were then incubated with anti-CD8 antibody for 20 minutes on ice (4°C) prior to fixation and analysis by flow cytometry. (Due to the small size of the Miltenyi microbeads, cells may be analyzed by flow cytometry with beads still attached).



In this experiment the antigen-specific population was enriched 220-fold to yield a population of cells suitable for flow cytometry, cell culture, generation of T cell lines, PCR cloning or gene profiling.



Column-Based Isolation of Antigen-Specific Cells Using Streptavidin Beads

Materials and equipment

- Pro5® MHC Class I Pentamer, specific for cells of interest.
- Streptavidin magnetic beads to detect the biotin label of the Pentamer, suitable for use with magnetic columns (e.g. Streptavidin Microbeads, Miltenyi Biotec #130-048-102)
- Streptavidin, conjugated to fluorescent label of choice

  Anti-CD8 antibody, conjugated with a different fluorescent label to streptavidin
- Wash buffer, de-gassed (remove air prior to use) (Appendix I)
- Fix solution (Appendix I)
- Magnetic stand e.g. MACS Multistand, Miltenyi Biotec #130-042-303
- Separation unit e.g. MiniMACS separation unit, Miltenyi Biotec #130-042-102
- Magnetic columns e.g. MS Columns, Miltenyi Biotec #130-042-201
- 12 x 75 mm polystyrene tubes for flow cytometry (e.g. BD Biosciences #352052)
- Benchtop refrigerated centrifuge with swing-out rotor and appropriate tube carriers

Procedure for washing cells

Dispense 1 ml wash buffer into each sample tube and spin at 400 x *g* for 5 minutes in a chilled centrifuge at 4°C. Check for presence of a cell pellet before discarding the supernatant. Resuspend cell pellets in residual liquid (~50 µl).

Procedure

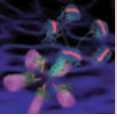
Note: procedure may need adjustment depending on the particular system used.

Procedure

Note: procedure may need adjustment depending on the particular system used.

1. Stain 1 x 10^7 cells with 50 µl (5 tests) biotin-labeled Pentamer for 10 minutes at room temperature (22°C).
2. Wash samples, centrifuge and discard the supernatant.
3. Resuspend cells in 90 µl wash buffer and add 10 µl streptavidin magnetic beads per tube (or as directed by the manufacturer).
4. Incubate for 15 minutes at manufacturer's recommended temperature (Miltenyi Biotec 4-8°C).
5. Wash cell-bead complexes, centrifuge and resuspend in 500 µl wash buffer (de-gassed).
6. Wash a column suitable for positive-selection with 500 µl wash buffer (de-gassed) and place on magnetic stand.
7. Load cell-bead complexes onto the column.

   Antigen-specific T cells labeled with Pentamer-bead complexes will be retained on the column (positive fraction).
8. Collect the negative fraction that elutes from the column, including 3 column washes of 500 µl each.
9. Remove column from magnet and flush out the positive fraction by adding 1 ml wash buffer onto the column and applying a plunger (provided with the column).
10. To obtain a purer antigen-specific cell population, the positive cell fraction may be passed over a second column.
11. Stain cells from pre-isolation, positive and negative fractions with anti-CD8 antibody and fluorescent streptavidin for flow cytometric analysis.



Tube-Based Isolation of Antigen-Specific Cells Using Streptavidin Beads

Materials and equipment

- Pro5® MHC Class I Pentamer, specific for cells of interest.
- Streptavidin magnetic beads to detect the biotin label of the Pentamer, suitable for use with an external magnet (e.g. Dynabeads® M-280 Streptavidin, Invitrogen #112-05D; LodeStars™ 2.7 Streptavidin, Varian Inc., #PL6727-1001
- Streptavidin, conjugated to fluorescent label of choice
- Anti-CD8 antibody, conjugated with a different fluorescent label to streptavidin
- Wash buffer, de-gassed (remove air prior to use) (Appendix I)
- Fix solution (Appendix I)
- Magnetic tube holder for tubes up to 2 ml (e.g. Dynal MPC®-S, Invitrogen #120-20D, or PL-MCS12, Varian Inc., #PL6700-0002 or for larger tubes (e.g. Dynal MPC®-1, Invitrogen #120-01D, or PL-MCS2, Varian Inc. #PL6700-0001)
- 12 x 75 mm polystyrene tubes for flow cytometry (e.g. BD Biosciences #352052)
- Benchtop refrigerated centrifuge with swing-out rotor and appropriate tube carriers

Procedure for washing cells

Dispense 1 ml wash buffer into each sample tube and spin at 400 x *g* for 5 minutes in a chilled centrifuge at 4ºC. Check for presence of a cell pellet before discarding the supernatant. Resuspend cell pellets in residual liquid (~50 µl).

Procedure

Note: procedure may need adjustment depending on the particular system used

1 For best results start with at least 1 x 10^7 lymphoid cells (PBMCs or splenocytes).

2 Wash cells with wash buffer and resuspend in 200 µl wash buffer.

3 Add 1 test (10 µl) biotin-labeled Pentamer per 2 x 10^6 cells.

4 Incubate at room temperature (22°C) for 10 minutes.

5 Wash the cells in wash buffer and resuspend in 500 µl wash buffer.

6 Add an optimally titrated amount of streptavidin beads

5 beads per cell is recommended.

7 Incubate on ice (4°C) for 30 minutes with mixing.

8 Bring the volume in the tube up to 2 ml with wash buffer then place in a magnetic tube holder.

9 Leave for 3-5 minutes.

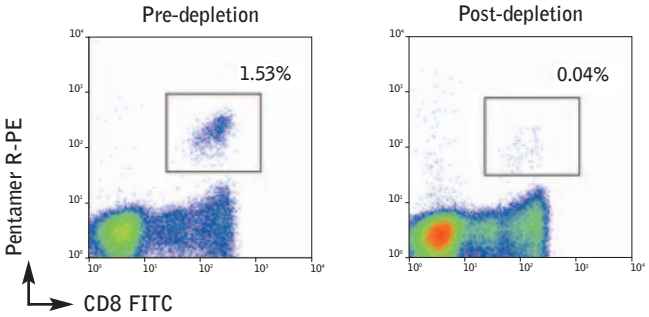
10 Wash the cell-bead complexes 3 times with wash buffer and discard supernatant (negative fraction).

