Multiplex Assay for Concurrent Diagnoses and Detection of HIV-1, HIV-2, and Recent HIV-1 Infection in a Single Test

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ABSTRACT

Reliable incidence assays that can accurately distinguish recent (occurring within the past year) from long-standing (> 1 year) HIV infection are crucial for providing up-to-date information on HIV transmission dynamics in a population. However, often these efforts are confounded by inaccurate HIV diagnosis and the presence of HIV-2 in the population being surveyed. Our study describes the development of a multiplex assay that simultaneously performs HIV diagnosis, HIV serotyping and detection of recent HIV-1 infection in a single well.

HIV diagnosis and HIV-2 serotyping were accomplished by coupling beads with an HIV-1 p24-gp41 fusion protein and HIV-2 peptide from gp36 immunodominant region, respectively. HIV-1 recent infection detection was accomplished by coupling beads with limiting amounts of multi-subtype gp41 immunodominant protein, recombinant immunodominant region, group M (rIDR-M). Assay conditions, including the concentration of coupled antigens, were systematically optimized using well-characterized specimens with known HIV-status (positive or negative), HIV-2 specimens, and recent or long-term HIV-1 classification based on LAg-Avidity EIA in a stepwise manner. Beads were then combined in a multiplex assay to evaluate its performance using large panel of specimens (n=1500) that included HIV-1 positive (n=570, recent = 78, long-term= 492), HIV-2 positive (n= 31) and seronegative individuals (n=899).

The diagnostic component of the assay performed with high sensitivity and specificity (99.8% and 99.7% respectively), while the HIV-2 serotyping sensitivity was 96.7% and specificity was 100%. There was a strong correlation (R= 0.84) between LAg-Avidity EIA and the multiplex assay for recent infection detection. The assay showed high inter- and intra- assay reproducibility with % coefficient of variation (CV) < 10% in the dynamic range of the assay.

The multiplex assay has the ability to diagnose HIV infection, perform serotyping and separate recent from long-term HIV infections, all in a single well. This novel assay has the potential to simplify HIV surveillance by reducing the multiple steps that are otherwise required.

MATERIALS AND METHODS

Specimens:

- Included stored plasma samples from our specimen bank collected over a period of last 10 years from various sources including the blood bank and commercials sources.
- The specimens were characterized by 3rd generation Genetic Systems EIA + Western Blot algorithm, Multispot HIV-1/2 for serotyping, with additional characterization of HIV-1 positive specimens as recent or long-term using the LAg-Avidity EIA.

Coupling procedure:

- All regents were equilibrated at room temperature (~1hr), except the EDC.
- Bead coupling was performed as recommended by the bead manufacturer with slight modifications.
- After coupling, the supernatant was removed and the mixed beads washed twice with 500 µl StabilGuard, and re-suspended in 1ml of same buffer and counted on a Luna Automatic Cell Counter.
- Three bead regions (12, 13, and 14) were coupled with three antigens (p24-gp41, rIDR-M, HIV-2 IDR respectively).

Assay Procedure:

- Coupled beads were vortexed and sonicated before preparing a working number from each bead region.
- Plasma samples were diluted 1:50 in assay buffer and added onto the plate wells containing the beads, then sealed and incubated while shaking for 1 hr at room temperature.
- After 1 hr, the plate was washed four times with wash buffer, rotating the plate after the second wash.
- Beads were resuspended in 100 µl of assay buffer containing 2 µg/ml of PE-conjugated goat anti-human antibody and incubated for 30 min at room temperature while agitating in the dark.
- Following incubation, the beads were washed and re-suspended in 100 µl of wash buffer.
- Data were acquired using the Luminex MagPix instrument according to the manufacturer's instructions with the background of each bead region determined from wells with no samples added.

Figure 1

Schematic representation of the Multiplex Assay

The scheme shows the serological parameters and logical approach that was used for diagnostics, serotyping and recent infection determination and the antigen used for each parameter.

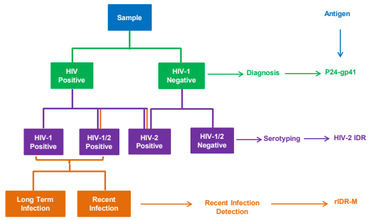


Table 1

Specimen panel used for the development, optimization and evaluation of the Multiplex Assay 1: Schematic representation of the Multiplex Assay

A) Specimens are obtained from different countries (Kenya, Ivory Coast, Thailand, Uganda, United States, Cameroon and South Africa) and commercially from Boca Biolistics. These specimens are well characterized using HIV ELISA and Western blot (for diagnosis), Multispot (for HIV-2) and LAg-Avidity EIA (for recency classification). B) Critical assay parameter showing the range of antigen concentrations tested, the antigen used and the optimal antigen concentration experimentally determined.* Represents specimens that are further classified as HIV-1 recent or HIV-1 long term by LAg-Avidity EIA.

A

Specimen	No.	HIV-1 or Dual	Negatives	Recent	LT	HIV-2	Purpose
Panel 1	10	5	5	N/A	N/A	0	HIV-1 diagnostic optimization
Panel 2	13	4	2	N/A	N/A	7	Serotyping optimization
Panel 3	15	15*	0	8	7	0	Incidence optimization
Panel 4	85	37*	45	26	11	3	Small panel combined evaluation
Panel 5	1500	570*	899	78	492	31	Large panel combined evaluation

B

Parameter	Purpose	Range Tested	Optimum Concentration
p24-gp41 Protein	HIV Diagnosis	50 µg/1.5x10^6 beads to 0.25 µg/1.5x10^6 beads	1 µg/1.5x10^6 beads
HIV-2 IDR Peptide	Serotyping	20 µg/1.5x10^6 beads to 0.5 µg/1.5x10^6 beads	10µg/1.5x10^6 beads
rIDR-M	Recent and Long term separation	1 µg/1.5x10^6 beads to 0.025 µg/1.5x10^6 beads	0.04 µg/1.5x10^6 beads

Figure 2

A scatter plot showing Multiplex results for panel 4 (N=85) demonstrating effective separation of specimens and distribution of MFI using beads coated with p24-gp41 (left), HIV-2 IDR (middle) and rIDR-M (right)

The horizontal lines (black) show the tentative cutoffs that separate the two groups within each parameter and the middle horizontal line within each group indicates the mean value for the group. N=85 MFI, mean fluorescent intensity.

