MACS molecular

3×3 Stimulation of peripheral blood mononuclear cells for isolation and expansion of infectious HIV

Special protocol

µMACS™ VitalVirus HIV Isolation Kit Order # 130-092-805

Contents

1. Description

1.1 Background

Stimulation of peripheral blood mononuclear cells (PBMCs) by various means leads to an upregulation of various cell surface receptors and transcription factors. A number of publications point out the advantageous use of stimulated and CD8-depleted PBMCs for infectious HIV assays: Parallel stimulation of T cells with 0.5 µg/mL phytohemagglutinin (PHA), 5 µg/mL PHA, and surface-immobilized anti-CD3 mAb increased the consistency of PBMC infection with HIV.[1,2] This very effective stimulation upregulates receptors required for HIV cell entry[3] as well as factors required for efficient HIV transcription and replication[4]. In addition, since CD8$^+$ T cells release HIV-suppressive factors[5] it is beneficial to remove this cell type before applying PBMCs to infectious HIV assays.
Until now, isolation of intact virus from plasma samples with low viral load (below 100,000 HIV-1 RNA copies/mL) has been difficult due to the relative insensitivity of standard isolation methods. The 3×3 stimulation protocol for donor PBMCs described below—combined with the µMACS™ VitalVirus HIV Isolation Kit using CD44 MicroBeads—leads to efficient isolation of infectious virions, especially from samples with low viral load.

1.2 Research applications

3×3 stimulated PBMCs can be used for neutralization assays, virus isolations, and virus productions.[1] The 3×3 stimulation protocol, combined with the µMACS VitalVirus HIV Isolation Kit, results in 100% successful isolation of intact virions in plasma samples with more than 10,000 RNA copies/mL and in 50% successful isolation of infectious HIV in samples with RNA copies/mL below 10,000. On average, the combined protocols enable one infectious dose per 1,385 virus particles (2,770 RNA copies) to be detected.

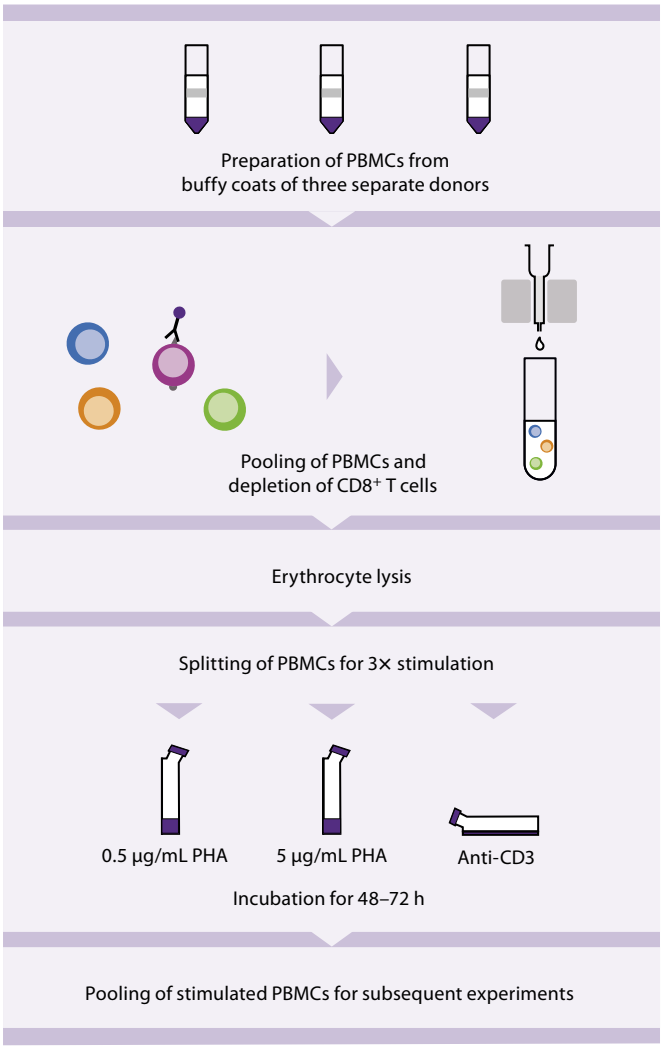

Figure 1: Scheme of 3×3 PBMC stimulation.

