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Ex vivo differentiation of resting CD4+ T cells coupled with the QVOA (dQVOA)

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Protocol status: Working
We use this protocol in our group and it is working This method was published with the following citation: Wonderlich ER, Subramanian K, Cox B, Wiegand A, Lackman-Smith C, Bale MJ, et al. (2019) Effector memory differentiation increases detection of replication-competent HIV-I in resting CD4+ T cells from virally suppressed individuals. PLoS Pathog 15(10): e1008074. https://doi.org/ 10.1371/journal.ppat.1008074

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Materials

MATERIALS

- EasySep™ Human Resting CD4+ T Cell Isolation Kit For processing 1 × 10^9 cells **STEMCELL Technologies Inc. Catalog #**17962
- Ficoll-Paque PLUS density gradient media **GE Healthcare Catalog #**17144002
- Gibco™ RPMI 1640 Medium GlutaMAX™ Liquid Supplement **Fisher Scientific Catalog #**61-870-036
- Gibco™ MEM Non-Essential Amino Acids Solution (100X) **Fisher Scientific Catalog #**11-140-076
- Gibco™ Penicillin-Streptomycin (10000 U/mL) **Fisher Scientific Catalog #**15-140-122
- Gibco™ HEPES (1M) **Fisher Scientific Catalog #**15-630-080
- Fetal Bovine Serum (FBS) - US Origin **Peak Serum, Inc Catalog #**PS-FB1
- EasySep Magnetic Particles **STEMCELL Technologies Inc. Catalog #**12001D
- Recombinant Human IL-2 Protein **R&D Systems Catalog #**202-IL
- Recombinant Human IL-6 Protein **R&D Systems Catalog #**206-IL
- Recombinant Human IL-7 Protein **R&D Systems Catalog #**207-IL
- Recombinant Human IL-10 Protein **R&D Systems Catalog #**217-IL
- Recombinant Human IL-15 Protein **R&D Systems Catalog #**247-ILB
- Remel™ PHA Purified **Thermo Fisher Scientific Catalog #**R30852801
- Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog #**14190144
- LIVE/DEAD™ Fixable Green Dead Cell Stain Kit for 488 nm excitation **Thermo Fisher Scientific Catalog #**L34969
- eBioscience™ CD16 Monoclonal Antibody (eBioCB16 (CB16)) FITC **Thermo Fisher Scientific Catalog #**11-0168-42
- eBioscience™ CD24 Monoclonal Antibody (eBioSN3 (SN3 A5-2H10)) FITC **Thermo Fisher Scientific Catalog #**11-0247-42
- PerCP/Cyanine5.5 anti-human CD8 **BioLegend Catalog #**344710
- Alliance HIV-1 P24 ANTIGEN ELISA Kit **Perkin Elmer Catalog #**NEK050B001KT
- Pacific Blue™ anti-human CD3 Antibody **BioLegend Catalog #**300330
- Brilliant Violet 510™ anti-human CD4 Antibody **BioLegend Catalog #**300546
- APC/Cyanine7 anti-human CD45RA Antibody **BioLegend Catalog #**304128
- PE Mouse Anti-Human CD27 **Becton Dickinson (BD) Catalog #**340425
- PE-Cy™7 Rat Anti-Human CCR7 (CD197) **Becton Dickinson (BD) Catalog #**557648
- APC Mouse Anti-Human CD25 **Becton Dickinson (BD) Catalog #**555434

Acetic acid glacial **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**ARK2183

Bovine Serum Albumin solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A8412

Recombinant Human TNF-alpha Protein **R&D Systems Catalog #**210-TA

Day -8

1 Thaw and prepare cryopreserved PBMC from cohort or clinical trial participants.
Typically, 50-100×10^6 cryopreserved PBMC are used to yield sufficient resting CD4+ T cells for each dQVOA.
For the referenced publication, a typical dQVOA used 8×10^6 rCD4+ T cells.

1.1 Identify 50 mL conical tubes with Experiment ID and add 10 mL cold FBS to each tube to thaw up to four vials that contain 1mL of DMSO-containing freeze-media. If needed, prepare additional 50 mL conical tubes with 10 mL cold FBS. The volume of DMSO-containing freeze-media being transferred to each 50 mL conical tube should not exceed 4 mL.