If desired, supernatant can be retained for flow cytometric analysis to confirm removal of antigen-specific cells, otherwise discard supernatant.

11 Isolated cell-bead complexes may be placed in cell culture, where beads should dissociate after a few days.

Figure 22

Antigen-specific cells were depleted from a peripheral blood suspension using a biotin-labeled Pro5® Pentamer and LodeStars™ 2.7 Streptavidin (Varian Inc.) exactly as detailed in the protocol. A sample of the original cell population (pre-depletion) and supernatant following isolation (negative fraction, post-depletion) were incubated with anti-CD8 FITC antibody plus Streptavidin R-PE to visualize antigen-specific cells. The antigen-specific population was reduced from 1.53% to 0.04%, confirming that bead isolation was successful (97.4% of antigen-specific cells removed).



The antigen-specific cells removed from the original population may be placed in cell culture, where beads will detach after several days. This pure population may also be used in other applications, such as PCR cloning or gene profiling.

Fluorescence Activated Cell Sorting (FACS)

Live Pro5® Pentamer-positive cells can be sorted and collected for further manipulation using a Fluorescence Activated Cell Sorter (FACS machine).

For this application it is necessary to remove the sodium azide preservative from the Pentamer reagent beforehand using centrifugal concentrators (e.g. Microcon® YM-10, Millipore #42406). Dilute the reagent to maximum volume with PBS and concentrate down to original volume. Repeat the procedure 2-3 times until the azide is sufficiently diluted. If azide is not removed it will kill the cells.

Protocol notes

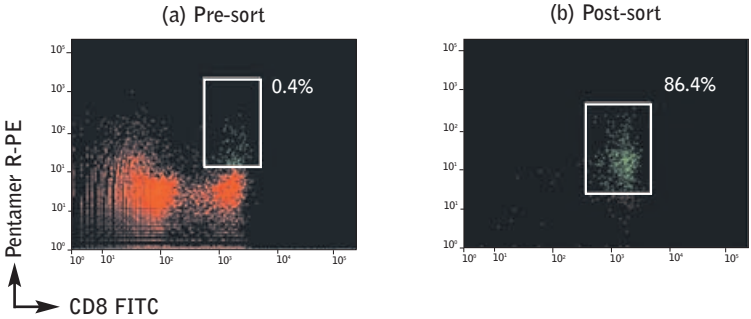
The protocol for staining cells for FACS is the same as for flow cytometry analysis, therefore the appropriate protocol for the Pentamer reagent in use should be followed (Section Four). However, the amount of Pentamer reagent used should be adjusted so that there is sufficient to stain a larger number of cells. For example, 1 test (10 µl) R-PE-labeled Pentamer is sufficient to stain 1-2 x 10^6 cells, but to stain 1 x 10^7 cells would require 5-10 tests (50-100 µl) Pentamer. Antibody volumes must also be scaled up for the number of cells to be sorted.

Following staining, the cells MUST NOT be fixed (fixing kills the cells), and sorting should be carried out immediately. If sorting a large number of cells, it is useful to filter the cells through a cell strainer and chill the tube during the procedure to prevent clumping.

FACS machine set-up is carried out in the same way as for flow cytometry (Section Five). Once the machine has been correctly prepared for use, the sort gates should be set around the live cells and the CD8-positive, Pentamer-positive population. It is advisable to set these gates slightly smaller than the desired population area to obtain a pure cell sample.

Figure 23

3 x 10^7 PBMCs were incubated for 10 minutes at room temperature (22ºC) with 20 tests (200 µl) R-PE-labeled Pentamer. The cells were then washed twice and incubated with 20 tests (20 µl) anti-CD8 FITC (clone LT8) for 20 minutes on ice. Following a further 2 washes, cells were resuspended in 2 ml wash buffer and filtered through a nylon mesh filter (e.g. Miltenyi #130-041-407) prior to sorting. The cells were sorted to obtain the Pentamer-positive, CD8-positive population, using a Becton Dickinson FACSAria machine.



23a: In the starting cell population there were 0.4% CD8-positive Pentamer-positive cells, as defined by the white gate that was used to sort the sub-population of interest.

23b: The sorted sample was analyzed by flow cytometry and shown to be 86.4% pure. Note that the gate used for defining this population is lower than the original sort gate. This is due to the R-PE fluorescence intensity of the labeled cells having been quenched by the laser beam during sorting.

Use of Pro5® Pentamers In Combination With Intracellular Cytokine Staining

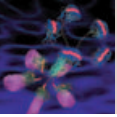
Co-staining $CD8^+$ T cells with Pro5® MHC Class I Pentamers and antibodies against intracellular cytokines has now become a routine procedure in many laboratories. This technique not only enables the frequency of antigen-specific T cells to be determined but also their effector function.

Production of cytokines plays an important role in the immune response. Examples include the induction of many anti-viral proteins by Interferon gamma (IFNγ), the induction of T cell proliferation by interleukin-2 (IL-2) and the inhibition of viral gene expression and replication by TNFα. Cytokines are not preformed factors; instead they are rapidly produced upon relevant stimulation. Intracellular cytokine staining relies upon the stimulation of T cells in the presence of an inhibitor of protein transport, thus retaining the produced cytokines inside the cell.

Cellular activation to trigger cytokine production generally results in down-regulation of the T cell receptor. For this reason, Pentamer staining is carried out prior to activation to ensure a good level of staining. Pentamers may be internalized with the T cell receptor during this period, but can still be detected in permeabilized cells.

