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Standardization of a cytometric p24-capture bead-assay for the detection of main HIV-1 subtypes.

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The prevailing method to assess HIV-1 replication and infectivity is to measure the production of p24 Gag protein by enzyme-linked immunosorbent assay (ELISA). Since fluorescent bead-based technologies offer a broader dynamic range and higher sensitivity, this study describes a p24 capture Luminex assay capable of detecting HIV-1 subtypes A–D, circulating recombinant forms (CRF) CRF01_AE and CRF02_AG, which together are responsible for over 90% of HIV-1 infections worldwide. The success of the assay lies in the identification and selection of a cross-reactive capture antibody (clone 183-H12-5C). Fifty-six isolates that belonged to six HIV-1 subtypes and CRFs were successfully detected with *p*-values below 0.021; limits of detection ranging from 3.7 to 3×104 pg/ml. The intra- and inter-assay variation gave coefficient of variations below 6 and 14%, respectively.

The 183-bead Luminex assay also displayed higher sensitivity of 91% and 98% compared to commercial p24 ELISA and a previously described Luminex assay. The p24 concentrations measured by the 183-bead Luminex assay showed a significant correlation ($R = 0.92$, $p < 0.0001$) with the data obtained from quantitative real time PCR.

This newly developed p24 assay leverages the advantages of the Luminex platform, which include smaller sample volume and simultaneous detection of up to 500 analytes in a single sample, and delivers a valuable tool for the field.

Abbreviations: C-Ab, capture antibody; FI, fluorescence intensity; HIV-IG, mix of human HIV immunoglobulins derived from pooled plasma of asymptomatic HIV-positive donors; IG-beads, beads coated with capture HIV-IG; mAB, monoclonal antibody; 183-beads, beads coated with the anti-p24 capture monoclonal antibody clone 183-H12-5C; 39/5.4A-beads, beads coated with the anti-p24 capture monoclonal antibody clone 39/5.4A; 4F6-bead, sbeads coated with the anti-p24 capture monoclonal antibody clone 4F6.

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1. Introduction

HIV p24 Gag protein is a commonly used marker of HIV replication clinically and scientifically. Although monitoring HIV-1 replication *in vitro* can be carried out by quantification of viral nucleic acid, the most common method to assess replication and infectivity is to measure p24 production by Enzyme-Linked Immunosorbent Assay (ELISA) (Patton et al., 2006; Schupbach, 2003). p24 is the structural protein of the viral capsid whose highly conserved amino acid sequence (Coplan et al., 2005) and abundance (Summers et al., 1992; Vogt and Simon, 1999) make it an ideal candidate for HIV detection.

Commercially available p24 ELISAs are reliable, specific, sensitive and widely used in the field; however, drawbacks include a narrow dynamic range and high cost. Currently, fluorescent bead-based technologies, like Luminex, offer a broader dynamic range, higher sensitivity and lower cost (Biancotto et al., 2009). Additionally, the Luminex platform requires a smaller sample volume than the ELISA and permits the simultaneous detection of up to 500 analytes in a single sample. The ability to screen up to 1000 samples per day confers an added advantage to this technique. The core technology is based on microspheres internally dyed with fluorophores that are pre-coated with antibodies to capture analytes of interest (C-Ab; capture antibody). Detection of the bound antibody–protein complex is based on the photometric reading of the laser-excited fluorescent detection antibody that sandwiches the captured antigen. As for all immunological assays, the success relies on the avidity and specificity of the C-Abs selected. Biancotto et al. (Biancotto et al., 2009) developed a sensitive assay for the detection of p24 using the Luminex technology that has been used in many HIV-1 subtype B studies (Balzarini et al., 2013; Introini et al., 2013; Merbah et al., 2012; Parrish et al., 2013; Saba et al., 2010; Vanpouille et al., 2012). However, the C-Ab described by Biancotto et al. lacks sensitivity for some HIV-1 non-B subtypes which account for almost 90% of HIV-1 infections worldwide (Hemelaar et al., 2011). Hence, having an assay that allows the assessment of virus production regardless of the subtype is important.

Here we present the development of a Luminex based assay using a p24 capture monoclonal antibody (mAb), that detects B and non-B subtypes. Assay performance was evaluated on a panel of 56HIV-1 isolates representing subtypes A, B, C, D, CRF01_AE and CRF02_AG, by comparing p24 concentrations measured with the Luminex assay developed by Biancotto et al. (Biancotto et al., 2009) and a commercially available p24 ELISA kit (Tang et al., 2010). Additionally, the performance of the assay was compared with real-time quantitative Polymerase Chain Reaction (qPCR) (Schupbach, 2003; Stefan et al., 2003).