1.2 Thaw cryovials of PBMC in a 37 °C water bath. When the cells are almost thawed (check every 10 seconds after one minute) transfer the vial contents to the 50 mL conical tubes containing the cold FBS. For a perfect thawing process, a pea-size frozen sphere should be present. Collect any residual PBMCs by rinsing the cryovial with 1 mL of RPMI-1640 using a P1000 pipet and combine. Q/S tube to 50mL with RPMI-1640.

1.3 Centrifuge at 300 x g for 10 minutes at 4 °C and discard supernatant. 10m

1.4 Resuspend PBMC in 5mL cold FBS and Q/S tube to 50mL with RPMI-1640.

1.5 Count PBMC using trypan blue.

1.6 Centrifuge at 300 x g for 10 minutes at 4 °C and discard supernatant.

1.7 Resuspend PBMC at 3×10^6 cells/mL in R10 media and place in appropriate tissue culture treated flask(s) for culture. (R10: RPMI-1640 w/ glutamax, 1% NEAA, 1% PenStrep, 1% 1M HEPES, 10% heat-inactivated FBS)

1.8 Incubate PBMC at 37 °C and 5% CO2 overnight (between 15-19 hours).

Day -7

2 Perform resting enrichment of CD4+ T cells per **StemCell protocol** with some noted adaptations for optimization.

2.1 Acquire PBMC from go to step #1.8 . Thoroughly resuspend PBMC in each flask and remove a sample to perform cell count.

2.2 Count PBMC with trypan blue.

2.3 (Optional: To assess resting CD4+ T cell enrichment purity and memory T cell subset distribution, take 2×10^5 cells as an "ex vivo pre-enrichment" sample and perform standard surface staining for flow cytometry.)

2.4 Evenly distribute cells into 50 mL conicals and centrifuge at 450 x g for 10 minutes at 4 °C . 10m

2.5 Carefully remove and discard supernatant and resuspend PBMC at 5×10^7 cells/mL in R10 media.

2.6 Add 50 µL of CD4 isolation antibody cocktail and 50 µL of CD25 antibody cocktail per 1 mL of cells. Mix well by pipetting up and down.

2.7 Incubate at Room temperature for 10 minutes. 10m

2.8 Vortex magnetic particles well (>30 seconds). Add 75 µL of magnetic particles per 1 mL of cells to each enrichment sample. Mix well by pipetting up and down.

2.9 Incubate at Room temperature for 5 minutes. 5m

2.10 Add R10 to Q/S tube to max volume per **StemCell protocol**.

2.11 Insert tube into appropriate magnet for 10 minutes at Room temperature . Through negative selection, the resting enriched CD4+ T cells will remain in the supernatant, unbound by the magnet. 10m

2.12 Carefully, without removing the tube from the magnet or disturbing the magnetic beads, use a pipet to transfer the resting enriched cell suspension to a new conical tube and take an aliquot for counting.

2.13 Count resting-enriched CD4+ T (rCD4+) cells using trypan blue.

2.14 (Optional: To assess resting CD4+ T cell enrichment purity and memory T cell subset distribution, take 2×10^5 cells as a "post-enrichment/pre-differentiation" sample and perform standard surface staining for flow cytometry.)

2.15 Centrifuge the rCD4+ cells at 450 x g for 10 minutes at 4 °C . 10m

2.16 Carefully remove and discard supernatant. Resuspend rCD4+ cells at 1×10^6 cells/mL in **freshly prepared** differentiation media. (Differentiation media: R10 containing 25ng/mL recombinant human IL-6, IL-7, IL-10, IL-15, and TNF-α, which are solubilized and stored per manufacturer recommendation.)

3 **Immediately, plate rCD4+ T cells in limiting dilution prior to any differentiation incubations**
Resting CD4+ T cells must be plated in limiting dilution before differentiation culturing or cellular proliferation may alter the ratio of infected cells. (DO NOT PERFORM DIFFERENTIATION CULTURING IN BULK.)
Typically, dQVOA is performed utilizing 8×10^6 rCD4+ cells. This generates 12 wells containing 5×10^5 rCD4+ cells (A wells), 6 wells containing 2×10^5 rCD4+ cells (B wells, one will be utilized for flow cytometric assessment of memory T cell differentiation), 2 wells containing 4×10^4 rCD4+ cells (C wells), and 2 wells containing 8×10^3 rCD4+ cells (D wells). A total of 22 wells are set up on Day -7. Two wells that do not contain patient-derived CD4+ cells will be generated on Day 0 (G wells). The numbers and cellular concentration of each well must be determined and plated at this point.

