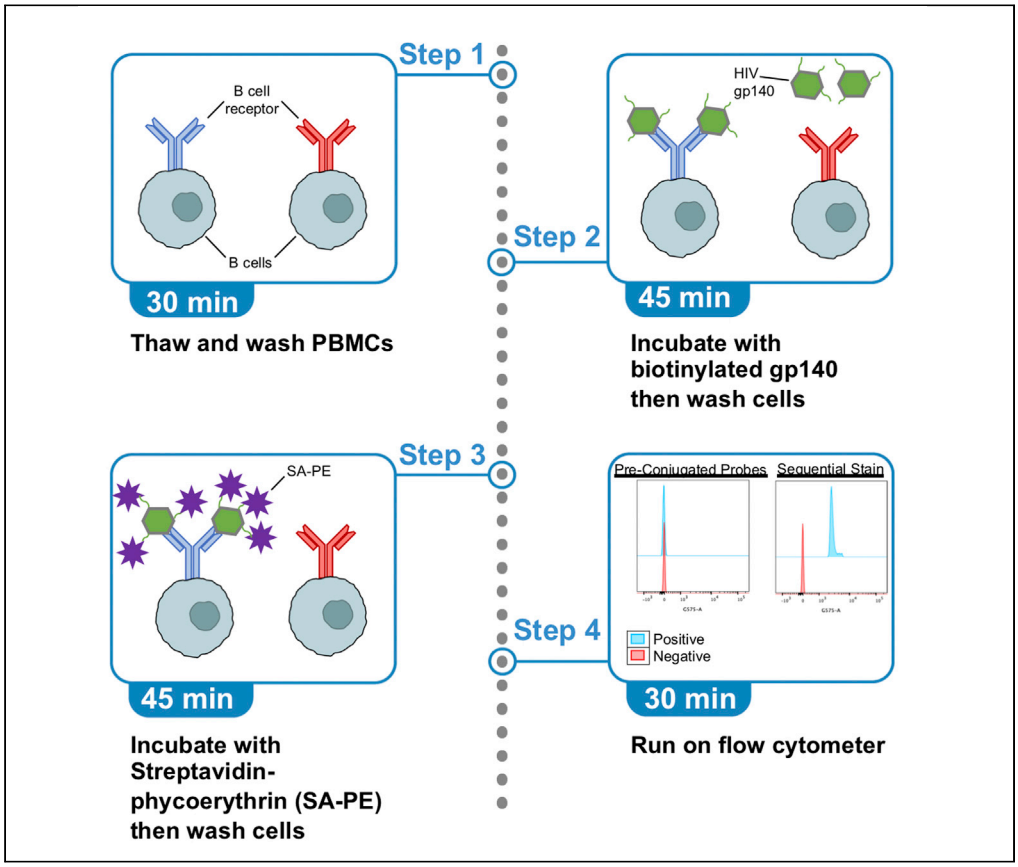
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Sequential staining of HIV gp140 to capture antigen-specific human B cells via flow cytometry



Protocols for efficient capture of antigen-specific B cells (ASBCs) are useful for understanding pathogen-specific B-cell responses during natural infection or vaccination. Fluorescently labeled tetramerized probes are classically used to capture ASBCs, but many occlude valuable epitopes available for B-cell receptor binding. Here, we describe a bead assay to confirm ASBC receptor accessibility on probes and a sequential staining process to capture HIV gp140-specific B cells from human peripheral blood mononuclear cells.

Samantha M. Townsley, David J. Leggat, Madhu Prabhakaran, Adrian B. McDermott, Shelly J. Krebs

stownsley@hivresearch.org (S.M.T.)
david.leggat@nih.gov (D.J.L.)
skrebs@hivresearch.org (S.J.K.)

Highlights

Robust protocol provides approaches to capture antigen-specific B cells

Use of bead assays confirms epitope availability on fluorescently labeled tetramers

Sequential staining can be used as an alternative approach to tetramerized probes

Townsley et al., STAR Protocols 2, 100771 September 17, 2021
https://doi.org/10.1016/j.xpro.2021.100771

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Sequential staining of HIV gp140 to capture antigen-specific human B cells via flow cytometry

Samantha M. Townsley,[1,2,5,4,*] David J. Leggat,[3,5,4,*] Madhu Prabhakaran,[3] Adrian B. McDermott,[3] and Shelly J. Krebs[1,2,6,*]

[1]U.S. Military HIV Research Program, Center of Infectious Disease Research, Walter Reed Army Institute of Research, Silver Spring, MD 20910, USA

[2]Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., Bethesda, MD 20817, USA

[3]Vaccine Research Center, NIAID, NIH, Bethesda, MD 20892, USA

[4]These authors contributed equally

[5]Technical contact

[6]Lead contact

*Correspondence: stownsley@hivresearch.org (S.M.T.), david.leggat@nih.gov (D.J.L.), skrebs@hivresearch.org (S.J.K.)
https://doi.org/10.1016/j.xpro.2021.100771

SUMMARY

Protocols for efficient capture of antigen-specific B cells (ASBCs) are useful for understanding pathogen-specific B-cell responses during natural infection or vaccination. Fluorescently labeled tetramerized probes are classically used to capture ASBCs, but many occlude valuable epitopes available for B-cell receptor binding. Here, we describe a bead assay to confirm ASBC receptor accessibility on probes and a sequential staining process to capture HIV gp140-specific B cells from human peripheral blood mononuclear cells.

For complete details on the use and execution of this protocol, please refer to Townsley et al. (2021).

