B-069

HIV p24 Immunoassay with the Sensitivity of PCR Methods

Martin Stengelin, Daisy Roy, Anahit Aghvanyan, John Kenten, George B. Sigal, Eli N. Glezer, and Jacob N. Wohlstadter

Meso Scale Diagnostics, LLC. Rockville, Maryland, USA

1 Abstract

Patients who have recently been infected with HIV contribute disproportionately to the spread of the disease. Viral loads are high in the first few weeks after infection, and newly infected patients are unlikely to be aware that they are infected and can spread the disease to others before they are diagnosed. Therefore, early detection of acute HIV infection is of great importance for public health. PCR methods are the gold standard with respect to sensitivity; they can detect as few as 60 HIV RNA copies per mL of serum or plasma (30 virus particles per mL). However, PCR technology is complex and expensive, and therefore not suitable for all settings. Immunoassays are simpler and cheaper, but the detection limit of current, 4th generation p24 immunoassays is only about 10 pg/mL, or approximately 250 million capsid proteins per mL. On a per virus basis, these immunoassays are several thousand times less sensitive than PCR testing, despite the fact that there are about 2,000 p24 capsid proteins per virus.

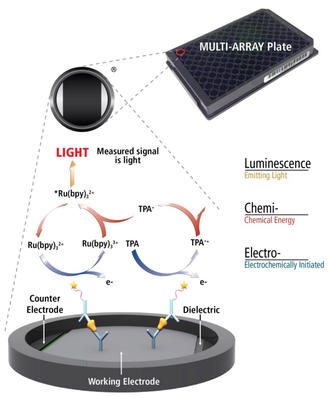
A next-generation electrochemiluminescence assay format based on MSD's MULTI-ARRAY® technology was developed and its performance characterized. The detection limit for this novel p24 immunoassay was approximately 1 fg/mL -- 10,000 fold more sensitive than current p24 immunoassays. A sensitivity of 1 fg/mL corresponds to less than 1 virus particle in our sample volume of 25 µL. The lower and upper limits of quantitation were 3 fg/mL and 38,000 fg/mL, respectively. Within-plate CV was 7%, and total CV 15%. Spike recovery and dilution linearity were between 80% and 120%. p24 was undetectable in the serum or plasma of 32 apparently healthy donors. A SeraCare p24 "Mixed Titer Panel" (12 samples) showed good correlation between our p24 assays and commercial p24 immunoassays. Two seroconversion panels were tested: SeraCare PRB948 (days 0 and 18, PCR negative; days 22 and 23, PCR positive) and PRB962 (days 0 and 2, PCR negative; days 7, 9, 14, and 17, PCR positive). In both cases, the MSD® p24 assay result was negative for all PCR-negative samples and positive for all PCR-positive samples, and infection was detected well before conventional p24 immunoassays.

In conclusion, we developed a next-generation p24 immunoassay that is 10,000 times more sensitive than the current limits of p24 ELISAs and comparable in sensitivity to PCR assays. The assay does not require specialized equipment and can be run on the MESO® QuickPlex SQ 120, and all MESO SECTOR® Imagers.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates.

We developed the S-PLEX™ assay platform, a next-generation MULTI-ARRAY technology with significantly higher sensitivity. S-PLEX assays do not require specialized equipment and can be run on the MESO QuickPlex SQ 120, and all MESO SECTOR Imagers.



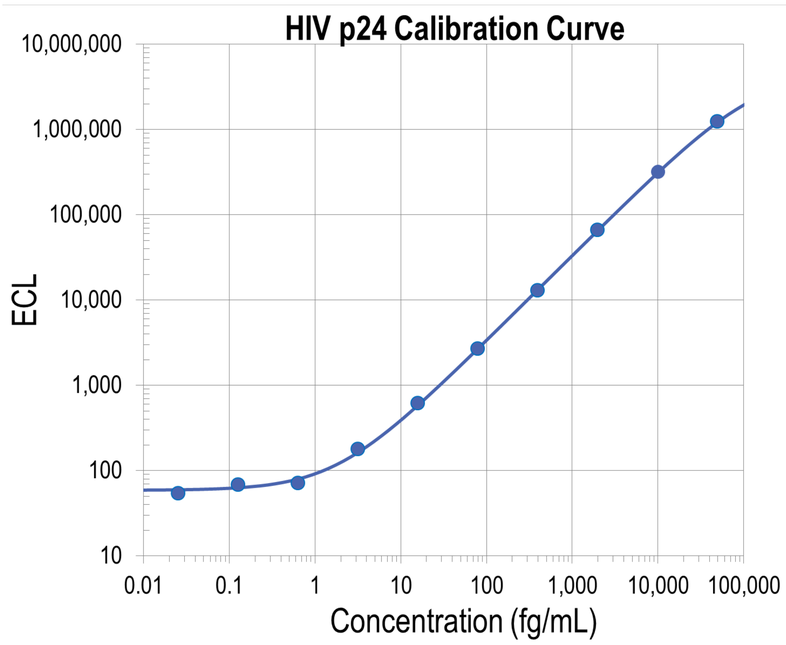
Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