To analyze the effector function of antigen-specific T cells, the cells are first stained with Pentamer, then stimulated with antigen. This is followed by staining with antibodies specific for extracellular markers (such as CD8), then by membrane permeabilization and intracellular cytokine staining.

The following protocols are applicable for Pentamer co-staining with antibodies against IFNγ, TNFα, MIP-1β, or IL-2. Certain cytokines such as IL-4 and IL-10 are difficult to detect by intracellular staining methods. In addition to this, frequencies of Pentamer-positive cells producing these cytokines may also be extremely low.



Pro5® Pentamer and Intracellular Cytokine Staining Protocol For Use With Human Cells

Materials and equipment

- Cell sample, eg. PBMCs
- Pro5® MHC Class I Pentamer conjugated to fluorescent label of choice (e.g. R-PE).
- Fluorescent-labeled anti-cytokine antibody eg. anti-human IFNγ-FITC; ProImmune (different fluorescent label to Pentamer)
- Fluorescent-labeled anti-CD8 antibody
- Peptide (stock at 50 mg/ml in DMSO - may be aliquotted and stored at -20°C)
- Leukocyte activation cocktail (LAC; optional) for use as a control for cytokine production (BD Biosciences #550583 - may be aliquotted and stored at -80°C)
- Cytokine IC control cells (optional) for use as a positive control when establishing the intracellular cytokine staining protocol (eBioscience #00-4509)
- PBS Wash (2% fetal calf serum, 0.1% sodium azide in Phosphate buffered saline)
- Permeabilization buffer (0.1% saponin, 1% fetal calf serum, 0.1% sodium azide in PBS)
- 4% Paraformaldehyde
- Fix buffer (Appendix I)
- R10 medium (Appendix I)
- Brefeldin A (Sigma #15870)
- 12 x 75mm round bottom tubes

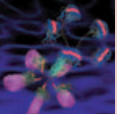
- Benchtop refrigerated centrifuge with swing out rotor and appropriate tube carriers
- Chilled microcentrifuge
- Humidified CO_2 incubator at 37°C
- Vortex mixer

Protocol applicable for intracellular staining of IFNγ, TNFα, MIP-1β, or IL-2 with Pro5® Pentamer

All procedures up to step 10 should be carried out in a suitable sterile tissue culture hood, using sterile buffers and aseptic conditions.

Procedure

1. Centrifuge Pro5® Pentamer in a chilled microcentrifuge at 14,000 x *g* for 5 minutes.
2. Prepare peripheral blood cells in PBS Wash at a cell concentration of 2×10^7 cells/ml.
3. Transfer the cell suspension to individual tubes in 50 µl aliquots.
4. Add relevant titrated fluorescently labeled Pentamers to the desired tubes, and incubate for 10 min at 22°C (non-stimulated single-color controls should not be stained at this stage). Add 10 µl PBS Wash to remaining tubes.



5 Add 500 µl PBS Wash to each tube. Centrifuge at 450 × *g* for 5 minutes at 10°C.

6 Aspirate supernatant. Agitate to disrupt cell pellets and resuspend in 200 µl R10 medium.

7 Dilute peptide stock 1:50 in R10 medium. Add 2 µl of this (10 µg/ml final) to each desired tube.

If using Leukocyte Activation cocktail (LAC) as a control, rapidly thaw this at 37°C in a water bath and add 0.33 µl of this to each desired tube. After use, discard the unused portion of Leukocyte Activation cocktail.

8 Place the tubes at 37°C in a humidified CO_2 incubator for 15 minutes to 1 hour.

9 Add Brefeldin A (10 µg/ml final) to the desired tubes (Note: LAC contains Brefeldin A) and return to the incubator. Incubate for 15 hours.

The optimal stimulation period for induction of a given cytokine is variable and has to be determined. The incubation period of 16 hours described above was determined to be optimal for human PBMCs stimulated with 10 µg/ml peptide to induce an IFNγ response. The incubation period will vary dependent on the method of stimulation and the cytokine response you wish to determine.

10 Remove tubes from the incubator. Centrifuge at 450 × *g* for 5 minutes at 10°C.

11 Aspirate supernatant. Resuspend desired cell pellets in 50 µl PBS Wash containing an optimally titrated amount of anti-CD8 antibody. Add 50 µl PBS Wash to remaining tubes.

Single-color controls should be stained at this stage. If additional

phenotyping of samples is desired, antibodies to other cell surface markers or receptors may also be added at this time.

12 Incubate for 20 minutes on ice.

13 Add 500 µl PBS Wash to each tube. Centrifuge at 450 × *g* for 5 minutes at 10°C.

14 Aspirate supernatant. Agitate to disrupt cell pellets.

15 Add 200 µl 4% paraformaldehyde to each sample tube. Vortex tubes. Incubate for 20 minutes on ice.

This step will fix the cell morphology of the activated cells.

The procedure can be stopped at this point. Repeat steps 13 and 14. Resuspend the cells in 100 µl/tube PBS Wash. Cover and store the cells at 4°C for up to 3 days. To proceed, repeat steps 13 and 14. Resuspend the cells in 200 µl/tube permeabilization buffer and proceed to step 17.

16 Add 200 µl permeabilization buffer to each tube.

17 Centrifuge at 450 × *g* for 5 minutes at 10°C. Aspirate supernatant.

18 Add 100 µl permeabilization buffer to the sample tubes that are to be stained with anti-cytokine antibody. Add 100 µl PBS Wash to the remaining tubes (i.e. Single-color controls).

19 Incubate for 5 minutes at room temperature.

20 Add an optimally titrated amount of FITC-conjugated anti-cytokine antibody to the desired sample tubes and mix.

21 Incubate for 20 minutes at room temperature.

22 Add 200 µl permeabilization buffer to each tube and

centrifuge at 450 × *g* for 5 minutes at 10°C. Aspirate supernatant and agitate tubes to disrupt the cell pellets.

23 Resuspend the cells in 200 µl fix solution. Vortex tubes.

It is important to vortex well when adding this fixative so that cells do not clump.