1.3 3×3 Stimulation of donor PBMCs for infectious virus assay

To isolate infectious HIV, the target cells, in this case PBMCs, need to be pretreated to increase the probability of cell infection and viral replication. First, PMBCs from three donors are depleted of CD8$^+$ T cells, which would otherwise inhibit viral replication[5] and lower the content of infectious HIV. Then, the three donor CD8$^-$ PBMCs are pooled and split into three fractions that are individually stimulated with 0.5 µg/mL PHA, 5 µg/mL PHA, and surface-immobilized anti-CD3 mAb. Finally, they are pooled again in high-IL–2 medium and are ready to be infected with viable HIV-virions.

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1.4 Reagent and instrument requirements

- CD8 MicroBeads, human (# 130-045-201)
- Erythrocyte lysis buffer (e.g. ACK Lysing Buffer, BioWhittaker)
- Phosphate-buffered saline (PBS) containing 2 mM EDTA (PE)
- Basic medium: RPMI 1640, 2 mM Glutamine, 10% FCS, 100 U/mL Penicillin/Streptomycin (P/S), 10 U rIL-2
- High-IL–2 medium: RPMI 1640, 2 mM Glutamine, 10% FCS, 100 U/mL P/S, 100 U rIL-2
- Medium for OKT3 hybridoma cells: RPMI supplemented with 10% FCS, 100 U/mL (P/S)
- For stimulation:
  Phytohemagglutinin (PHA)
  - 0.5 µg/mL PHA basic medium (PHA 0.5)
  - 5 µg/mL PHA basic medium (PHA 5)
  Supernatant from OKT3 murine hybridoma cell culture producing anti-human CD3 mAb, ATCC (CRL-8001)
- Only for anti-CD3 mAb stimulation: overnight pretreatment of cell culture flasks (75 cm^2) with 8 mL per flask of OKT3 hybridoma supernatant or 10 mL per flask of purified OKT-3 monoclonal antibody in PBS (10 µg/mL)
- Appropriate cell culture flasks (e.g. 75 cm^2) and plates
- 50 mL conical tubes

1.5 Related products

- CD8 Antibodies
- MACS® Cell Analysis Reagents
- MultiMACS™ VitalVirus HIV Isolation Kit (12×8, # 130-092-806)
- MultiMACS VitalVirus HIV Isolation Kit (4×96, # 130-092-807)
- µMACS™ VitalVirus HIV Isolation Kit (# 130-092-805)
- µMACS VitalVirus HIV Isolation Starting Kit (# 130-092-833)

2. Protocol for PBMC stimulation

Before starting

▲ Pre-warm media necessary for cell culture to 37 °C.

Part of the protocol is the stimulation of one of three cell fractions with surface-immobilized anti-CD3 mAb. Cell culture flasks have to be prepared a day before the stimulation experiment.

1. To immobilize anti-CD3 mAb OKT3 onto cell culture flasks (75 cm^2), coat flask with 8 mL OKT3 hybridoma supernatant or 10 mL of purified OKT-3 antibody in PBS overnight.
   ▲ **Note:** Flasks should be incubated horizontally.
2. Remove coating solution prior to stimulation.

2.1 PBMC preparation

1. Prepare PBMCs from three buffy coats from healthy, HIV-negative blood donors. A general protocol about density gradient centrifugation is available on www.miltenyibiotec.com.
2. Transfer each PBMC layer to a fresh 50 mL conical tube.
3. Wash once with 50 mL of PBS/2 mM EDTA (PE) with centrifugation at 300×g for 10 minutes. Carefully aspirate the supernatants completely.
4. Resuspend each cell pellet in 50 mL of PE and measure granulocyte content, it should be below 1×10^6 per mL.
5. Centrifuge at 150×g for 20 minutes to remove platelets. Carefully aspirate the supernatants completely.
   ▲ **Note:** If the supernatant remains cloudy after this step the platelet content is too high; a further wash is necessary.
6. Combine cells from all three samples in one 50 mL tube in an appropriate volume of PE and count cells. Dilute cell suspension to 10^8 cells/mL for $CD8^+$ T Cell Depletion, section 2.2.