BEFORE YOU BEGIN

Background

This protocol describes a quality control test for the utility of different antigens as probes that can be used to isolate and characterize antigen-specific B cells. The procedure is divided into three steps; 1) assessing the antigens as tetramers, 2) sequential staining of antigens that fail as tetramers, and 3) use of sequential staining of antigens to capture antigen-specific B cells.

Biotinylate gp140 protein

Timing: 2 h

1. Thaw your gp140 protein of interest on ice. Biotinylate your gp140 using the FluoReporter Mini-Biotin-XX Protein Labeling Kit (Invitrogen) following the manufacturer's protocol to achieve 2–3 biotin molecules per antigen: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fmp06347.pdf&title=Rmx1b1JlcG9ydGVyIE1pbmktYmlvdGluLVhYIFByb3RlaW4gTGFiZWxpbmcgS2l0
   a. Store biotinylated gp140 at −80°C.

Tetramer preparation

Timing: 2 h

2. Thaw biotinylated gp140 on ice and aliquot 125 ng of gp140 into a 0.5 mL microtube.
   a. Dilute streptavidin-phycoerythrin (SA-PE) 1:125 in 1× PBS. Using a 10 μL tip and a P2 pipettor, add 1.15 μL diluted SA-PE to the 125 ng of gp140. Mix by pipetting up and down immediately >5 times vigorously.

   Optional: This is a general calculation for gp140 proteins. If desired, an exact calculation can be done on a per gp140 basis. Using the smallest volume possible, add enough SA-PE to your gp140 of interest to make a 4:1 molar ratio of biotinylated gp140:SA-PE. This will yield a tetramer with 4 gp140 epitopes bound to an SA-PE (Morris et al., 2011).
   b. Incubate at room temperature (20°C–22°C) for 30 min in the dark. Mix by pipetting up and down every 10 min for a total of 3 mixes.
   c. After the final mix, transfer tetramers to ice. Incubate for 1.5 h in the dark.
   d. Bring the volume of the tetramer up to 6.25 μL with 1× PBS to get a final concentration of 20 ng/μL of gp140. Keep on ice in the dark.

⚠ CRITICAL: Tetramers should be made fresh on the day of use.

Culture medium and buffer preparation

Timing: 30 min

3. See materials and equipment for preparation of needed materials.
4. Pre-warm R10 media at 37°C using a water bath. Generally, 20 min is sufficient to warm 500 mL of media.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC anti-human/mouse Integrin β7 Antibody, clone FIB504 (1:200 final dilution)	BioLegend	Cat# 321214 RRID:AB_830858
BV510 Mouse anti-human CD3, clone SP34-2 (1:200 final dilution)	BD Biosciences	Cat# 740187 RRID:AB_2739940
Brilliant Violet 510 anti-human CD14 Antibody, clone M5E2 (1:100 final dilution)	BioLegend	Cat# 301842 RRID:AB_2561946
Brilliant Violet 510 anti-human CD56 (NCAM) Antibody, clone HCD56 (1:100 final dilution)	BioLegend	Cat# 318340 RRID:AB_2561944
BV510 Mouse anti-human CD16, clone 3G8 (1:200 final dilution)	BD Biosciences	Cat# 563830 RRID:AB_2744296
BV510 Mouse anti-human CD4, clone SK3 (1:100 final dilution)	BD Biosciences	Cat# 562970 RRID:AB_2744424
Brilliant Violet 510 anti-human CD8a Antibody, clone RPA-T8 (1:100 final dilution)	BioLegend	Cat# 301048 RRID:AB_2561942
Brilliant Violet 570 anti-human CD20 Antibody, clone 2H7 (3:200 final dilution)	BioLegend	Cat# 302332 RRID:AB_2563805
Brilliant Violet 605 anti-human CD27 Antibody, clone O323 (1:50 final dilution)	BioLegend	Cat# 302830 RRID:AB_2561450
Brilliant Violet 711 anti-human CD10 Antibody, clone HI10a (1:20 final dilution)	BioLegend	Cat# 312226 RRID:AB_2565876
PE-Cy7 Mouse anti-human CD21, clone B-ly4 (1:50 final dilution)	BD Biosciences	Cat# 561374 RRID:AB_10681717
CD19-ECD, clone J3-119 (1:25 final dilution)	Beckman Coulter	Cat# IM2708U RRID:AB_130854
Ax680 anti-human CD38, clone OKT10 (1:200 final dilution)	NIH	N/A
PE-Cy5 Mouse anti-human IgM, clone G20-127 (1:20 final dilution)	BD Biosciences	Cat# 551079 RRID:AB_394036
BV421 Mouse anti-human IgG, clone G18-145 (3:100 final dilution)	BD Biosciences	Cat# 562581 RRID:AB_2737665