2. Materials and methods

2.1. HIV-1 isolates

The 56 virus isolates (Table 1) belong to the International Panel of HIV-1 Isolates (Brown et al., 2005) representing the six major globally prevalent strains A, B, C, D, CRF01_AE and CRF02_AG. Available through the NIH AIDS Reagent Program (Cat #11412), they constitute a resource for standardization for studies using different HIV-1 subtypes and are wildly used (Jones et al., 2012; Rosa Borges et al., 2010; Stoddart et al., 2015; Zhai et al., 2013). To ensure consistency of our measurement over time, a 500 μl or 1 ml vial of virus stock was thawed, divided into 100 μl aliquots and stored at −80 °C.

2.2. p24 quantification by ELISA

To measure the p24 concentration of the isolates, a commercial p24 capture ELISA kit (Advance Bioscience Laboratories, Rockville, MD, USA) was used per the manufacturers' instruction. A modified version of this ELISA was also used to test the binding abilities of the C-Ab candidates (Table 2) to the p24 of the different HIV-1 subtypes. The wells of a flat-bottom polystyrene 96-well plate (Thermo Scientific, Boston, MA, USA) were coated with 100 μl of bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) containing 4 μg/ml of capture-antibody, and incubated overnight at 4°C. The coating solution was then removed and other protein binding sites were blocked by adding 250 μl per well of a blocking solution (1X PBS, 0.5% skim milk powder and 0.1% Tween20, pH 7.4), and incubated at room temperature (RT) for 2 h. After the blocking solution was removed, the assay was completed by using the commercial reagents from the p24 capture ELISA cited above and following the instruction of the manufacturer.

2.3. Beads coupling reaction and validation

The C-Ab yielding the best result was selected for coupling to beads. The content of a vial of carboxylated magnetic beads region 43 (1.25×10^7 beads, 1 ml, region 43, 6.5 μm in diameter, Luminex Corp. Austin, TX, USA) was transferred into a low binding microcentrifuge tube (Eppendorf, Hamburg, Germany) and centrifuged at $8000 \times g$ for 2 min. After a wash in 200 μl of H_2O, the beads were pelleted, resuspended in 160 μl of 100 mM NaH_2PO_4 (Sigma–Aldrich, St. Louis, MO, USA) pH 6.2 and activated for 20 min under agitation by adding 20 μl of 50 mg/ml of Sulfo-NHS (sulfo-*N*-hydroxysuccinimide) (Pierce Biotechnoloy Inc., Rockford, IL, USA) and 20 μl of 50 mg/ml of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) (Pierece Biotechnoloy Inc). The solution of Sulfo-NHS and EDC were freshly prepared and checked for the correct pH before each coupling procedure. The activated beads were washed twice in 250 μl of 100 M of MES (2[N-Morpholino] ethanesulfonic acid) (Fisher Scientific, Pittsburgh, PA, USA) solution pH 6, centrifuged and resuspended in 250 μl of 100 M of MES. 110 μg of C-Ab diluted in PBS were added and the total volume brought to 1 ml with 100 mM MES pH6. The coupling reaction proceeded for 2 h at RT with a gently stirring at 4 °C overnight. The coupled beads were then centrifuged, resuspended and incubated under constant stirring at RT for 30 min in 500 μl of PBS-TBN (1X PBS, 0.1% BSA, 0.02% Tween 20 and 0.05% Sodium Azide). After two washes with 500 μl of PBS-TBN, the coupled beads were finally resuspended in 1 ml of PBS-TBN. The bead concentration was determined with a hemocytometer, and the C-Ab-beads were stored at 4 °C in the dark. During the coupling procedure, the beads were protected from the light as much as possible. All centrifugation steps were performed at $\geq 8000 \times g$ for 2 min. After each wash the beads were vortexed at maximum speed for 30 s and sonicated for 15 s.

The coupling procedure was then validated by incubating the protein-coupled beads with serially diluted PE-labeled antibody solutions, ranging from 6.25 μg/ml to 200 μg/ml for 1 h. The beads were then washed and resuspended in 200 μl of BD FACSFlow™ (BD Biosciences, San Diego, CA, USA) and acquired on a LSRII flow cytometer (BD Biosciences, San Diego, CA, USA) using DIVA software version 8.0.1 (BD Biosciences, San Diego, CA, USA), and analyzed with FlowJo version 9.8.2 (Tree Star, Ashland, OR).

2.4. p24 Luminex capture assay

The p24 Luminex capture assay was performed in a flat bottom 96-well plate. The washes were performed using an automatic magnetic washer; and the plates were kept from the light during incubation. The magnetic beads were diluted in the assay buffer (20 mM Tris Hcl pH6, 0.1% BSA and 0.05% Tween 20) to a concentration of 60,000 beads/ml and 50 μl (3000 beads) were added in each well. The samples and the p24 standards were lysed by adding a 10% volume of a 10X lysis solution (10X Triton X-100 and 0.05%