The performance of the HIV p24 assay was characterized.
Essentially all experiments had the following plate layout:
- Point-symmetrical plate layout; calibrators, QC samples and unknowns measured in duplicates.
- 7 calibrator levels + zero calibrator; 7x serial dilutions.
- 3 QC samples spanning the assay range and a plasma pool control (QC-4).

Performance characterization included determination of limit of detection, upper and lower limit of quantitation; within plate and total reproducibility, spike recovery and dilution linearity.
Serum and plasma samples from apparently healthy donors and from well-characterized HIV patients were tested.

3 Assay Range



	ECL
Average Zero	51
SD Zero (n=8)	18
LOD (2.5 SD above Zero): 1.3 fg/ml	

Determination of LLOQ and ULOQ

	fg/ml (expected)	ECL counts	fg/ml measured	CV (n=8)	Accuracy
ULOQ	37,500	963,683	34,041	9%	91%
	25,000	760,589	25,338	7%	101%
	16,500	477,434	14,793	6%	90%
LLOQ	24	866	25	8%	102%
	12	452	12	13%	99%
	6.1	260	6.1	10%	101%
	3.0	172	2.8	18%	93%
	1.5	113	1.5	49%	97%

- **Assay Range:**
  - **LOD: 1.3 fg/mL**
  - **LLOQ: 3.0 fg/mL**
  - **ULOQ: 37,500 fg/mL**
- **A detection limit of 1.3 fg/ml for a 25 ul sample corresponds to approximately 650 P24 molecules**
- **Each Virus particle produces ~ 2000 copies of P24 protein.**

4 Spike Recovery, Dilution Linearity

Dilution Linearity

	Dilution factor	Expected (fg/mL)	Measured (fg/mL)	% recovery
Sample # 11 EDTA Plasma	100%		58,355	
	50%	29,177	34,192	117%
	25%	14,589	16,740	115%
	12.5%	7,294	7,900	108%
Sample # 12 EDTA Plasma	100%		45,383	
	50%	22,691	24,147	106%
	25%	11,346	12,079	106%
	12.5%	5,673	6,169	109%
Sample # 21 EDTA Plasma	100%		64,128	
	50%	32,064	32,198	100%
	25%	16,032	14,960	93%
	12.5%	8,016	7,465	93%

	Dilution factor	Expected (fg/mL)	Measured (fg/mL)	% recovery
Sample # 22 EDTA Plasma	100%		11,332	
	50%	5,666	5,567	98%
	25%	2,833	2,588	91%
	12.5%	1,417	1,358	96%
Sample # 23 EDTA Plasma	100%		7,359	
	50%	3,680	3,597	98%
	25%	1,840	1,781	97%
	12.5%	920	904	98%
Sample # 24 EDTA Plasma	100%		9,442	
	50%	4,721	4,781	101%
	25%	2,360	2,281	97%
	12.5%	1,180	1,224	104%

Average Recovery: 102%

Spike Recovery

	Spike	Expected (fg/mL)	Measured (fg/mL)	% recovery
Serum-1	unspiked		2	
	5,000	5,002	6,241	125%
	3,333	3,335	3,286	99%
	714	716	834	116%
Serum-2	unspiked		0	
	5,000	5,000	6,128	123%
	3,333	3,333	3,309	99%
	714	714	856	120%
Serum-3	unspiked		0	
	5,000	5,000	5,006	100%
	3,333	3,333	2,720	82%
	714	714	695	97%