24 The samples are now ready for data acquisition and analysis on a flow cytometer but may be stored overnight at 4°C in the dark prior to analysis.

Notes

1 Incubation period: The optimal stimulation period for induction of a given cytokine is variable and has to be determined. The incubation period of 16 hours described above was determined to be optimal for human PBMCs stimulated with 10 µg/ml peptide to induce an IFNγ response. The incubation period will vary dependent on the method of stimulation and the cytokine response you wish to determine.

2 Peptide concentration: Investigate the effect on cytokine response of titrating your peptide.

3 Pentamer staining: Cellular activation to trigger cytokine production also results in the down-regulation of the T cell receptor, which can make it difficult to perform Pentamer staining subsequently. For this reason, we recommend Pentamer staining prior to activation.

4 Antibody concentrations: Investigate the effect of titrating your anti-CD8 and anti-cytokine antibodies. Titration of anti-CD8 will prevent any antibody-mediated blocking of or competition with the Pro5® Pentamer-binding site.

5 Protein Transport Inhibitor: Brefeldin A is an inhibitor of intracellular protein transport. Incubation of cells in culture with Brefeldin A leads to blockade of protein transport to the Golgi complex and accumulation of proteins in the endoplasmic reticulum. Brefeldin A is effective for enhanced detection of a majority of intracellular cytokines; however, it is advised that you investigate the use and efficacy of this reagent as well as other protein transport inhibitors such as Monensin in your specific assay system.

6 Background staining: When performing Pentamer staining, if the background staining is unusually high, it may be necessary to first block the cells with Fc block or 10% mouse serum prior to Pentamer staining. Further suggestions for

7

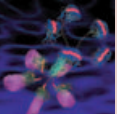
reduction of background staining can be found on page 102.

To carry out intracellular cytokine staining without Pentamer co-staining omit steps 1 and 4.

Pro5® Pentamer and Intracellular Cytokine Staining Protocol For Use With Murine Cells

Materials and equipment

- Cell sample, eg. murine splenocytes or lymph node cells
- Pro5® MHC Class I Pentamer conjugated to fluorescent label of choice (e.g. R-PE)
- Fluorescent-labeled anti-cytokine antibody eg. anti-Murine IFNγ-FITC; ProImmune (different fluorescent label to Pentamer)
- Fluorescent-labeled anti-CD8 antibody
- Peptide (stock at 50 mg/ml in DMSO - may be aliquotted and stored at -20°C)
- Leukocyte activation cocktail (LAC; optional) for use as a control for cytokine production (BD Biosciences #550583 - may be aliquotted and stored at -80°C)
- PBS Wash (2% fetal calf serum, 0.1% sodium azide in Phosphate buffered saline)
- Permeabilization buffer (0.1% saponin, 1% fetal calf serum, 0.1% sodium azide in PBS)
- 4% Paraformaldehyde
- Fix buffer (Appendix I)
- R10 medium (Appendix I)
- Brefeldin A (Sigma #15870)
- 12 x 75mm round bottom tubes
- Benchtop refrigerated centrifuge with swing out rotor and appropriate tube carriers



- Chilled Microcentrifuge
- Humidified CO_2 incubator at 37°C
- Vortex mixer

Protocol applicable for intracellular staining of IFNγ with Pro5® Pentamer

All procedures up to step 10 should be carried out in a suitable sterile tissue culture hood, using sterile buffers and aseptic conditions.

Procedure

1 Centrifuge Pro5® Pentamer in a chilled microcentrifuge at 14,000 x *g* for 5 minutes.

2 Prepare murine splenocytes by lyzing red cells then wash and resuspend in PBS Wash at a cell concentration of 2 × 10^7 cells/ml.

3 Transfer the cell suspension to individual tubes in 50 µl aliquots.

4 Add relevant titrated fluorescently labeled Pentamers to the desired tubes, and incubate for 10 min at 22ºC (non-stimulated single-color controls should not be stained at this stage). Add 10 µl PBS Wash to remaining tubes.

5 Add 500 µl PBS Wash to each tube. Centrifuge at 450 × *g* for 5 minutes at 10°C.

6 Aspirate supernatant. Agitate to disrupt cell pellets and resuspend in 200 µl R10 medium.

7 Dilute peptide stock 1:50 in R10 medium. Add 4 µl of this (20 µg/ml final) to each desired tube.

If using Leukocyte Activation cocktail (LAC) as a control, rapidly thaw this at 37°C in a water bath and add 0.33 µl of this to each desired tube. After use, discard the unused portion of Leukocyte Activation cocktail.

8 Place the tubes at 37°C in a humidified CO_2 incubator for 15 minutes to 1 hour.

9 Add Brefeldin A (10 µg/ml final) to the desired tubes (Note: LAC contains Brefeldin A) and return to the incubator. Incubate for 24 hours.

10 Remove tubes from the incubator. Centrifuge at 450 × *g* for 5 minutes at 10°C.

11 Aspirate supernatant. Resuspend desired cell pellets in 50 µl PBS Wash containing an optimally titrated amount of anti-CD8 antibody. Add 50 µl PBS Wash to remaining tubes.

Single-color controls should be stained at this stage. If additional phenotyping of samples is desired, antibodies to other cell surface markers or receptors may also be added at this time.

12 Incubate for 20 minutes on ice.

13 Add 500 µl PBS Wash to each tube. Centrifuge at 450 × *g* for 5 minutes at 10°C.

14 Aspirate supernatant. Agitate to disrupt cell pellets.

15 Add 200 µl 4% paraformaldehyde to each sample tube. Vortex tubes. Incubate for 20 minutes on ice.

This step will fix the cell morphology of the activated cells.

The procedure can be stopped at this point. Repeat steps 13 and 14. Resuspend the cells in 100 µl/tube PBS Wash. Cover and store the cells at 4°C for up to 3 days. To proceed, repeat steps 13 and 14. Resuspend the cells in 200 µl/tube permeabilization buffer and

proceed to step 17.