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Purified Mouse Anti-human IgG, clone G18-145	BD Biosciences	Cat# 555784 RRID:AB_396119
VRC01	(Zhou et al., 2010)	N/A
Biological samples		
Human peripheral blood mononuclear cells	(Townsley et al., 2021)	N/A
Chemicals, peptides, and recombinant proteins		
RPMI 1640 Medium	Gibco	Cat# 11875-085
Fetal Bovine Serum (FBS)	Gemini Bio Products	Cat# 10438018
LIVE/DEAD Fixable Aqua Dead Cell Stain	Thermo Fisher	Cat# L34957
Benzonase	Novagen	Cat# 70664-3
Streptavidin, R-Phycoerythrin Conjugate (SA-PE)	Thermo Fisher	Cat# S21388
Streptavidin, Allophycocyanin (APC) Conjugate (SA-APC)	Thermo Fisher	Cat# S32362
FluoReporter Mini-Biotin-XX Protein Labeling Kit	Invitrogen	Cat# F6347
DPBS (PBS) without Ca & Mg	Quality Biological	Cat# 114-057-101
Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat# 00-5523-00
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Biosciences	Cat# 552843
Mouse IgG Isotype Control	Invitrogen	Cat# 10400C
RV217.10204v0A founder gp140	(Townsley et al., 2021)	N/A
Experimental models: Cell lines		
Ramos cell lines	ATCC	Cat# CRL-1596
Software and Algorithms		
FlowJo 9.9.6 and FlowJo 10.7.1	FlowJo, LLC	https://www.flowjo.com
Other		
5 mL Polypropylene round-bottom FACS tube	Fisher	Cat# 352063
Falcon 15 mL Conical Centrifuge Tube	Fisher	Cat# 14-959-70C
Stericup Quick Release GP Sterile Vacuum Filtration System	Fisher	Cat# S2GPU05RE
0.5 mL Sarstedt screw cap microtube	Fisher	Cat# NC0912963
Invitrogen Countess Automated Cell Counter	Fisher	https://www.fishersci.no/shop/products/invitrogen-countess-automated-cell-counter-101-boxes-slides-3/p-4931442
BD Symphony A5 SORPARIA	BD Biosciences	Special Order Research Product

MATERIALS AND EQUIPMENT

R10 media		
Reagent	Final concentration	Amount
RPMI 1640 Media	90%	450 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 1 month.		

FACS wash buffer		
Reagent	Final concentration	Amount
DPBS (PBS) without Ca & Mg	98%	490 mL
Fetal Bovine Serum (FBS)	2%	10 mL
Total	**n/a**	**500 mL**
Store at 4°C for up to 1 month.		

⚠ CRITICAL: R10 media and FACS wash buffer should be filtered through a 0.2 μm membrane before use.

1:500 LIVE/DEAD Fixable Aqua Dead Cell Stain		
Reagent	Final concentration	Amount
LIVE/DEAD Fixable Aqua Dead cell stain	n/a	2 µL
DPBS (PBS) without Ca & Mg	n/a	998 µL
Total	**n/a**	**1,000 µL**

⚠ CRITICAL: Always prepare fresh 1:500 LIVE/DEAD Fixable Aqua Dead Cell Stain

1:125 SA-PE		
Reagent	Final concentration	Amount
Streptavidin, R-Phycoerythrin Conjugate (SA-PE)	8 µg/mL	4 µL
FACS wash buffer	n/a	496 µL
Total	**n/a**	**500 µL**

Optional: Can use streptavidin-allophycocyanin (SA-APC) instead of SA-PE, or can combine SA-PE and SA-APC (4 µL of each) with 492 µL FACS wash buffer to select double positive events to reduce background.

⚠ CRITICAL: Always prepare fresh 1:125 SA-PE

Fc block		
Reagent	Final concentration	Amount
Mouse IgG Isotype Control	100 µg/mL	15 µL
FACS wash buffer	n/a	135 µL
Total	**n/a**	**150 µL**

⚠ CRITICAL: Always prepare fresh Fc block

Antibody Master Mix		
Reagent	Final concentration	Amount
FITC anti-human/mouse Integrin β7 Antibody	2.5 µg/mL	0.5 µL
BV421 Mouse anti-human IgG	0.09 µg/mL	3 µL
BV510 Mouse anti-human CD3	1 µg/mL	0.5 µL
Brilliant Violet 510 anti-human CD14 Antibody	1 µg/mL	1 µL
Brilliant Violet 510 anti-human CD56 (NCAM) Antibody	1 µg/mL	1 µL
BV510 Mouse anti-human CD16	1 µg/mL	0.5 µL
BV510 Mouse anti-human CD4	0.25 µg/mL	1 µL
Brilliant Violet 510 anti-human CD8a Antibody	1 µg/mL	1 µL
Brilliant Violet 570 anti-human CD20 Antibody	1.5 µg/mL	1.5 µL
Brilliant Violet 605 anti-human CD27 Antibody	2 µg/mL	2 µL
Brilliant Violet 711 anti-human CD10 Antibody	1.25 µg/mL	5 µL
Ax680 anti-human CD38	n/a	0.5 µL
CD19-ECD	0.4 µg/mL	4 µL
PE-Cy5 Mouse anti-human IgM	0.625 µg/mL	5 µL
PE-Cy7 Mouse anti-human CD21	1 µg/mL	2 µL
gp140 (100 µg or 100 µg tetramer)	1 µg/mL	5 µL
FACS wash buffer	n/a	66.5 µL
Total	**n/a**	**100 µL**

⚠ CRITICAL: Always prepare fresh Antibody Master Mix. The amount of antibody used should be determined by performing titrations of each lot of antibody.

STEP-BY-STEP METHOD DETAILS

Bead assay of pre-conjugated fluorescently labeled gp140 tetramer probes

Timing: 1.5 h

This protocol provides step-by-step instructions on performing a bead assay with gp140 tetrameric probes in order to validate their integrity, quality, and the magnitude of detecting antigen-positive B cells using flow cytometry. These probes can be used to target and characterize B cells expressing B cell receptors (BCRs) specific for HIV gp140. This protocol makes use of polystyrene anti-mouse Ig κ microparticles typically used to optimize fluorescent compensation settings for flow cytometry assays. These beads will bind to any mouse κ light chain. This protocol uses these beads to first capture mouse anti-human antibody before incubating with human antibodies specific for gp140. These beads can then be used to capture fluorescently labeled gp140.

1. In the biological safety cabinet, add 50 μL of Anti-mouse Ig, κ beads to two FACS tubes, one for the positive control (VRC01) and one for the negative control (irrelevant or no mAb linker).