2.2 $CD8^+$ T cell depletion

1. Deplete $CD8^+$ T cells from the PBMCs using CD8 MicroBeads, human (# 130-045-201, see product datasheet for protocol) otherwise, this T cell subset will inhibit HIV replication.[5]
   ▲ **Note:** Either process all PBMCs or choose the number of PBMCs dependent on the downstream application (e.g. neutralization assay, virus isolation, or virus production).
2. After magnetic depletion of the $CD8^+$ cells, pellet the negative fraction at 150×g for 10 minutes. Carefully aspirate the supernatant.

2.3 Erythrocyte lysis

1. Add 5 mL of erythrocyte lysis buffer (e.g. ACK Lysing Buffer, BioWhittaker), resuspend pellet carefully, and incubate at room temperature for 5 minutes.
2. Add 45 mL of PBS and centrifuge at 150×g for 10 minutes. Carefully aspirate the supernatant.
3. Resuspend cell pellet in 50 mL of PBS and count the cells. Centrifuge at 150×g for 10 minutes. Carefully aspirate the supernatant.
4. Resuspend pellet in 3 mL of basic medium (RPMI 1640 supplemented with 10% FCS, 10 U/mL rIL-2, 2 mM glutamine, and 100 U/mL P/S).

2.4 Stimulation of PBMCs with 0.5 µg/mL PHA, 5 µg/mL PHA, and OKT3

▲ During stimulation, culture flasks should be incubated vertically; only flasks for OKT3-coating and -stimulation are kept horizontally.

▲ Prepare required media: Supplement basic stimulation medium with 0.5 µg/mL PHA and 5 µg/mL PHA.

▲ Discard OKT3 medium from cell flasks that are used for subsequent OKT3-stimulation.

Before starting

1. Stimulation with PHA (0.5 µg/mL): Adjust 1 mL of PBMCs from section 2.3, step 4, to 4×10^6/mL in basic stimulation medium, supplemented with 0.5 µg/mL PHA, and incubate for 72 hours.
2. Stimulation with PHA (5 µg/mL): Adjust 1 mL of PBMCs from section 2.3, step 4, to 4×10^6/mL in basic stimulation medium, supplemented with 5 µg/mL PHA, and incubate for 72 hours.
3. Stimulation with surface immobilized anti-CD3 mAb: Remove coating solution from cell flask incubated overnight with OKT3 medium. Adjust 1 mL of PBMCs from section 2.3, step 4, to 4×10^6 cells/mL in basic stimulation medium and transfer cells into the OKT3-coated cell flasks. Incubate for 72 hours.

2.5 Using 3×3 stimulated PBMCs for infection assays

1. After 48–72 hours of stimulation, pellet cells from the three different stimulation conditions at 150×g for10 minutes.
2. Resuspend cells in fresh high-IL–2 stimulation medium (RPMI 1640, 10% FCS, 100 U/mL IL-2, 2 mM glutamine, 100 U/mL P/S) at the cell density corresponding to the size of culture plate or flask required for downstream culturing (table 1).
3. Combine equal amounts of PBMCs from the three stimulations for subsequent use in neutralization assays, titrations, virus productions, etc.

   ▲ **Note:** PBMCs stimulated in this way can be used for fresh HIV infection—and subsequent HIV isolation— for a maximum of 48 hours; after this timepoint, the susceptibility of stimulated PBMCs to HIV infection decreases.
4. After 6–8 days, fresh high-IL2 stimulation medium may be added to the cell cultures.

Vessel	No. of 3×3 stimulated PBMCs	Sample volume
96-well plate	1×10^5 cells / well	200 µL
24-well plate	1×10^6 cells / well	1 mL
75 cm^2 cell flask*	2×10^6 cells / mL	–

* Keep flask in an upright position.

Table 1: Suggested cell densities to seed 3×3 stimulated PBMCs for downstream experiments.

3. References

1 Trkola, A. *et al.* (1999) J. Virol. 73: 8966–8974.
2 Trkola, A. *et al.* (2003) J. Virol. 77: 13146–13155.
3 Bleuel, C.C. *et al.* (1997) PNAS 94: 1925–1930.
4 Rohr, O. *et al.* (2003) J. Leukoc. Biol. 74: 736–749.
5 Cocchi, F. *et al.* (1996) Science 270: 1811–1815.

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