16 Add 200 µl permeabilization buffer to each tube.

17 Centrifuge at 450 × *g* for 5 minutes at 10°C. Aspirate supernatant.

18 Add 100 µl permeabilization buffer to the sample tubes that are to be stained with anti-cytokine antibody. Add 100 µl PBS Wash to the remaining tubes (i.e. Single-color controls).

19 Incubate for 5 minutes at room temperature.

20 Add an optimally titrated amount of FITC-conjugated anti-cytokine antibody to the desired sample tubes and mix.

21 Incubate for 20 minutes at room temperature.

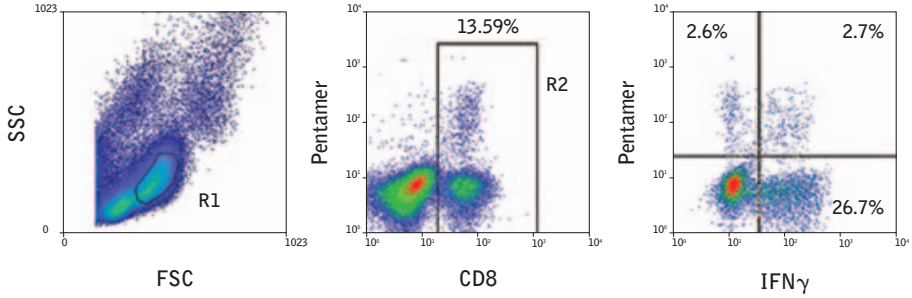
22 Add 200 µl permeabilization buffer to each tube and centrifuge at 450 × *g* for 5 minutes at 10°C. Aspirate supernatant and agitate tubes to disrupt the cell pellets.

23 Resuspend the cells in 200 µl fix solution. Vortex tubes. It is important to vortex well when adding this fixative so that cells do not clump.

24 The samples are now ready for data acquisition and analysis on a flow cytometer but may be stored overnight at 4°C in the dark prior to analysis.

See page 90 for additional notes for Pro5® Pentamer and intracellular cytokine staining protocol (also applicable to protocol for use with murine cells).

Figure 24: Example gating strategy for Pro5® Pentamer and intracellular cytokine staining



1 Create a 2D density plot showing FSC vs. SSC. Draw a tight region around the lymphocyte population (R1).

2 Create a second density plot, gated only on events encircled within R1, showing CD8 staining on the x-axis and Pentamer staining on the y-axis. Draw a region around all of the CD8-positive cells (R2).

3 Create a third density plot, gated on both R1 and R2, showing cytokine staining on the x-axis and Pentamer staining on the y-axis. Draw a quadrant that clearly separates positive cells from negative cells on both axes, and use quadrant statistics to obtain quantitative information about the frequency of antigen-specific, cytokine-positive T lymphocytes.

Figure 25: Example staining data for Pro5® Pentamer and intracellular cytokine staining

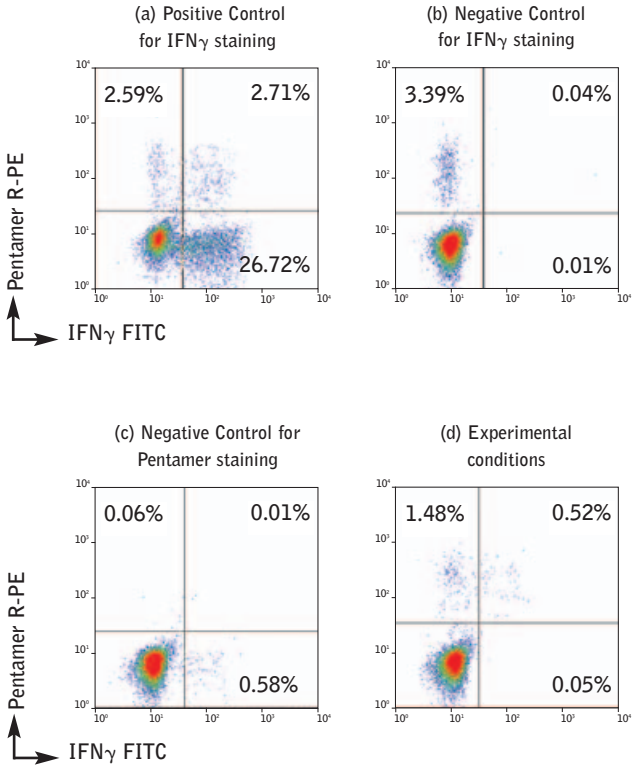


Figure 25

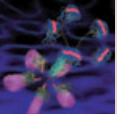
The figures illustrate IFNγ versus Pro5® Pentamer staining of live lymphocytes. PBMCs were incubated with either a negative control (non-specific) Pentamer A*0201/GLCTLVAML (EBV) or a Pentamer specific for the cells of interest B*0801/RAKFKQLL (EBV), then stimulated with PMA and ionomycin (nonspecific activation) or RAKFKQLL peptide (specific peptide activation) for 16 hours in the presence of Brefeldin A. Fixation, permeabilization and staining for IFNγ were carried out exactly as detailed in the protocol for use with human cells. The results show that IFNγ secretion is specific for activated cells (a, c & d), and that 26% of Pentamer positive cells have secreted IFNγ during a 16 hour incubation period (d).

25a: PBMCs stained with a Pentamer specific for the cells of interest, B*0801/RAK, then stimulated with PMA and ionomycin (non-specific activation) for 16 hours in the presence of Brefeldin A.