   Optional: Additional positive control tubes using other mAbs (PGT128, 4E10, etc.) can be included to test the probe's specific reactivity to antibodies that recognize various epitopes. Alternatively, Ramos cells stably transfected to express VRC01 IgM (positive control) or irrelevant antibodies such as anti-influenza or anti-coronavirus antibodies or no antibody (negative control) can be used in place of beads (Benjamin et al., 1982; Lingwood et al., 2012).

2. Add 4 mL of R10 media (see materials and equipment) to the FACS tubes containing beads.
3. Vigorously vortex (3 s on setting 8–10) the two FACS tubes in R10 media and centrifuge at 300 × *g* for 5 min at room temperature (20°C–22°C).
4. Using a P1000 pipettor, aspirate the top 3 mL of R10 media from the FACS tubes.
5. Vigorously vortex the FACS tubes.
6. Centrifuge the stock of Purified Mouse Anti-human IgG for 1 min at 1,000 × *g*.
7. Add 1 μg mouse anti-human IgG to all of the positive and negative control FACS tubes. Incubate for 15 min at room temperature.

   ⚠ CRITICAL: The anti-human IgG antibody used must be a mouse κ antibody to be captured by the Anti-mouse Ig κ beads.

8. Add 4 mL R10 media to FACS tubes containing beads. Vigorously vortex and centrifuge at 300 × *g* for 5 min at room temperature.
9. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
10. Repeat steps 8 and 9.
11. Vigorously vortex then centrifuge the stock of VRC01 for 1 min at 1,000 × *g*.
12. Add 1 μg of VRC01 mAb to the positive control tube. Incubate for 15 min at room temperature.

    Optional: If available, add 1 μg of an irrelevant human mAb to the negative control tube.

13. Add 4 mL R10 media to FACS tubes containing beads. Vigorously vortex and centrifuge at 300 × *g* for 5 min at room temperature.
14. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
15. Repeat steps 13 and 14.
16. Add 5 μL of tetramer probe mix to all tubes. Incubate for 15 min at 4°C in the dark.

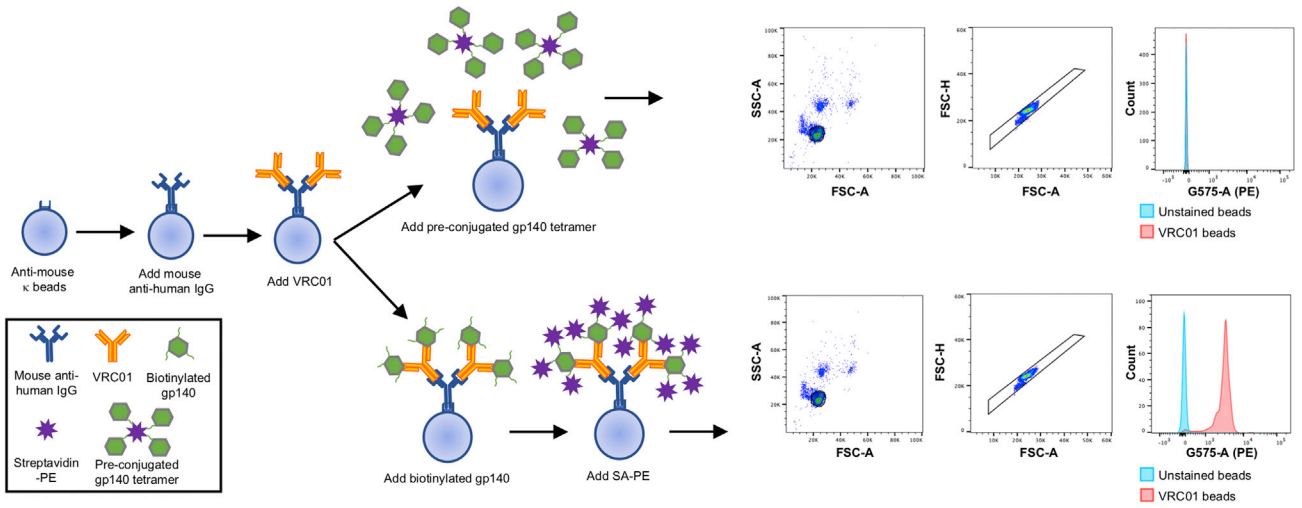


Figure 1. Quality control bead assay using pre-conjugated tetramer staining or sequential staining

(Top) Example of failed gp140 tetramer binding and (bottom) successful use of sequential staining of gp140 in a bead assay. Components for bead assays are listed in the black box. Anti-mouse κ beads are used to capture mouse anti-human IgG. VRC01 is then captured by the mouse anti-human antibody. Beads are then used to test pre-conjugated gp140 tetramer probes (top panel). If the probes do not bind to VRC01 bound beads (red histogram) as a distinct population from unstained negative control beads (blue histogram), sequential staining should be performed using biotinylated gp140 incubated with VRC01 conjugated beads followed by incubation with SA-PE (bottom panel).

17. Fill FACS tube with 4 mL R10 media. Vigorously vortex and centrifuge at 300 × *g* for 5 min at room temperature.
18. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
19. Repeat steps 17 and 18 four more times.
20. Store the test and negative control tubes in the dark at 4°C until the beads can be tested on a flow cytometer.
21. Compare the negative control to the positive control. If the positive control MFI is distinctly above the negative control MFI (Figure 1), these probes can be used to isolate probe-specific B cells as previously described (Morris et al., 2011). If the probes do not bind to the positive control beads as a distinct population from the negative control (see troubleshooting; problem 1), epitopes may be occluded from BCR binding. Therefore, test the ability of the antigen to be used in the sequential staining method below. If there are high levels of background, positive samples are negligibly positive, or there are few bead events see troubleshooting: problem 2, problem 3, or problem 4, respectively.

