Rapid Cell Separation using Pro5® Pentamers and Magnetic Beads

Magnetic cell separation is a simple technique used to isolate pure populations of antigen-specific lymphocytes for use in all kinds of in vitro and in vivo experiments. The advantages of magnetic cell separation are its speed and simplicity – it can be carried out on your own bench and there is no requirement to book and set up a flow cytometry-based sort. Magnetic cell separation can also be used as a pre-enrichment step before flow cytometry sorting to reduce both the number of cells to be sorted and the necessary sorting time.

To get the best results from magnetic cell separation, you need to use highly specific reagents, such as ProImmune’s Pro5® MHC Class I Pentamers. The staining reagent is used in conjunction with a magnetic bead system to gently pull out antigen-specific lymphocytes from a cell preparation. Miltenyi Biotec’s MACS® system is perhaps the best-known magnetic separation system, but other companies, including StemCell Technologies™, Invitrogen®, and R&D Systems®, offer similar products.

Improved magnetic separation with Pro5® Pentamers:

Professor Paul Moss of the University of Birmingham, UK, has used Pentamers to isolate CMV-specific T cells for potential use in stem cell transplant patients, who can fall victim to opportunistic CMV infection.

Figure 1: Anti-biotin magnetic bead-based separation of CMV epitope-specific T cells from donors using A*02:01/NLVPMVATV multimers. The purity of selection is much greater with ProImmune Pentamers versus tetramers. As a consequence cells prepared using Pentamers will be much more effective when used in adoptive transfer than cells separated using tetramers, which will contain many more non-specific T cells. The viability of the selected cells was also shown to be excellent. Read more about Prof. Moss and his work on our website.

PROIMMUNE’S PRO5® MHC CLASS I PENTAMERS ARE FOR RESEARCH USE ONLY AND ARE NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.
**Magnetic Cell Separation Methodology**

There are two main methods for magnetic bead separation (see table). With both methods, cells are mixed with paramagnetic beads (these only exhibit magnetic properties when placed within a magnetic field) specific for a particular target (for example, anti-FITC).

<table>
<thead>
<tr>
<th></th>
<th>Tube-based methods</th>
<th>Column-based methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Materials required</strong></td>
<td>Beads, magnet</td>
<td>Beads, magnet, magnet stand, columns</td>
</tr>
<tr>
<td><strong>Magnet size</strong></td>
<td>Different magnet sizes available, according to tubes inserted.</td>
<td>Different magnet sizes available, according to sample volume used.</td>
</tr>
<tr>
<td><strong>Flexibility</strong></td>
<td>Same magnet for all purposes, many bead specificities available.</td>
<td>Different magnets required for some columns. Various columns for enrichment or depletion and for different sample volumes. Many bead specificities available.</td>
</tr>
<tr>
<td><strong>Positive or negative isolation?</strong></td>
<td>Can do both, but may need to detach the beads from cells for further analyses following positive selection.</td>
<td>Can do both, and can proceed with further analyses straight from column</td>
</tr>
<tr>
<td><strong>Unusual buffer?</strong></td>
<td>No</td>
<td>No, but de-gassed buffer is recommended.</td>
</tr>
<tr>
<td><strong>Centrifugation steps</strong></td>
<td>None</td>
<td>One</td>
</tr>
<tr>
<td><strong>Washing steps</strong></td>
<td>None, although beads may need washing prior to use.</td>
<td>One</td>
</tr>
<tr>
<td><strong>Automation available?</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

With the tube-based method, target-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 target-bead complexes. Separation is gentle and does not require centrifugation or columns. In the column-based method, target-bead complexes pass through a separation column by gravity flow, which is placed in a strong, permanent magnet. The column matrix serves to create a high-gradient magnetic field that retains bead-bound complexes while non-labeled cells flow through. Following removal of the column from the magnetic field, the retained cells are eluted.
Example Experiments

To demonstrate how magnetic separation can be used, we performed two example.