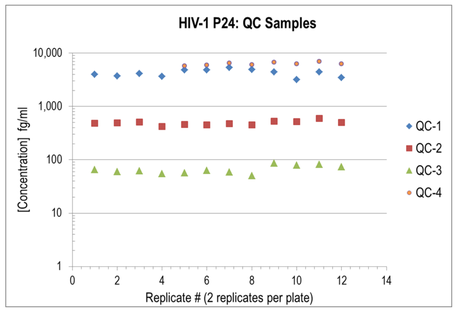
	Spike	Expected (fg/mL)	Measured (fg/mL)	% recovery
EDTA Plasma 1	unspiked		2	
	5,000	5,002	6,130	123%
	3,333	3,335	3,175	95%
	714	716	858	120%
EDTA Plasma 2	unspiked		0	
	5,000	5,000	6,262	125%
	3,333	3,333	2,922	88%
	714	714	851	119%
EDTA Plasma 3	unspiked		0	
	5,000	5,000	5,188	104%
	3,333	3,334	2,667	80%
	714	715	686	96%

	Spike	Expected (fg/mL)	Measured (fg/mL)	% recovery
Heparin Plasma 1	unspiked		1	
	5,000	5,001	5,284	106%
	3,333	3,335	2,690	81%
	714	716	862	120%
Heparin Plasma 2	unspiked		0	
	5,000	5,000	6,135	123%
	3,333	3,333	3,205	96%
	714	714	881	123%
Heparin Plasma 3	unspiked		1	
	5,000	5,001	4,199	84%
	3,333	3,334	2,679	80%
	714	715	732	102%

Average Recovery: 105%

Six HIV positive EDTA plasma samples were diluted as shown in the table. Samples diluted linearly, with an average dilution linearity of 102%.
Three serum samples, EDTA plasma samples, and heparin plasma samples from apparently healthy donors were spiked with calibrator at three concentrations. Average spike recovery was 105%.

5 Reproducibility



Total CV; 6 plates; 2 replicates per Plate			
	fg/mL	CV	n
QC-1	4,244	13%	12
QC-2	491	8%	12
QC-3	66	17%	12
QC-4	6,332	5%	8

QC-1, 2, 3: rec. p24; QC-4: Spiked Plasma pool.

HIV-1 P24 Assay; within-plate CV (n=96)

[Conc.]	Mean ECL	CV (n=96)
10pg/ml	295,335	7%

Whole Plate CV: ECL counts for 96 replicates of a mid range calibrator (10 pg/ml).

333,537	278,865	295,348	286,558	318,018	287,561	257,034	309,721	308,572	277,433	307,304	278,524
304,161	320,139	302,337	296,168	322,579	306,977	317,682	302,516	316,901	281,837	322,686	273,612
316,821	337,945	304,127	295,517	300,448	300,158	307,463	300,635	294,298	312,831	299,507	281,355
312,662	323,977	295,799	300,579	299,019	317,188	308,631	270,976	301,888	312,011	304,816	255,255
280,527	285,899	305,544	274,642	295,909	264,909	296,464	268,319	290,612	283,193	289,586	275,027
299,799	330,341	323,214	307,029	308,032	281,304	287,800	278,741	298,375	291,906	285,243	251,487
292,060	303,698	324,902	287,602	289,646	286,517	296,795	286,262	326,346	294,733	303,094	251,454
269,410	306,508	319,818	271,024	287,883	306,295	302,689	262,087	256,849	262,925	288,708	257,017

Six plates were run over a period of 10 days. Each plate included an 8-point calibration curve (duplicates) and two replicates each of four QC samples. The plate layout was point-symmetrical with calibrators in columns 1 and 12, and QC samples in columns 2 and 11. Total CV ranged from 5% to 17%.
To assess within-plate reproducibility, one 96-well plate was run at a single mid-range calibrator concentration. Within-plate CV was 7%.

6 Serum/Plasma from Cases and Controls

Apparently Healthy Donors	Average ECL ± SD	ECL Range	HIV p24 Concentration (fg/mL)
Plasma (n=22)	135 ± 39	72 to 200	<4 fg/mL
Serum (n=10)	108 ± 29	75 to 155	<4 fg/mL

Serum and plasma samples from 32 apparently healthy donors were tested. All measured HIV p24 concentrations were below the detection limit (4 fg/mL in this experiment). The table above shows the ECL range.
The table on the right shows data obtained with a SeraCare p24 "Mixed Titer Panel" (12 samples). Columns 2, 3, and 4 show results reported by Seracare for three commercial methods (Numbers marked in red are positive). Columns 5 and 6 show results obtained with the S-PLEX assay. S-PLEX results correlate well with results obtained using commercial methods.