Note: We recommend collecting 20,000 events for all samples tested to ensure an accurate result.

Bead assay of sequentially stained gp140 probes

Timing: 2 h

This protocol provides step-by-step instructions on performing a sequential staining bead assay with gp140 probes that do not function well as tetrameric probes in the prior method. This assay will validate the ability to use gp140 in a sequential staining technique to capture B cells expressing antigen-specific BCRs. This step can be skipped if the antigen of interest works well as a tetramer

22. In the biological safety cabinet, add 50 μL of anti-mouse κ beads to two FACS tubes, one for the positive control (VRC01) and one for the negative control (irrelevant or no mAb).

Optional: Additional positive control tubes using other mAbs (PGT128, 4E10, etc.) can be included to test the probe's specific reactivity to antibodies that recognize various epitopes. Alternatively, Ramos cells stably transfected to express VRC01 IgM (positive control) or irrelevant antibodies/no antibody (negative control) can be used in place of beads (Benjamin et al., 1982; Lingwood et al., 2012).

23. Add 4 mL of R10 media (see materials and equipment) to the FACS tubes containing beads.
24. Vigorously vortex (3 s on setting 8–10) the FACS tubes in R10 media. Centrifuge at 300 × *g* for 5 min at room temperature.
25. Using a P1000 pipettor, aspirate the top 3 mL of R10 media from the FACS tubes.
26. Vigorously vortex the FACS tubes.
27. Centrifuge the stock of mouse anti-human IgG for 1 min at 1,000 × *g*.
28. Add 1 μg mouse anti-human IgG to all of the positive and negative control FACS tubes. Incubate for 15 min at room temperature.
29. Fill FACS tubes containing beads with 4 mL R10 media. Vortex and centrifuge at 300 × *g* for 5 min at room temperature.
30. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
31. Repeat steps 29 and 30.
32. Vigorously vortex then centrifuge the stock of VRC01 for 1 min at 1,000 × *g*.
33. Add 1 μg of VRC01 mAb to the positive control tube. Incubate for 15 min at room temperature.

Optional: If available, add 1 μg of an irrelevant mAb to the negative control tube.

34. Fill FACS tubes with 4 mL R10 media. Vigorously vortex and centrifuge at 300 × *g* for 5 min at room temperature.
35. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
36. Repeat steps 34 and 35.
37. Add 100 ng of biotinylated gp140 to each tube. Incubate for 15 min at 4°C.
38. Fill FACS tubes with 4 mL R10 media. Vigorously vortex and centrifuge at 300 × *g* for 5 min at room temperature.
39. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
40. Add 100 μL of 1:125 SA-PE (stock concentration of 1 μg/μL, see materials and equipment) to all FACS tubes. Incubate for 15 min at 4°C in the dark.

Optional: A mixture of 1:125 SA-PE and 1:125 SA-APC can be used to select double positive beads to reduce background.

41. Fill FACS tube with 4 mL R10 media. Vigorously vortex and centrifuge at 300 × *g* for 5 min at room temperature.
42. Using a P1000 pipettor, aspirate all but 50–100 μL R10 media.
43. Repeat steps 41 and 42 four more times.
44. Store the test and negative control tubes in the dark at 4°C until the beads can be tested on a flow cytometer.
45. Compare the negative control to the positive control. If the MFI of the positive control is distinctly above the negative control MFI (Figure 1), this method can be used to isolate probe-specific B cells using the sequential staining method below (Townsley et al., 2021). If there are high levels of background, positive samples are negligibly positive, or there are few bead events see troubleshooting: problem 2, problem 3, or problem 4, respectively. If there are no positive events, see troubleshooting: problem 5.

Note: We recommend collecting 20,000 events for all samples tested to ensure an accurate result.

Sequential staining protocol to characterize or isolate gp140-specific B cells

Timing: 2.5 h

This protocol provides step-by-step instructions using the sequential stain technique with HIV gp140 probes that do not function well as tetramer probes in a B cell flow cytometry panel. This assay can be used to phenotype and isolate B cells expressing BCRs that bind HIV gp140.

46. In the biological safety cabinet, add 9 mL pre-warmed R10 media (see materials and equipment) to a 15 mL conical tube for each vial of up to 10 million PBMCs.

    Optional: If available, include a normal, healthy donor PBMC sample as a control. If desired, cells can be counted using a cell counter such as the Countess Automated Cell Counter according to the manufacturer's protocol: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0019566_Countess_3_Auto_Cell_Counter_UG.pdf

47. Add 500 units of Benzonase to each 15 mL conical tube.
48. Thaw PBMC in a 37°C water bath before transferring into designated 15 mL conical tube. Centrifuge at 300 × *g* at room temperature for 5 min.

    ⚠ CRITICAL: The institute's standard protocols and guidelines should be followed and personal protective equipment used when PBMC from HIV infected donors are used.