Column-based separation

In the first experiment we used column-based magnetic separation to enrich antigen-specific T cells from PBMC. 1x10⁷ PBMC were incubated with 5 tests (50μl) R-PE labeled A*02:01/EBV Pentamer (GLCTLVAML) for 10 minutes at room temperature (22°C), followed by 20μl 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). Isolated cells were incubated with anti-CD8 antibody for 20 minutes on ice prior to fixation and analysis by flow cytometry. Owing to the small size of the Miltenyi microbeads, cells may be analyzed by flow cytometry with beads still attached.

Tube-based separation

Figure 6 shows the results of an experiment in which antigen-specific cells were depleted from a peripheral blood suspension using a biotin-labeled Pro5® Pentamer and streptavidin microbeads (LodeStars™ 2.7 Streptavidin; Polymer Laboratories) following the protocol available on our website (see below). A sample of the original cell population (pre-depletion) and supernatant following isolation (post-depletion) was incubated with anti-CD8-FITC antibody plus streptavidin-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).

Protocols for both of these methods can be downloaded from our website.
Magnetic Cell Separation Case Studies

Hepatitis B Virus: Functional analysis of HBV-specific T cells

Understanding the precise mechanisms causing liver damage in Hepatitis B infection is crucial if this debilitating disease is to be treated effectively. Intrahepatic cytokine production triggers the recruitment of mononuclear cells, which sustain acute and chronic liver damage. Using virus-specific CD8+ T cell clones and primary human hepatocytes, Gehring et al. analyzed the modulation of CD8+ T cell function following recognition of peptide pulsed or virally infected hepatocytes.

The T cell clones were generated from peripheral blood mononuclear cells (PBMC) from HLA-A2+ HBV infected donors by labeling the cells with R-PE-conjugated HBC18-27-specific Pro5® Pentamer and purifying via magnetic cell sorting using anti-R-PE microbeads. Separated cells were cloned by limiting dilution assay and clones were expanded using allogeneic irradiated PBMC feeder cells.

Limiting the amount of viral antigen in infected human hepatocytes preferentially stimulated CD8+ T cell degranulation, shown by CD107alpha expression without concommitant TNF-alpha expression. This degranulation may lead to hepatocyte damage that is directly caused by virus specific T cells in chronic hepatitis patients. The data suggest a mechanism where virus-specific CD8 T-cell function is influenced by the quantity of virus produced within hepatocytes.

Epstein Barr Virus: life-saving use of Pentamers and magnetic cell separation for a transplant patient.

Three months after receiving a cord blood transplant for acute myeloid leukemia, a young patient presented at the Karolinska University Hospital Huddinge, Stockholm, with Epstein-Barr virus (EBV) associated lymphoma, high blood EBV titers and lesions in the lungs, liver, adrenal gland and kidneys indicative of post-transplant lymphoproliferative disorder (PTLD). The patient was unresponsive to standard PTLD treatments and as no other options were available, adoptive EBV-specific CTL transfer from the patient’s mother was performed.

After only 36 hours, the frequency of Pentamer-positive antigen-specific T cells detected in the patient increased from 0.3% of total CD8+ cells to 4.4%, indicating that the donor cells had expanded rapidly in vivo. Furthermore, following the CTL transfusion, EBV titers fell back to normal levels within days. Prior to CTL infusion a CT scan showed considerable growth of lymphoma in the lungs, liver, adrenal gland and kidney. 189 days later, a comparative CT scan showed only remnant streaks in the thorax, no sign of lesions in the liver and only minor lesions in the kidney. Additionally the left adrenal gland, which had been very enlarged, returned to normal size. At 12 months post-transplant, after being admitted with EBV virus in the tonsils, blood and colon, the patient was given a second infusion, using Pro5® Pentamers to isolate both A*02:01/GLCTLVAML and additional A*02:01/CLGGLLTMV EBV specific cells (2x10⁴ CTL/kg). By 72 hours post-infusion, all signs of tonsillitis and enteritis had been cured.