Sample ID	BioMerieux HIV Ag VIDAS p24 (pg/mL)	Perkin Elmer HIV Ag p24 (signal/cut-off)	Zeptometrix HIV Ag p24 (signal/cut-off)	MSD p24 S-PLEX (pg/mL)	MSD p24 S-PLEX (ECL counts)
PRA204 (B)-10	<3	0.5	0.1	0.00	174
PRA204 (B)-20	<3	0.6	0.2	0.00	150
PRA204 (B)-23	14	2.4	2.4	7	237,726
PRA204 (B)-24	15	3	3	9	306,728
PRA204 (B)-22	17	3	0.8	10	347,517
PRA204 (B)-12	60	11	14	>38	1,601,078
PRA204 (B)-21	68	14	18	>38	1,422,070
PRA204 (B)-11	85	18	16	>38	1,674,519
PRA204 (B)-13	170	47	41	>38	1,902,237
PRA204 (B)-15	192	45	36	>38	1,884,816
PRA204 (B)-17	>400	42	61	>38	1,897,359
PRA204 (B)-09	>400	>42	75	>38	1,915,873

The two tables below show results for two Seroconversion panels obtained from SeraCare. Each panel contains a series of plasma samples from a single donor before and after HIV seroconversion, and results from five or six commercial HIV assays (four p24 immunoassays and a Roche PCR assay). Numbers marked in red indicate a positive result. The last two columns show p24 concentrations and ECL counts for the S-PLEX assay. For both seroconversion panels, the S-PLEX assay is as sensitive as PCR: turning positive between day 18 and day 20 for panel 1, and between day 2 and day 7 for panel 2.

Seroconversion Panel I	Days Since 1st Bleed	Abbott BBI HIV-1 Ag (signal/cut-off)	Coulter BBI HIV-1 Ag (signal/cut-off)	Dupont BBI HIV-1 Ag (signal/cut-off)	Innogenetics RL29 HIV-1 Ag (signal/cut-off)	Roche PCR HIV RNA BBI (copies/mL)	MSD p24 S-PLEX (pg/mL)	MSD p24 S-PLEX (ECL counts)
PRB948-01	0	0.4	0	0.1	0.4	BLD	0.001	121
PRB948-02	18	0.4	0	0.1	0.4	BLD	0.001	100
PRB948-03	20	0.5	0.2	0.5	1.3	$3x10^4$	3	97,688
PRB948-04	23	5	23	15	31	$6x10^5$	>38	1,736,809

Seroconversion Panel II	Days Since 1st Bleed	Coulter ELISA HIV-1 Ag (signal/cut-off)	Perkin Elmer ELISA HIV-1 Ag (signal/cut-off)	Roche Elecsys ELISA HIV-1 Ag (signal/cut-off)	Zeptometrix ELISA HIV-1 Ag (signal/cut-off)	Roche Ultra sensitive HIV-1 RNA (copies/mL)	Roche Standard HIV-1 RNA (copies/mL)	MSD p24 S-PLEX (pg/mL)	MSD p24 S-PLEX (ECL counts)
PRB962-01	0	0.3	0.3	0.1	0.1	<50	n/a	0.002	149
PRB962-02	2	0.2	0.2	0.2	0.2	<50	n/a	0.001	120
PRB962-03	7	0.2	0.2	0.2	0.2	n/a	$7.6x10^2$	0.021	778
PRB962-04	9	0.6	0.3	0.3	0.3	n/a	$7.7x10^3$	0.2	7,603
PRB962-05	14	>40	30	23	10	n/a	$7.0x10^5$	>38	1,808,344
PRB962-06	17	>40	>49	155	24	n/a	$1.2x10^7$	>38	1,863,699

7 Conclusion

We developed a next-generation p24 immunoassay that is 10,000 times more sensitive than the current limits of p24 ELISAs and comparable in sensitivity to PCR assays. The assay does not require specialized equipment and can be run on the MESO QuickPlex SQ 120, and all MESO SECTOR Imagers.

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