25b: PBMCs stained with a Pentamer specific for the cells of interest, B*0801/RAK, and incubated for 16 hours in the prescence of Brefeldin A only

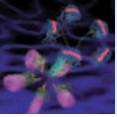
25c: PBMCs stained with a negative control (non-specific) Pentamer, A*0201/GLC and incubated with RAKFKQLL peptide (specific peptide activation) for 16 hours in the presence of Brefeldin A

25d: PBMCs stained with a Pentamer specific for the cells of interest, B*0801/RAK, then stimulated with RAKFKQLL peptide (specific peptide activation) for 16 hours in the presence of Brefeldin A



Section Eight

Troubleshooting Guide



Lack of Staining

1 The antigen-specific population is not present

In addition to staining by flow cytometry, it is advisable to use an alternative method to verify the presence or absence of an antigen-specific population. Functional assays such as ELISPOT, intracellular cytokine staining or other cytokine secretion assays are recommended.

2 The antigen-specific population is below the threshold of detection

The threshold of detection of antigen-specific events depends on the type of cells used and their method of preparation. For human PBMCs the threshold is around 0.02%. The threshold of detection for mouse splencoytes is likely to be in the region of 0.1%. Detecting a smaller population is possible but thorough protocol optimization will be required.

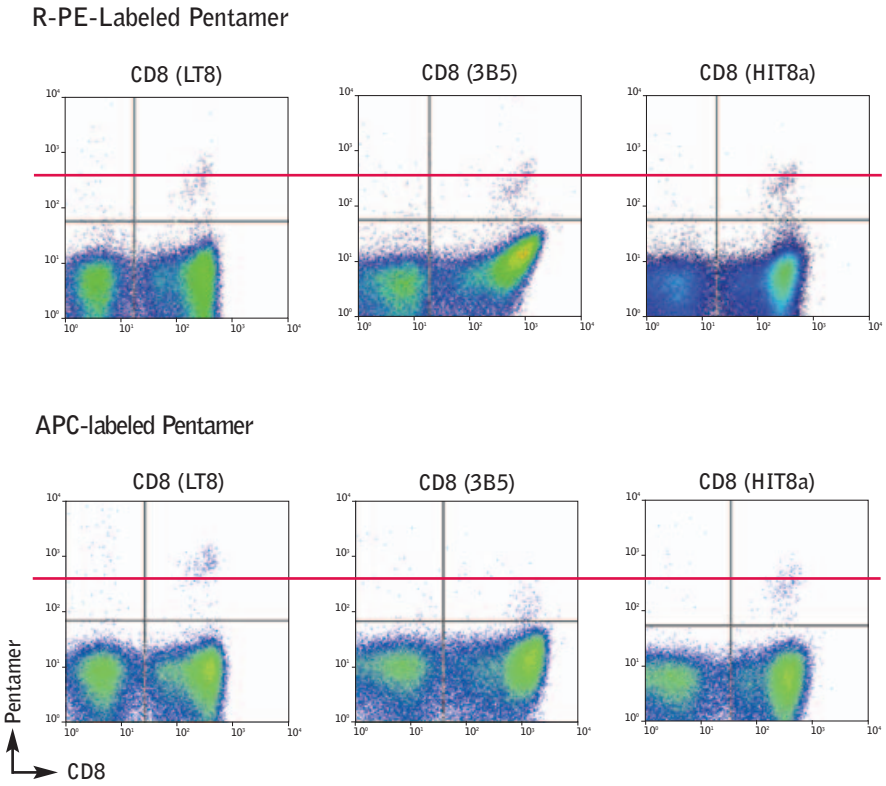
An improvement in results may be achieved by enriching the population of T cells. This may be done using magnetic beads (Section Seven) or by stimulation of cells in culture using peptides or antibodies. It is important to note that cells will have a different phenotypic profile following stimulation.

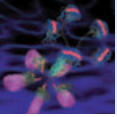
3 Use of sub-optimal anti-CD8 antibody clone

The anti-CD8 antibody used for co-staining should be selected carefully as some clones interfere with Pentamer binding to the T cell receptor (TCR). ProImmune has carried out extensive testing of Pentamers with different anti-CD8 clones and staining conditions. Clones LT8 (human) and KT15 (mouse) are recommended, as they do not block binding of Pentamers to the TCR. Both of these antibody clones are available from ProImmune.

Figure 26

The figure compares staining of R-PE- and APC-labeled Pentamers with three different anti-CD8 clones. Optimal staining is seen when the preferred clone LT8 is used, compared with clones 3B5 and HIT8a, particularly when using APC-labeled Pentamers. The red line aids comparison of the Pentamer positive populations in the plots. The staining is consistently bright when anti-CD8 clone LT8 is used.





4 Insufficient events acquired

When looking at rare populations, such as antigen-specific T cells, it is important to collect as many cells as possible. If expecting a result of only 0.1% a sample size of 500,000 cells would show 500 dots on the density plot. It is important to bear in mind the expected frequency of events to be collected and adjust the starting sample size accordingly (page 26).

5 Reagent has expired

A considerable amount of time and effort is spent on cellular staining experiments, so it is important to make sure that all your reagents are within their expiry date. ProImmune guarantees all Pro5® MHC Pentamers for six months from delivery when stored according to instructions. All ProVE® MHC Pentamers are guaranteed for three months from delivery when stored according to instructions. Unlabeled and biotin-labeled Pentamers may be stored for longer periods at -80ºC.

Non-Specific Staining

Non-specific staining is defined as events seen in the CD8-negative, Pentamer-positive quadrant of antigen-specific cells, or Pentamer-positive staining of negative control cells.

Common reasons for non-specific staining

1 Too much Pentamer for the number of cells

One test of labeled-Pentamer (10µl) should stain 1-2 x 10^6 antigen-specific cells. However, it is recommended that the Pentamer is titrated prior to use (page 48).

2 Temperature used for staining was too high

Pentamer staining should be carried out at room temperature (22°C) where possible. Staining at temperatures above this will increase non-specific staining (page 46).

3 Incubation time with Pentamer was too long

Pentamer staining for 10 minutes at room temperature (22°C) is sufficient. However, if staining at 4°C the incubation time may be increased to 30 minutes. Incubation for longer periods may increase background (page 46).