3.1 rCD4+ T cells are already resuspended at 1×10^6 cells/mL of differentiation media.

To generate A wells: Transfer 500 µL (5×10^5) of rCD4+ cell suspension to each well of a 24-well plate. Be sure to leave empty wells between samples to minimize cross contamination. Q/S each well up to 1mL with 500 µL of differentiation media.

3.2 rCD4+ T cells are already resuspended at 1×10^6 cells/mL of differentiation media.
To generate B wells: Transfer 200 µL (2×10^5) of rCD4+ cell suspension to each well of a 96-well round-bottom plate. Be sure to leave empty wells between samples to minimize cross contamination. (200 µL is the final volume, no need to Q/S B wells)

3.3 rCD4+ T cells are already resuspended at 1×10^6 cells/mL of differentiation media.
To generate C wells: Transfer 40 µL (4×10^4) of rCD4+ cell suspension to each well of a 96-well round-bottom plate. Be sure to leave empty wells between samples to minimize cross contamination. Q/S each well up to 200uL with 160 µL of differentiation media.

3.4 rCD4+ T cells are already resuspended at 1×10^6 cells/mL of differentiation media.
To generate D wells: Transfer 8 µL (8×10^3) of rCD4+ cell suspension to each well of a 96-well round-bottom plate. Be sure to leave empty wells between samples to minimize cross contamination. Q/S each well up to 200uL with 192 µL of differentiation media.

3.5 Add sterile liquid (i.e. PBS or water) to any empty wells in the 24- and 96-well plates to minimize evaporation from assay wells.

3.6 Differentiate rCD4+ cells by incubating at 37 °C and 5% CO2 for 4 days. If the media turns yellow, prepare fresh differentiation media and replace half of the volume in each well as appropriate.

Day -3

4 Replenish media and cytokines by replacing half of the volume of each well of the dQVOA.
Any culture supernatant being removed from the dQVOA may be saved for analysis.

4.1 To replenish A wells: Without disturbing cells, remove 500 µL of media from each well in the 24-well plate. Add 500 µL of **freshly prepared** differentiation media to

each well. Take care to add slowly, without splashing.

4.2 To replenish B/C/D wells: Without disturbing cells, remove 100 µL of media from each well in the 96-well plate. Add 100 µL of **freshly prepared** differentiation media to each well. Take care to add slowly, without splashing.

4.3 Continue to differentiate CD4+ cells by incubating at 37 °C and 5% CO2 for an additional 3 days. If the media turns yellow, prepare fresh differentiation media and replace half of the volume in each well as appropriate.

Day -1

5 (Optional: To assess memory T cell subset distribution and any other parameters after ex vivo differentiation, sacrifice one B well as a "post-differentiation" sample and perform standard surface staining for flow cytometry.)

Day 0

6 Generate γ-irradiated allogeneic PBMC as a part of the stimulation method for dQVOA.

6.1 Irradiate HIV-naive buffy coats from two donors by exposing to 5000R in a Cs6 source irradiator. Immediately place irradiated buffy coats on ice.

6.2 Process each buffy coat into PBMC through standard density gradient centrifugation (i.e. ficoll, histopaque, LSM, etc).

6.3 After final wash step of PBMC processing, centrifuge γ-irradiated allogeneic PBMC at 350 x g for 10 minutes at 4 °C . 10m

6.4 Carefully remove and discard supernatant and resuspend γ-irradiated allogeneic PBMC at 2.5×10^6 cells/mL in **freshly prepared** stimulation media. (Stimulation media: R10 containing 0.5µg/mL REMEL PHA and 100 IU/mL recombinant human IL-2) Place γ-irradiated allogeneic PBMC on ice immediately to prevent cell adhesion and store on ice until ready for addition to dQVOA.

7 Set up stimulations and longterm outgrowth cultures in the dQVOA.
Small scale differentiation cultures are transferred to larger culture dishes **without splitting or cross-contaminating**.

This step is done to allow enough room during the addition of γ-irradiated allogeneic PBMC in a 10:1 stimulation ratio.
A table describing steps 7.1 through 7.3 is found in step 7.4.