49. Discard supernatant into a 10% bleach solution, flick tube to break up cell pellet.
50. Add 1 mL of R10 media to cell pellet and move the 1 mL cell suspension into a polystyrene FACS tube. Wash the walls of the 15 mL conical tube with up to 2.5 mL R10 media and transfer to same FACS tube.
51. Vigorously vortex (3 s on setting 8–10) FACS tubes and centrifuge at 300 × *g* at room temperature for 5 min. Discard supernatant into a 10% bleach solution, flick tube to break up cell pellet, and add 4 mL 1× PBS.
52. Repeat step 51 for two additional washes.
53. Resuspend cells in 100 μL of 1:500 LIVE/DEAD Fixable Aqua Dead Cell Stain (see materials and equipment). Incubate for 2–3 min at room temperature in the dark.
54. Add 4 mL FACS wash buffer (see materials and equipment) to neutralize Aqua. Vigorously vortex and centrifuge at 300 × *g* at room temperature for 5 min.
55. Discard supernatant into a 10% bleach solution, flick tube to break up cell pellet.
56. Resuspend cells in 100 μL of Fc block (see materials and equipment). Incubate for 10 min at room temperature.
57. Add 4 mL FACS wash buffer. Vigorously vortex and centrifuge at 300 × *g* at room temperature for 5 min.
58. Discard supernatant into a 10% bleach solution, flick tube to break up cell pellet.
59. Resuspend cells in 100 μL of Antibody Master Mix containing fluorescently labeled antibodies and biotinylated gp140 monomeric protein (see materials and equipment). Incubate for 30 min at 4°C in the dark.
60. Add 4 mL FACS wash buffer. Vigorously vortex and centrifuge at 300 × *g* at room temperature for 5 min.
61. Discard supernatant into a 10% bleach solution, flick tube to break up cell pellet.
62. Repeat steps 60 and 61 for two additional washes.
63. Resuspend cells in 100 μL of 1:125 SA-PE (see materials and equipment). Incubate for 30 min at 4°C in the dark.

    Optional: A mixture of 1:125 SA-PE and 1:125 SA-APC can be used to select double positive cells to reduce background.

64. Add 4 mL FACS wash buffer. Vigorously vortex and centrifuge at 300 × *g* at room temperature for 5 min.
65. Discard supernatant into a 10% bleach solution, flick tube to break up cell pellet.
66. Repeat steps 64 and 65 for two additional washes.
67. Resuspend cells after final wash in 250 μL cold FACS wash buffer or R10 media. Keep cells at 4°C in the dark until cells can be run on a flow cytometer capable of detecting the fluorescently labeled antibodies and the PE labeled tetramer (often measured with a G575 detector). For these purposes, we used a BD Symphony A5 (Liechti and Roederer, 2019).
68. Cells can be sorted into plates for sequencing and analyzed with FlowJo software using Townsley *et. al* gating strategy (Townsley et al., 2021). If included, use healthy B cells to determine the negative population and set positive gates for gp140-reactive B cells (Figure 2). If there are few cell events, see troubleshooting: problem 4.

Pause point: The stained cells could be kept for 12–24 hours at 4°C before flow cytometer analysis. We recommend fixing the cells with 100 μL of diluted FoxP3 fixation/permeabilization solution from the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's protocol: https://www.thermofisher.com/us/en/home/references/protocols/cell-and-tissue-analysis/protocols/staining-intracellular-antigens-flow-cytometry.html - fixation. Wash cell suspension with 4 mL of FACS wash buffer, centrifuge at 300 × *g* at room temperature for 5 minutes, and resuspend in 250 μL FACS wash buffer. Keep at 4°C in the dark for up to 24 hours.

⚠ CRITICAL: Prepare all required controls for flow cytometry analysis and run on flow cytometer the same day as PBMC samples. Controls include compensation controls (one for each fluorophore) for proper compensation calculations on the cytometer or in FlowJo. An antibody conjugated to PE can be used as a compensation control for SA-PE stained gp140.

⚠ CRITICAL: Fixed cells cannot be used for sequencing analysis. Run the fresh, unfixed cells for sequencing analysis. Ensure sufficient separation of all populations by viewing the NxN plots of compensated PBMC samples (Figure 3). We recommend running additional Fluorescence Minus One (FMO) controls on the panel to ensure good separation of each fluorophore.

Note: The number of HIV antigen-specific B cells is dependent on sample size, variability from the patient's contracted virus, time from initial infection, and antiretroviral therapy treatment. We recommend collecting a minimum of 1 milliion PBMC events to ensure accurate results and simultaneously running a bead assay with the cell staining protocol to ensure antigen probe integrity.

EXPECTED OUTCOMES

A positive result from the bead assay will show a distinct positive population with a higher MFI above the negative population (Figure 1). The greater the distinction, the better the resolution and sensitivity that will translate to identifying antigen-specific B cells. The negative healthy control PBMC sample will indicate the background level of the probe staining. Depending on the fluorophore used, there is often a low level of non-specific background (Figure 2A). A positive sample should demonstrate an increased number of events in the positive gating area above the non-specific background from the negative control sample (Figures 2B and 2C). For example, using individual founder gp140s, a median of 0.03% (range= 0.007%–0.148%) $CD19^+$ B cells were $gp140^+$ compared to a median of 0.12% (range= 0.015%–0.379%) and 0.14% (range= 0.021%–0.407%) $CD19^+$ B cells that were $gp140^+$ 1 month and >1 year, respectively, after HIV infection in a therapy-naïve cohort (N=25) (Townsley et al., 2021). The background and the positive response are experimentally

