P24 ELISA Protocol

Sandwich ELISA using commercial antibodies against the HIV-1 capsid protein, p24. The assay reads alkaline phosphatase activity using a substrate that emits light. This is more sensitive and has a greater dynamic range than calorimetric assays. The assay is more economical than the Abbot p24 assay and faster. It requires a microtiter plate luminometer.

MATERIALS:

- **Sheep Anti-HIV-1 p24 Gag Antibody** – Primary antibody. Affinity purified, Aalto Bioreagents Cat. #D7320, 2 mg at 1 mg/mL. Comes lyophilized. Reconstitute with 2 mL double distilled water and store aliquots at 4˚C. $787 + S&H per quantity.

- **HIV1 p24 Monoclonal Antibody (5(05-001)) –** ThermoFisher cat#MA1-71515. Secondary antibody. Recombinant Mouse monoclonal antibody to p24 (aa48-62) peptide. Protein G purified in PDS, pH7.4. 1mg/mL Stored at -20C.

- **Mix-n-Stain Alkaline Phosphatase antibody labeling kit –** Biotium cat# 92315. Used to conjugate p24 secondary to alkaline phosphatase. 1 labeling set per kit. Store -20C.

- **Recombinant HIV-1 p24** – Use for p24 standard curve. Intracel Cat. #14101, 100 µg. Reconstitute with 1 mL ddH_20 to give final concentration of 100 µg/mL. Make several 10 µg/mL aliquots in 1% FBS / 0.1% Empigen / TBS and store in the 'p24 Reagents' box in the common -20˚C.

- **Mouse Serum** – Sigma-Aldrich Cat. #M5905-10ML, 10 mL. Stored as 1 mL aliquots in the common -20˚C freezer in the 'p24 Reagents' box. As those run out, more can be found in the -80˚C, stocks rack, box #250 and possibly #251.

- **Sheep Serum** – Sigma-Aldrich Cat. #S2263-100mL, USA origin, sterile filtered, suitable for cell culture, 100 mL. Stored as 10 mL aliquots in -20˚C restriction enzyme freezer.

- **Empigen BB Detergent** – Sigma-Aldrich cat# 45165-250ML, CAS 66455-29-6. Stock is ~35% solution and is stored in the chemical room.

- **DynaLight Substrate with Rabid Glow Enhancer –** Molecular Probes cat#4475406. Alkaline phosphatase substrate 100mL. Stored at 4C.

- **Costar Black 96-Well Flat Bottom Plates** – Corning Costar Cat. #3961.

- **10X TBS:**

ddH_2O	About 3000 mL; Final Vol 5L
NaCl	421 g
Trizma Base	151 g

Titrate to pH 7.5 w/ concentrated HCl.

- **10X $NaHCO_3$, pH 8.5**

ddH_2O	500 mL
$NaHCO_3$	42 g

- **0.1% Tween-20/1X PBS (PBST)**

1X PBS	500 mL
Tween-20	0.5 mL

- **0.1% Tween-20/1X TBS (TBST)**

1X TBS	500 mL
Tween-20	0.5 mL

- **10% Tween-20/1X TBS**

1X TBS	500 mL
Tween-20	50 mL

- **2% Non-Fat Dry Milk TBS**

1X TBS	800 mL
Non fat dry milk	16 g

Used as the blocking solution.

- **2% Empigen/TBST**

1X TBST	30 mL
Empigen (~35%)	2 mL

- **0.1% Empigen/TBST**

1X TBST	500 mL
Empigen (~30%)	2.5 mL

- **1% FBS / 0.1% Empigen / TBST**

Fetal Bovine Serum	100 µL
0.1% Empigen/TBST	10 mL

PLATE PREPARATION:

Day 1

1. This protocol yields 40 coated plates. Alter volumes as necessary if more or less plates are desired.
2. Make 10X $NaHCO_3$, and then make 400 mL of 1X $NaHCO_3$ by diluting the 10X stock.
3. Reconstitute sheep anti-HIV-1-p24 gag antibody in 2 mL ddH_2O to make 1 mg/mL stock. Always reconstitute this way. If some stock will be unused, wrap the top of the bottle with parafilm and store at 4˚C.
4. Add 2.0 mL of reconstituted antibody to 200 mL 1X $NaHCO_3$ and mix.
5. Add 100 µL of diluted antibody to all the wells of 20 black 96 well plates. Save any antibody left in the reservoir by covering with parafilm.
6. Cover and incubate for 1 hr at rm temp.

a. During the wait, make 5% milk/TBS blocking solution by dissolving 40 g of milk in 800 mL of 1X TBS .

7. Collect the antibody solution from all 20 plates and put in the reservoir containing the leftover antibody solution. It will be reused to coat 20 more plates.
8. Wash the already-coated plates twice with 1X TBST.
9. Block the plates overnight in the 4˚C cold room by adding 200 µL of blocking solution (milk/TBS) per well.
10. Add 100 µL diluted antibody from the reservoir to each well of 20 fresh black 96 well plates.
11. Cover and incubate 1 hr at rm temp.
12. Wash the plates twice with 1X TBST.
13. Block plates overnight in the 4˚C cold room by adding 200 µL blocking solution (milk/TBS) per well.