7.1 To generate full-sized A wells: Carefully **without splitting or cross-contaminating** resuspend each A well in the 24-well plate and transfer the whole volume from each well (1 mL) of differentiated CD4+ cell culture to its own fresh well in a 6-well plate. Be sure to skip wells to minimize cross contamination. Add 2 mL (5×10^6) of γ-irradiated allogeneic PBMC to each A well. Q/S each A well up to 8mL with 5 mL of stimulation media.

7.2 To generate full-sized B/C/D wells: Carefully **without splitting or cross-contaminating** resuspend each B/C/D well in the 96-well round bottom plate and transfer the whole volume from each well (0.2 mL) of differentiated CD4+ cell culture to its own fresh well in a 24-well plate. Be sure to skip wells to minimize cross contamination. Add 1 mL (2.5×10^6) of γ-irradiated allogeneic PBMC to each B/C/D well. Q/S each B/C/D well up to 2mL with 0.8 mL of stimulation media.

7.3 To generate G wells: Add 1 mL (2.5×10^6) of γ-irradiated allogeneic PBMC to each G well. Q/S each G well up to 2mL with 1 mL of stimulation media. G wells serve as a negative control for potential HIV-contamination or cross-contamination sources. Be sure to skip wells to minimize cross contamination.

7.4 Table explicitly outlining the volumes of each reagent entering each well of the stimulation portion of dQVOA:

Well type	Differentiated CD4+ cells from previous culture plate (mL)	γ-irradiated allogeneic PBMC at 2.5x10^6 cells/mL of stimulation media (mL)	Q/S with stimulation media (mL)	Total volume/well (mL)	Plate type
A wells	1	2	5	8	6-well
B/C/D wells	0.2	1	0.8	2	24-well
G wells	N/A	1	1	2	24-well

7.5 Add sterile liquid (i.e. PBS or water) to any empty wells in the 6- and 24-well plates to minimize evaporation from assay wells.

7.6 Incubate dQVOA 6- and 24-well plates at 37 °C and 5% CO2 overnight (>17 hours of stimulation). 17h

Day 1

8 Remove PHA to minimize cytotoxicity and replace with feeding media (Feeding media: R10 containing 100 IU/mL recombinant human IL-2. Note: TCGF is **not** included as a reagent in dQVOA.)
Any culture supernatant being removed from the dQVOA may be saved for analysis.

8.1 For A wells: Without disturbing cell layer, remove 6 mL of media from each well in the 6-well plates. Add 6 mL of feeding media to each well. Take care to add slowly, without splashing.

8.2 For B/C/D/G wells: Without disturbing cell layer, remove 1.5 mL of media from each well in the 24-well plate. Add 1.5 mL of feeding media to each well. Take care to add slowly, without splashing.

8.3 Incubate dQVOA 6- and 24-well plates at 37 °C and 5% CO2 for 4 days. If the media turns yellow, replace half of the volume of feeding media in each well as appropriate.

Day 5

9 Without disturbing or removing any cells, replenish feeding media by replacing half of the volume of each well of the dQVOA.
For A wells remove and replace 4 mL of feeding media. For B/C/D/G wells remove and replace 1 mL of feeding media.
Any culture supernatant being removed from the dQVOA may be saved for analysis.

Day 8

10 Without disturbing or removing any cells, replenish feeding media by replacing half of the volume of each well of the dQVOA.
For A wells remove and replace 4 mL of feeding media. For B/C/D/G wells remove and replace 1 mL of feeding media.

Any culture supernatant being removed from the dQVOA may be saved for analysis.

Day 11

11 Harvest supernatants for endpoint testing and analysis. Freeze all vials at -80 °C until ready for endpoint analysis. Harvesting supernatants prior to day 11 of outgrowth may yield a lower reservoir measurement.
(Optional: At the end of the assay, cell pellets may be harvested, washed in PBS, and stored as a pellet for future analysis.)

Endpoint Analysis

12 PerkinElmer Alliance p24 antigen ELISA kits are used per manufacturer specifications. Each supernatant is assessed in duplicate ELISA wells. 3.25 pg/mL is used as the cutoff value to determine whether a well is considered positive or negative for HIV outgrowth. Typical wells from dQVOA have p24 concentrations that are either negative or beyond the high end of the standard curve, thus binary (+/-) scoring is used.

IUPM Calculation

13 The maximum-likelihood method is used to calculated the infectious units per million rCD4+ T cells (IUPM) using IUPMStats v1.0 (found here: http://silicianolab.johnshopkins.edu/ and published here: https://doi.org/10.1101/018911)