Fluorescence-linked Antigen Quantification (FLAQ) Assay for Fast Quantification of HIV-1 p24^{Gag}

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[Abstract] The fluorescence-linked antigen quantification (FLAQ) assay allows a fast quantification of HIV-1 p24^{Gag} antigen. Viral supernatant are lysed and incubated with polystyrene microspheres coated with polyclonal antibodies against HIV-1 p24^{Gag} and detector antibodies conjugated to fluorochromes (Figure 1). After washes, the fluorescence of microspheres is measured by flow cytometry and reflects the abundance of the antigen in the lysate. The speed, simplicity, and wide dynamic range of the FLAQ assay are optimum for many applications performed in HIV-1 research laboratories.

Materials and Reagents

1. Sphero Protein A Polystyrene Particles (6-8 µm) (Spherotech, catalog number: PAP-60-5)
2. Human anti-p24^{Gag} HIV-1 IIIB polyclonal antibodies (ImmunoDX, catalog number 2503)
3. Normal Human IgG (Sigma-Aldrich, catalog number: I2511)
4. p24^{Gag} recombinant protein (Abcam, catalog number: ab43037)
5. Anti-p24^{Gag} KC57 clone conjugated to fluorescein isothiocyanate (FITC) or to *phycoerythrin* also called RD1 (Beckman Coulter, catalog numbers: 6604665 and 6604667)
6. 1x phosphate-buffered saline (PBS) without calcium and magnesium (Corning, catalog number: 21-031-CV)
7. Bovine serum albumin (BSA) (Axenia Biologix, catalog number: S200)
8. Triton X-100 (Thermo Fisher Scientific, catalog number: BP151-500)
9. 16% paraformaldehyde (PFA) solution (Electron Microscopy Sciences, catalog number: 15710)
10. Fluorescence-activated cell sorting (FACS) staining buffer (see Recipes)

Equipment

1. Eppendorf centrifuge

2. Rotating mixer
3. 96-well V-bottom plate (optional) (Thermo Fisher Scientific, catalog number: 12-565-216)
4. Thermo Fisher lids for 96-well microplates (Thermo Fisher Scientific, catalog number: 14-245-53A)
5. Plate sealers (VWR International, catalog number: 62402-921)
6. Eppendorf tubes
7. Multichannel pipetman (optional)
8. Pipetman
9. Tips (1 ml, 200 µl, and 10 µl)
10. Reagent reservoirs (optional)
11. Tissue culture centrifuge for Eppendorf tubes or for 96 well plates (optional)
12. 37 °C incubator
13. Flow cytometer

    Note: The FLAQ requires a flow cytometer equipped with a blue laser excitation (488 nm) and one measurement parameter. The photomultiplicator tube (PMT) with a 525/50 nm or a 585/42 band pass filter is used for the detection of FITC or RD1 (phycoerythrin) signals, respectively.

Software

1. Forecyt (Intellicyt)
2. FlowJoX software (Tree Star) or other FACS analysis software

Procedure

A. Preparation of the FLAQ beads coated with $p24^{Gag}$ HIV-1 IIIB polyclonal antibodies
   1. Pipet 1 ml of Sphero Protein A Polystyrene Particles (referred hereafter as beads) into an Eppendorf tube.
   2. Centrifuge for 2 min at 12,000 rpm to pellet the beads.
   3. Remove supernatant.
   4. Add 1 ml of 1x PBS to beads pellet and centrifuge at 12,000 rpm for 2 min.
   5. Resuspend the beads pellet with 80 µl of the commercial vial of human anti-$p24^{Gag}$ HIV-1 IIIB polyclonal antibodies sold at the concentration 1 mg/ml. Pipet three times up and down and incubate for 15 min at room temperature on a rotating mixer.
   6. Centrifuge for 2 min at 12,000 rpm to pellet the beads.
   7. Remove and keep aside the antibody-containing supernatant.
   8. Add 1 ml of 1x PBS to the beads pellet.

9. Centrifuge for 2 min at 12,000 rpm.
10. Add another 1 ml of 1x PBS.
11. Centrifuge for 2 min at 12,000 rpm.
12. Add to the beads pellet 100 µl of a 1x PBS solution containing 4.8 mg/ml of Human normal IgG blocking reagent. Mix well by pipetting up and down 3 times.
13. Incubate for 15 min at room temp on the rotating mixer.
14. Centrifuge for 2 min at 12,000 rpm to pellet the beads.
15. Discard the supernatant.
16. Wash the beads twice with 1 ml of 10 mg/ml of BSA in PBS, spinning 2 min at 12,000 rpm each time.
17. Resuspend the beads in 1 ml of 10 mg/ml of BSA in PBS and store these “FLAQ beads” at 4 °C.
18. Repeat steps 1-17 using the supernatant containing $p24^{Gag}$ HIV-1 IIIB polyclonal antibodies from step 7 (Note 1).