Day 2

1. Freeze and store coated plates at -20˚C until use.

P24 ELISA

1. Prepare 1-2 L TBST. Use this for making empigen/TBST solutions and for washing plates.

2. Prepare 0.1% empigen/TBST and 1% FBS/0.1% empigen/TBST. Make enough to prepare p24 standards and serially dilute viral supes in sample preparation. If p24 standards are already made, it's only necessary to make enough 0.1% empigen/TBST to use as the negative control (800 µL for 1 plate) and not necessary to make any 1% FBS/0.1% empigen/TBST.

3. Prepare 2% empigen/TBST. Use for solubilizing the virus sample.

4. Prepare p24 standards (if not already done):

    a. Dilute p24 standard in 1% FBS/0.1% emp/TBST from 10 ng/µL to 40 ng/100 µL (25-fold dilution).
    b. Use 40 ng/100 µL p24 to make serial dilutions with 1% FBS/0.1% empigen/TBST (a series of 4-fold dilutions) to:
        i. 0.1 ng/µL
        ii. 0.025 ng/µL
        iii. 6.25 pg/µL
        iv. 1.56 pg/µL
        v. 0.39 pg/µL
        vi. 0.0976 pg/µL
        vii. 0.024 pg/µL
        viii. 0.006 pg/µL

    These 8 dilutions will be your standards in the assay.

5. Solubilize virus by adding 100 µL virus to 100 µL 2% empigen/TBST. Let all solutions sit for 1 hr at rm temp.

6. Thaw p24 plate(s) in the bacterial incubator at 37˚C when there is about 30 min. left until the solutions are ready.

7. Serially dilute solubilized virus to desired final concentrations in 0.1% empigen/TBS.

    a. Make 2 or 3 dilutions per sample in the range of 1:10 to 1:1000 (not accounting for 1:2 with empigen). Commonly used: 1:10, 1:100 and 1:1000.
    b. Use a 96 well plate and multichannel pipettemen to make dilutions.
    c. Make enough of each dilution to run triplicates to average in the readout – just over 300 µL of each dilution.

8. Discard blocking solution (milk) from thawed plates.

9. Wash plates twice with 1X TBST using the plate washer. Dry plates by patting upside down onto paper towels.

10. Add 100 µL of serially diluted p24 standards into the wells in column 1.

11. Add 100 µL of 0.1% emp/TBST to each well in column 2 as a negative control.

12. Add 100 µL /well of virus in the rest of the plate. Make sure to record the layout used.

13. Incubate plate for 2 hr at rm temp.

14. When the 2 hrs are nearly elapsed, prepare the p24 alkaline phosphatase-conjugate mouse antibody solution, 1:5000.

Solution	For 1 plate…	For 1.5 plates…	For 2 plates…
1X TBST	8.6 mL	12.9 mL	17.2 mL
Sheep Serum	2.2 mL	3.3 mL	4.4 mL
Mouse Serum	220 µL	330 µL	440 µL
10% Tween/TBS	55 µL	82.5 µL	110 µL
Mouse Ab	2.2 µL	3.3 µL	4.4 µL

15. After the 3 hrs have elapsed, aspirate the samples in the wells and wash twice with TBST.

16. Add 100 µL per well of Mouse Ab solution.

17. Incubate at rm temp for 1 hr.

18. Wash 2 times with 0.1% Tween/**PBS** (**PBST**).

19. Rinse wells once with PBS

20. Add 50 µL per well of DynaLight substrate, cover plates with foil, and incubate at rm temp for 10 min.

21. Cover plate with a plastic cover and read it using the Perkin Elmer plate reader, Lumi protocol. Export data and save on a flash drive to analyze.

Notes:

1. Virus must be solubilized with detergent to disolve the lipid envelope and to dissociate the caspid (p24). Capsid monomers can then be bound by the α-p24 antibody on the plate.
2. the use of black-bottom plates prevents loss of luminescence from the bottom of the plate.
3. For each plate, use 100 µL of antibody solution and 10 mL of sodium bicarbonate solution, mixed together. For 20 plates, use the volumes listed above. For 40 plates, double the volumes… etc.
4. If not using the entire vial of antibody at one time, wrap top with parafilm so that it won't evaporate or spill.
5. When using the plate washer, prime first, twice with ddH_2O followed by wash solution.
6. Several steps can be done ahead of time in bulk to minimize labor. Stacks of plates can be coated, blocked, and stored at -20˚C. Also, p24 standard serial dilutions can be stored in aliquots at -20˚C.
7. Samples must be read in the linear range of the assay. High-titer virus needs to be diluted 1:100 and 1:1000.
8. The assay is linear from about 0.6 pg to 25 ng (or maybe higher).
9. To analyze the data, use an Excell spreadsheet that compares the value of the sample to the standard curve.
10. 96-well plates are not all alike and need to be tested for background and spillover. We found Costar plates to be best.
11. The secondary antibody is very dilute to save money.
12. There have been some background problems. This seems to result from occasional nonspecific binding of the secondary antibody to the wells. Perhaps different blocking agents should be tried to help reduce this.