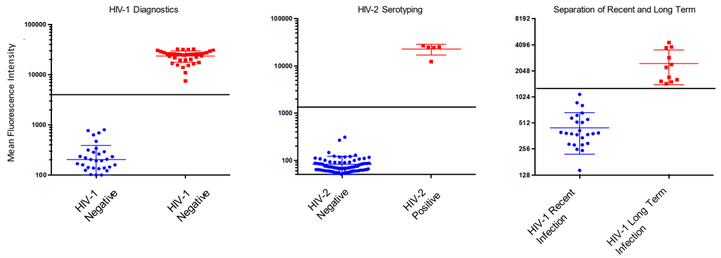


Figure 3

A scatter plot showing the distribution and antibody reactivity levels of p24-gp41 (left), HIV-2 IDR (middle) and rIDR-M (right) with Panel 5 (N=1500).

The horizontal lines (black) show the tentative cutoffs that separate the two groups within each parameter and the middle horizontal line within each group indicates the mean value for the group.

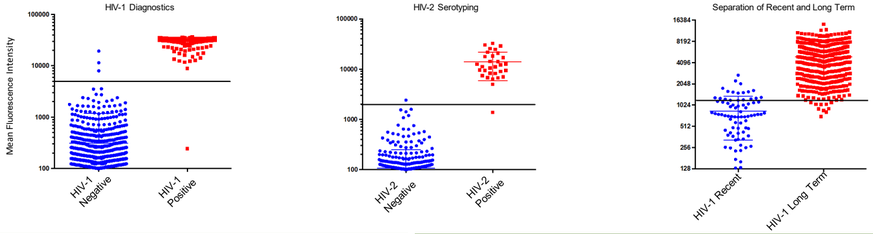


Table 2

A) Comparing the performance of the diagnostic and serotyping components of the multiplex assay with results from the standard algorithm (N=1500); EIA, Western Blot and Multispot and B) Performance of the multiplex assay at a cutoff of 1250 MFI U compared with the LAg-Avidity EIA at a cutoff of 1.5 ODn for positives (N=570) Those shown in red represent concordant results.

A)

Luminex Multiplex	Reference HIV Status by EIA/Western Blot/Multispot					
	HIV-1	HIV-2	Negative	Total	Sensitivity	Specificity
HIV-1	**569**	0	3	572	**99.8**	**99.7**
HIV-2	0	**30**	1	31	**96.8**	**99.9**
Negative	1	1	**895**	897		
Total	570	31	899	**1500**		

B)

Luminex Multiplex @1250 MFI U	LAg Avidity @ 1.5 ODn		
	Recent	Long Term	Total
Recent	**63**	15	78
Long Term	15	**477**	492
Total	78	492	**570**

Figure 4

A scatter plot showing correlation between LAg-Avidity EIA and multiplex assay to distinguish recent and long-term infection with red line showing polynomial best fit. Vertical arrow represents LAg cutoff of 1.5 and horizontal line represents Luminex cutoff of 1250 MFI.

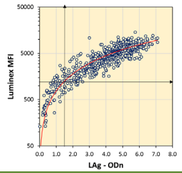


Figure 5

Three-dimensional representation of the multiplex data for panel 5 (n=1500) showing the partitioning of the different analytes using cutoffs of 4000 MFI U for HIV-1 positive and negative; 2000 MFI U for HIV-2 positive and negative specimens; and 1250 MFI U for recent and long term infections. Three-dimensional plot with shadows on the wall (small dots) allows for proper visualization of the separations between the different parameters. The z-axis MFI is log transformed.

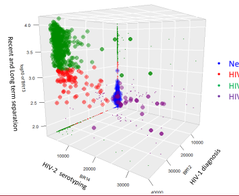
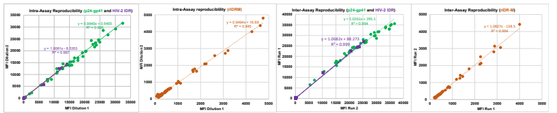


Figure 6

Assay reproducibility shown as correlation in MFI. Shown is the concordance between MFI values obtained from analysis of a mixture of negative and positive specimens from panels 4 and 5. Note that specimens used for inter-assay reproducibility and intra-assay reproducibility are not necessarily the same. Line of best-fit is represented by the dotted lines with statistics shown. Intra-assay reproducibility is from three different dilutions with two dilutions compared. Inter-assay data is from two independent runs on different days. Green, purple and brown represent single well consistencies of p24-gp41, HIV-2 IDR and rIDR-M, respectively.



CONCLUSIONS

- The study shows that HIV diagnosis, serotyping and separation of recent from long term infections can be simultaneously performed using a single assay in a single well.
- The assay is robust and precise with consistently low inter- and intra- assay variability.
- Simultaneous classification of individuals as positive or negative, if positive, HIV-1 or HIV-2, and if HIV-1, recent or long term would have a major implications in HIV surveillance.
- This methodology is suitable for adding new biomarkers; therefore it presents a great opportunity to revolutionize multi-disease detection.

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