B. Measuring $p24^{Gag}$ content in viral preparations

1. Resuspend the Abcam $p24^{Gag}$ protein in FACS buffer at 8 µg/ml. Aliquot and freeze.
2. Prepare 200 µl of the Abcam $p24^{Gag}$ protein standard by serial dilution ½ in FACS buffer with 0.5% Triton-X100. Typically we perform 8 serial dilutions ranging from 10 ng/ml to 78.12 pg/ml.
3. Harvest viral supernatants and prepare dilutions in FACS buffer supplemented with 0.5% Triton-X100 (Note 2). 160 µl per sample will be required.
4. Prepare a mix containing the following per sample:
   0.5 µl KC57 conjugated to RD1 or FITC
   0.5 µl FLAQ beads
   39 µl FACS buffer 0.5% Triton X-100
5. Transfer 160 µl of the standards and the viral supernatants to a 96 well V-bottom plate.
6. Add 40 µl of the reagent mix to each well and mix.
7. Incubate at 37 °C for 1 h.
8. Centrifuge each plate at 2,000 rpm for 2 min. Do not use plate sealers at this step to avoid well-to-well contamination due to the low superficial tension of the FACS buffer supplemented with 0.5% Triton X-100.
9. Discard the supernatants and wash beads once with 150 µl of FACS buffer.
10. Resuspend the beads in each well in 100 µl of FACS buffer containing 1% PFA.
11. Acquire samples on a flow cytometer, which will create files with an FCS extension. FCS files are flow cytometry standard files containing all the specifications needed to completely describe experimental data flow sets.

12. Analyze the fcs files to obtain the median fluorescence intensities of the beads by the FITC or PE detectors for experiments with KC-57 conjugated with FITC or RD1, respectively.
13. Plot the median fluorescence in the PE channel (x axis) against the concentration of $p24^{Gag}$ in the standard (y axis) in Excel. After adding the trendline, determine the equation that fits the standard curve the best and calculate the concentration (y value) of the unknown.

Representative data

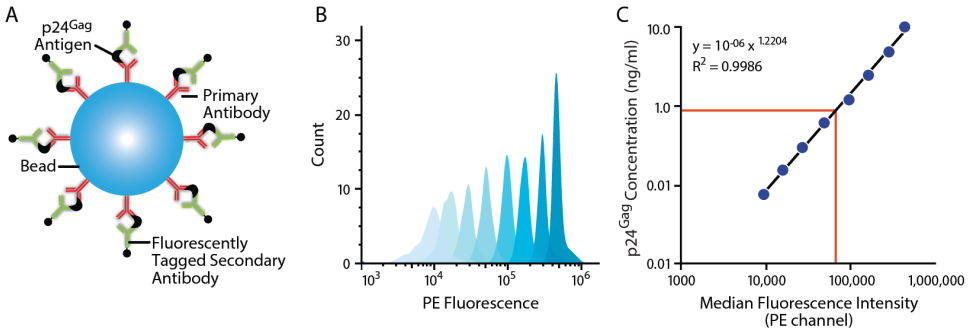

Figure 1. Representative data. A. Principle of the FLAQ assay. B. Gating strategy. The population of beads is first gated on Forward Scatter area (FSC-A) and Side Scatter area (SSC-A). Overlay of the population of beads for the standard (10 ng/ml to 78.12 pg/ml). C. Standard Curve.

Notes

1. We can reuse the antibody at least 5 times, generating 5 ml of beads. The median fluorescence is slightly lower for each reuse.
2. The presence of triton ensures good dilution of viral preparations. Supernatants from transfected 293T should contain between 500 and 1,000 ng/ml of $p24^{Gag}$ protein while supernatants from infected cultures (SupT1 or PBMCs) range from 20 to 200 ng/ml. Usually three dilutions per sample are analyzed to ensure that the measurement will fall within the dynamic range of the assay.

Recipes

1. Fluorescence-activated cell sorting (FACS) staining buffer
   1x phosphate-buffered saline (PBS)

2% fetal bovine serum
Stored at 4 °C

Acknowledgments

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References

1. Hayden, M. S., Palacios, E. H. and Grant, R. M. (2003). Real-time quantitation of HIV-1 p24 and SIV p27 using fluorescence-linked antigen quantification assays. *AIDS* 17(4): 629-631.