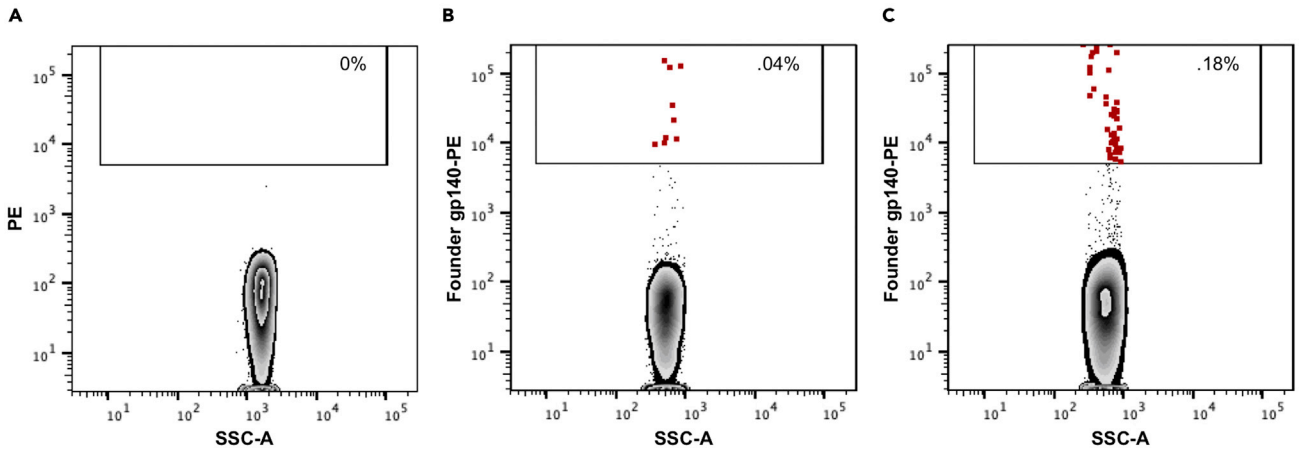


Figure 2. Flow cytometry capture of gp140-specific B cell populations

(A) Gating of CD19$^+$ IgD$^-$ B cells to identify background levels of PE positivity from a sample lacking PE probe staining.

(B and C) Representative gating of sequentially stained PBMC with biotinylated gp140 followed by SA-PE. (B) Lack of gp140-specific CD19$^+$ IgD$^-$ B cells from an uninfected donor prior to infection or (C) presence of gp140-specific CD19$^+$ IgD$^-$ B cells from the same donor 1 month after HIV infection are shown. The frequency of gp140-specific B cells (red cells) is noted.

specific and depend on factors including sample size and cell frequency. It is normal that IgD positive B cells show higher levels of non-specific probe positive events (background) whereas IgD negative B cells show lower background levels and a clearer distinction between the true antigen-specific B cells and the background events (Figure 2). Distinct populations should be apparent with all marker combinations in compensated samples (Figure 3).

LIMITATIONS

Multiple antigen-specific proteins can be tetramerized and labeled using separate fluorophores to distinguish each of them via flow cytometry, allowing for the individual selection of multiple distinct antigen-specific B cell populations (Morris et al., 2011). The suggested flow panel includes antibodies that allow for the phenotyping of a subset of B cells. Additional antibodies can be included to further characterize B cell subpopulations. A disadvantage of this sequential staining protocol is that labeling will not distinguish between multiple antigen-specific populations by flow cytometry with this technique alone if more than one antigen is desired. However, this method could be used to label multiple antigens using the same fluorophores to identify the total antigen-specific population. An alternative approach includes using two or more fluorophores to remove fluorophore-specific events or other background identified in a single channel by selecting double positive events. To have distinction between multiple antigens would require the use of probes that do not rely on a SA/biotin interaction such as direct conjugation with NHS chemistry. Therefore, it is recommended to use directly labeled probes, when possible, to eliminate background and distinguish multiple antigen-specific populations when used in combination with the sequential staining protocol. However, the sequential staining protocol is recommended when antigen-specific probes fail in the pre-conjugated bead assay described here.

TROUBLESHOOTING

Problem 1

Tetramer probe fails to bind to VRC01-bound beads as a distinct population compared to negative control beads (step 21).

Potential solution

Alternate biotinylation methods can be employed to attempt to produce a tetramer with intact, exposed epitopes of interest. Reduce the target number of biotins per gp140 from 2-3 to 1-2 (see