The use of the Pro5® Pentamers was central to developing this novel and successful method for treatment of EBV-associated complications following stem cell transplantation and offers new life-saving strategies for rapid treatment of PTLD patients.


2011: Three years after treatment, the patient is thriving, and Michael Uhlin and his co-workers continue to explore the potential offered by adoptive T-cell therapy. Read editorial in Immunotherapy. [PubMed ID: 21322751]
Help With Magnetic Separation

Q. ProImmune Pentamers are available with R-PE, APC and Biotin tags - which are best to use in magnetic cell separation?

A. Anti-fluorochrome, streptavidin, and anti-biotin microbeads are widely available. Traditionally the combination of streptavidin beads and biotin label has been favored, but all will work. The advantage of using R-PE or APC labeling in conjunction with microbeads is that you can check your purity immediately after separation using flow cytometry.

Q. What should I look out for when preparing my cells?

A. Make sure your cell preparation is as clean as possible. Good red-cell lysis is essential, and make sure your cells are well-filtered. We recommend using a 40µM filter, and several filtration steps if you notice fatty agglomerations – this can be an issue when using whole-tissue cell suspensions from mouse.

Q. How long do I incubate my magnetic beads and Pentamer-stained cells, and at what temperature should I perform the incubation?

A. Correct incubation conditions are important to get the most specific magnetic labeling. Incubation times and temperatures may vary but will have been optimized by the manufacturer of your beads and you should follow their recommendations as closely as possible. Note that there is a slight difference between ‘on ice’ and ‘4°C’, with the latter meaning you can just put your tubes in the fridge.

Q. How much Pentamer and how many microbeads should I use?

A. Always begin by performing a titration of the Pentamer to see which concentration gives you the best staining by flow cytometry. ProImmune recommends using 10µl (one test) for 1-2x10^6 cells, but you may be able to use less. Whichever concentration of microbeads your manufacturer recommends should also be your starting point.

Q. Are my cells stressed by the separation procedure?

A. Your cells undergo a little mechanical stress during the separation, but spend far less time out of culture (typically only around half an hour) than they would during flow-cytometry sorting. You can check cell viability at any step of the procedure by using trypan blue staining to give you confidence in your protocol. Try resting your cells in rich culture medium for at least an hour before using them in further experiments.

Q. Can I use several rounds of sorting to purify my cells further?

A. It’s always possible if you really want to, although a single separation with a staining reagent as specific as ProImmune’s Pro5 Pentamers should be fine. Some researchers choose to use a negative selection step (for example, depletion of B cells and CD4^+ cells) followed by positive enrichment with their specific staining reagent.

Q. Do I have to take the magnetic beads out afterwards?

A. It depends on your bead system. For example, Miltenyi MACS® beads are very small – so will not interfere with flow cytometry, and are composed of a biodegradable matrix of iron oxide and polysaccharide so will degrade after a few days in culture or in their new host. Always check your manufacturer’s protocol.

Any other Questions?

Email us at enquiries@proimmune.com

Visit www.proimmune.com

Call our technical support team:

+1 888 505 7765 (US and Canada)

+44 870 042 7279 (rest of world)
**Publications**

Below is a selection of publications citing the use of Pro5® Pentamers in conjunction with magnetic cell separation. A full list of all Pentamer Citations is available online.

**Cancer**


**Viruses (Influenza, CMV, EBV, Hepatitis, HIV)**


Bengsch, B. et al. (2007). Analysis of CD127 and KLRG1 expression on hepatitis C virus-specific CD8+ T cells reveals the existence of different memory T-cell subsets in the peripheral blood and liver. J. Virology. 81: 945-953. [PubMedID:17079288]


Jarvis, LB. et al. (2008) Human leukocyte antigen class I-restricted immunosuppression by human CD8+ regulatory T cells requires CTLA-4-


Infectious disease


Basic Immunology