4 Pentamer was not centrifuged prior to staining

The Pentamer should be centrifuged at 14,000 x *g* for 3-5 minutes to isolate any aggregates.

5 Insufficient washing between staining steps

Cells should be washed with a 10x volume of buffer between staining steps (some specificities may require 2 washes between steps).

6 Buffer contains phenol red

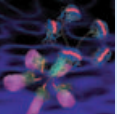
If using RPMI 1640, HBSS or DMEM as a medium in which to stain cells it is recommended that this does not contain phenol red, which can contribute to autofluorescence and thus appear as non-specific staining on analysis plots.

7 Pentamer binds non-specifically to the cells

In very rare cases the Pentamer may bind non-specifically to the cell surface. This can be eliminated by first blocking the cells with 4 μg/ml pure mouse IgG or 10% human or mouse serum.

8 Inaccurate setting of the live lymphocyte gate

Dead or dying cells incorporate fluorescent entities and must be excluded correctly (page 57).



Technical Support

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	Fax:	+1 775 206 4635
All other countries	Telephone:	+44 870 042 7279
	Fax:	+44 870 712 0588
All countries	E-mail:	enquiries@proimmune.com
	Web:	www.proimmune.com

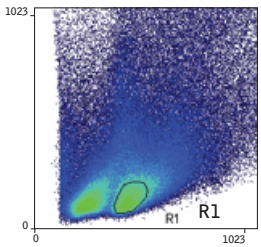
To help us to provide technical assistance with the minimum possible delay, please have answers ready to the following questions when you call or write.

1. Product code and batch number of reagent, e.g. Pentamer F001-2A, batch JP/1234-01; if you have purchased more than one of the same product also note the batch numbers of the other products and whether or not the same problems were encountered.
2. Type of cells stained e.g. human/mouse, PBMC, splenocytes, lymph nodes, fresh/frozen.
3. How many cells were stained per sample?
4. Was the Pentamer centrifuged before use (14,000 x *g* for 3-5 minutes)?
5. Volume of Pentamer and/or Fluorotag/Biotag (µl) used per sample, and total staining volume.
6. Temperature at which cells were incubated with Pentamer/Fluorotag/Biotag.
7. Time of incubation with Pentamer/Fluorotag/Biotag.
8. Order in which Pentamer and anti-CD8 antibody were added to cells e.g. Pentamer first then antibody, or other.
9. Which anti-CD8 antibody was used (clone and fluorescent label)?
10. What functional evidence do you have to indicate the presence of antigen-specific cells, e.g. ELISPOT, Chromium release assay?

In order to resolve any Pentamer staining issues as quickly as possible, it is useful for our technical support team to view staining data showing the problem. The data shown in figure 27 gives an example of the type of data that allows us to diagnose the problem. Data may be sent in MS PowerPoint or PDF format. Raw data files (FCS or LMD) are also accepted.

Figure 27: Example data to supply to technical support team

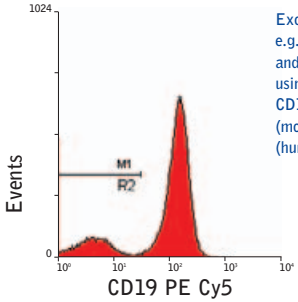
(a)



Live lymphocyte gate, set around the highest density cell population.

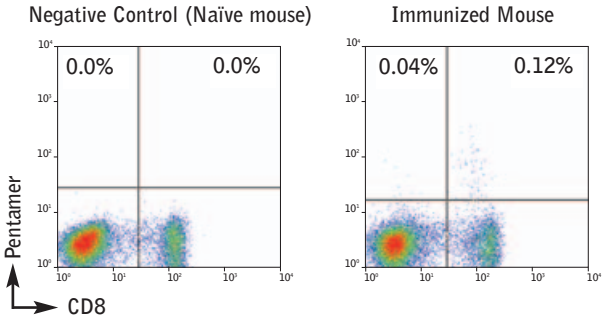
Note that cells which have been stimulated may need a larger gate to accommodate blast-like cells.

(b)



Exclusion gating, e.g. for B cells and/or NK cells, using antibodies to CD19 and CD49b (mouse) or CD56 (human).

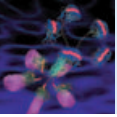
(c)



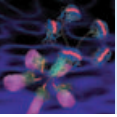
Density plot showing Pentamer vs CD8 with statistics showing % live lymphocytes that are Pentamer positive.

(d) Details of reagents used

	Reagent	Channel	Label
Pentamer	H-2Kb/SIINFEKL	FL2	R-PE
CD8 antibody	Rat anti-mouse CD8 (clone KT15)	FL1	FITC
Other antibodies	Rat anti-mouse CD19 (clone 6D5)	FL3	PE Cy5
Cell Type	Splenocytes from immunized mice		



Appendices



APPENDIX I - Solution Components

Wash Buffer

0.1% BSA, 0.1% sodium azide in PBS

Fix Buffer

1% heat-inactivated fetal calf serum (HI-FCS), 2.5% formaldehyde in PBS

Ammonium Chloride Lyzing Solution

0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, or a commercial preparation e.g. PharM Lyse™, BD Biosciences #555899

R10 Medium

RPMI 1640, containing 10% HI-FCS, 10mM HEPES, 2mM L-Glutamine, 50mM 2-ME, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin

APPENDIX II - Optimal Titration of Antibodies

1 Make a serial dilution of antibody stock in wash buffer (Appendix I), figure 28 a & b.

2 Make a 150 µl suspension of cells of the appropriate type (to include both positive and negative populations for the antibody under test) at 2 x 10^8 cells/ml. Add 25 µl to each tube figure 28c.

3 Incubate for 20 minutes on ice (4ºC), shielded from light.

4 Wash with a 10x volume of wash buffer and centrifuge.

5 Aspirate supernatant and resuspend cells in Fix solution (Appendix I)

6 Analyze by flow cytometry, gated on viable cells.

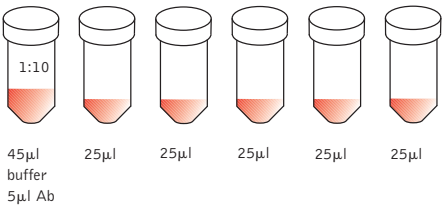
7 Print a page with the appropriate histograms, figure 29.