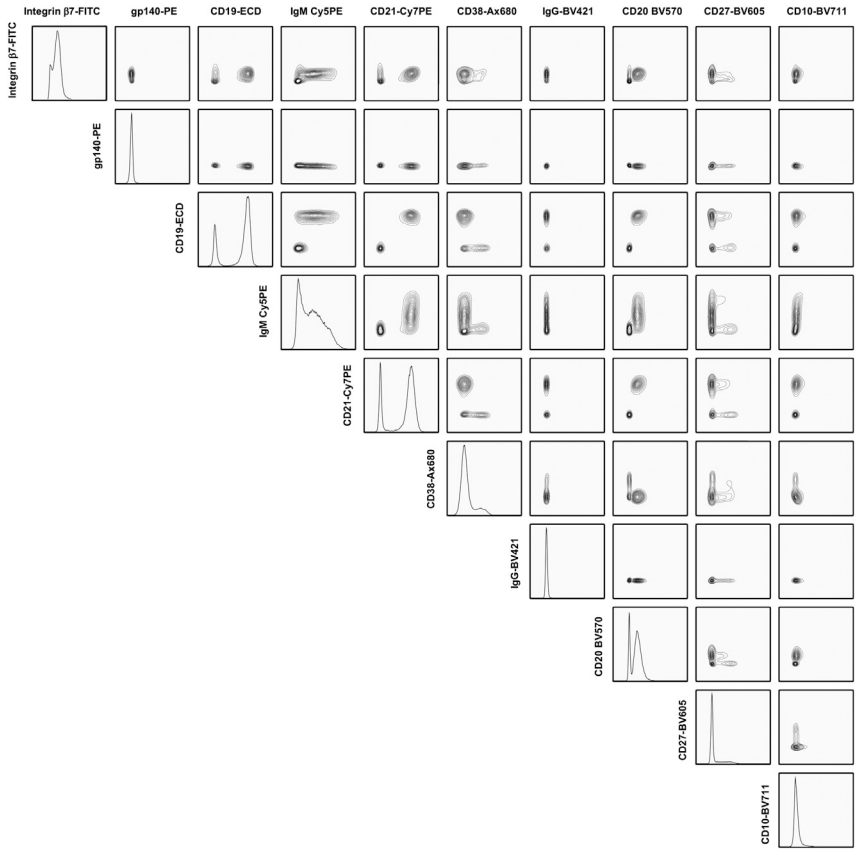


Figure 3. Flow cytometry panel NxN plots

NxN plots were generated to visually assess the separation of populations for each marker. Data plotted for evaluation was gated on lymphocytes, singlets, and live subsets. Because LIVE/DEAD Fixable Aqua Dead Cell Stain was used for live stain and falls in the same channel as BV510, cells that were BV510^{+} for markers CD3, CD14, CD16, CD56, CD4, or CD8 were also excluded.

before you begin section, step 2) and test the new biotinylated gp140 as a tetramer. Alternatively, an Avi-tagged gp140, if available, can be used to target the biotin to the Avi tag site using a biotinylation kit specific for Avi-tagged proteins. All attempted tetramers should be tested using the bead assay of pre-conjugated fluorescently labeled gp140 tetramer probes protocol here.

Problem 2

High levels of background on positive and negative control beads (step 21 or 45).

Potential solution

High levels of background can be reduced by blocking of reagents with R10 media before use and by more stringent washing. We recommend washing beads with R10 two times prior to adding mouse anti-human IgG and adding two additional final washes prior to acquiring data on the flow cytometer.

Problem 3

Positive samples from the bead assays show diffuse or only slightly positive peaks compared to negative controls (step 21 or 45).

Potential solution

Additional washes, especially final washes, can improve resolution between positive and negative bead samples. We advise you double the number of washes at each step to improve positive peaks.

Alternative streptavidin fluorophores are available and can impact background and sensitivity depending on individual flow cytometer configurations. If alternative streptavidin fluorophores are used, make sure to use an equal amount in the case of lower concentrations by the manufacturer.

Problem 4

There are few beads or cell events captured on the flow cytometer (steps 21, 45, or 68).

Potential solution

Bead assays yielding low event levels may try fewer wash steps or more careful aspiration during wash steps. We advise that you aspirate your cells or beads with a P1000 pipettor, being careful to not disturb the bead or cell pellet. Longer centrifuge steps of up to 10 min can also improve bead and cell retention.

Problem 5

No positive events from bead assay of sequentially stained gp140 probes (step 45).

Potential solution

The ability of beads to interact with antibodies in a low volume of solution is crucial to the success of the bead assays. Ensure that the beads are in less than 100 uL of R10 media prior to adding mouse anti-human IgG or VRC01 antibody. If you are unsure about the volume of media, a longer incubation time of up to an hour can be used. Check for biotinylated gp140 epitope integrity on MSD or ELISA assays using a range of anti-gp140 antibodies such as VRC01, PGT121, 10E8, etc. If biotinylated gp140 does not bind to these antibodies, reduce the target number of biotins per gp140 from 2-3 to 1-2 and test the new biotinylated gp140 in the bead assay of sequentially stained gp140 probes protocol.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shelly Krebs (skrebs@hivresearch.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

We thank John Mascola for providing the VRC01 antibody and Sarah Andrews for technical input. The investigators have adhered to the policies for protection of human subjects as prescribed in AR70-25. This work was supported by a cooperative agreement (W81XWH-11-2-0174) between the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. and the U.S. Department of Defense. The views expressed are those of the authors and should not be construed to represent the positions of the U.S. Army of the Department of Defense.

AUTHOR CONTRIBUTIONS

Conceptualization and investigation, S.M.T., D.J.L., and M.P.; supervision, A.B.M. and S.J.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Benjamin, D., Magrath, I.T., Maguire, R., Janus, C., Todd, H.D., and Parsons, R.G. (1982). Immunoglobulin secretion by cell lines derived from African and American undifferentiated lymphomas of Burkitt's and non-Burkitt's type. J. Immunol. *129*, 1336–1342.

Liechti, T., and Roederer, M. (2019). OMIP-060: 30-parameter flow cytometry panel to assess T cell effector functions and regulatory T cells. Cytometry A *95*, 1129–1134.

Lingwood, D., McTamney, P.M., Yassine, H.M., Whittle, J.R., Guo, X., Boyington, J.C., Wei, C.J., and Nabel, G.J. (2012). Structural and genetic basis for development of broadly neutralizing influenza antibodies. Nature *489*, 566–570.

Morris, L., Chen, X., Alam, M., Tomaras, G., Zhang, R., Marshall, D.J., Chen, B., Parks, R., Foulger, A., Jaeger, F., et al. (2011). Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-specific single B cell sorting. PLoS One *6*, e23532.

Townsley, S.M., Donofrio, G.C., Jian, N., Leggat, D.J., Dussupt, V., Mendez-Rivera, L., Eller, L.A., Cofer, L., Choe, M., Ehrenberg, P.K., et al. (2021). B cell engagement with HIV-1 founder virus envelope predicts development of broadly neutralizing antibodies. Cell Host Microbe *29*, 564–578.e9.

Zhou, T., Georgiev, I., Wu, X., Yang, Z.Y., Dai, K., Finzi, A., Kwon, Y.D., Scheid, J.F., Shi, W., Xu, L., et al. (2010). Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science *329*, 811–817.