8 Choose a titration.

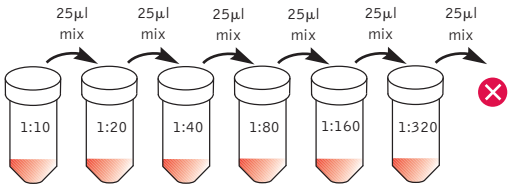
Use the histograms to select the appropriate dilution; negative cells should be completely contained in the first log decade; the positive peak should be at optimum fluorescence (i.e. positive cells remain higher up the log scale).

Figure 28

(a) Prepare tubes for dilution series



(b) Make antibody dilutions



(c) Add 5 x 10^6 cells in 25 µl buffer

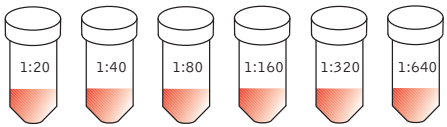
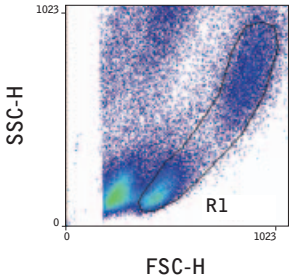
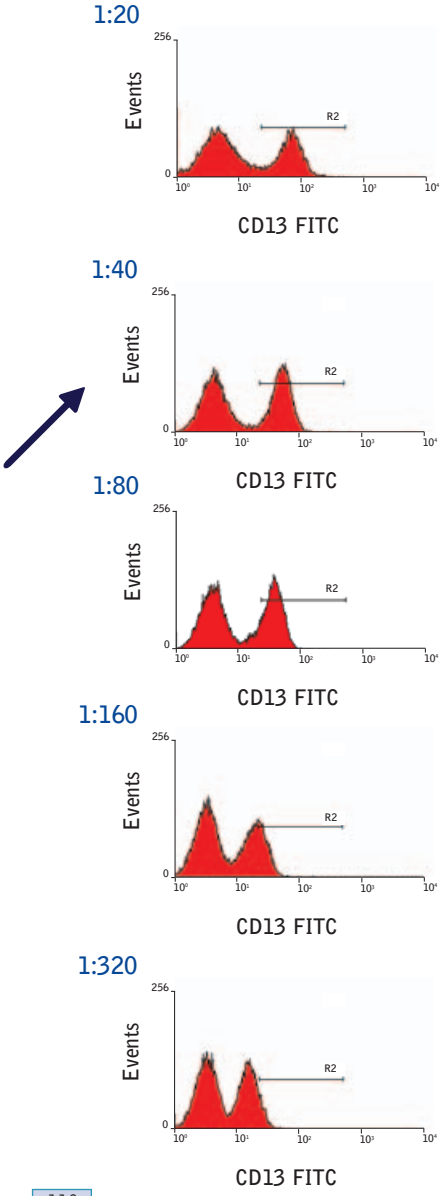


Figure 29
Titration of anti-human CD13-FITC on PBMCs

Live gate R1, note: CD13 is expressed on monocytes but cells are also gated on lymphocytes in order to view a negative population.

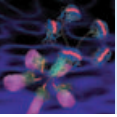


Negative cells should be completely contained in the first log decade; the positive peak should be at optimum fluorescence.



APPENDIX III - Table of Fluorochromes

	Typical Filters	Fluorochromes			Viability Probe
HeNe Laser 633 nm / Red Diode 635 nm	780/60	APC-Alexa Fluor® 750 (775 nm)	APC-Cy7 (774 nm)		
	720/40	APC-Cy5.5 (694 nm)	Alexa Fluor® 700 (723 nm)		
	660/20	APC (660 nm)	Alexa Fluor® 647 (668 nm)	Cy5 (666 nm)	
Argon (Blue) Laser 488 nm / Green Laser 543 nm	780/60	PE-Cy7 (767 nm)	PE-Alexa Fluor® 750		
	695/40	PE-Cy5.5 (694 nm)	PE-Alexa Fluor® 700 (723 nm)	PerCP-Cy5.5 (690 nm)	
	670/14	PE-Cy5 (670 nm)	PerCP (678 nm)		7-AAD (647 nm)
	610/20	PE-Alexa Fluor® 610 (628 nm)	PE-Texas Red® (615 nm)		Propidium Iodide (PI) (620 nm)
	575/26	R-PE (575 nm)	Cy3 (566 nm)		
	530/30	Alexa Fluor® 488 (519 nm)	FITC (525 nm)	Green Fluorescent Protein	
Violet Laser 405 nm	545/75	Cascade Yellow™ (558 nm)			
	585/42	Pacific Orange™ (552 nm)			
	440/40	Alexa Fluor® 405 (421 nm)	Pacific Blue™ (445 nm)	Cascade Blue® (420 nm)	
UV Laser 355 nm	440/40				DAPI (461 nm)



APPENDIX IV

Publications citing ProImmune Pentamers

Over 700 publications citing Pentamers are listed on our website at www.proimmune.com, indexed by research area.

APPENDIX V

Abbreviations

APC	Allophycocyanin
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CMV	Cytomegalovirus
DMSO	Dimethyl Sulphoxide
EBV	Epstein-Barr Virus
ELISPOT	Enzyme-Linked Immunospot assay
FACS	Fluorescent Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FL	Fluorescence Parameter
FSC	Forward Scatter
IFNγ	Interferon gamma
IL	Interleukin
MHC	Major Histocompatibility Complex
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
RBC	Red Blood Cell
R-PE	R-Phycoerythrin
SA	Streptavidin
SSC	Side Scatter
TCR	T Cell Receptor

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