Novel Multiplexed Serology Assays for Detection of IgG Antibodies against Monkeypox (mpox) and Vaccinia Viruses

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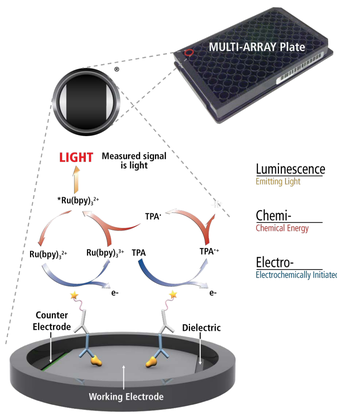
1 Abstract

Monkeypox (mpox) virus (MPXV) spreads through skin-to-skin contact, causing painful lesions. The 2022 outbreak resulted in the Congo's worst surge on record, with the subsequent world-wide spread causing nearly 20,000 suspected cases and 820 suspected deaths. Effective tools for understanding MPXV immune responses are needed for developing timely and effective MPXV-specific vaccines, as well as for understanding the immune correlates of protection from natural infection and/or vaccination. Despite a rapid increase in the number and availability of serology assays that can detect antibodies against MPXV, there is limited information available on their performance and validation status. In addition, most of these assays are low throughput and measure responses to a single antigen, which cannot capture the breadth of antibody responses to MPXV. Here we present a validated, quantitative, multiplexed serology assay to measure antibody responses towards 5 MPXV and 5 vaccinia virus (VACV) variant recombinant proteins. Vaccinia is included in the panel due to its common ancestry to MPXV and the prevalence of smallpox vaccination, which is expected to prevent or reduce the severity of MPXV infection. Viral antigens that elicit strong T cell and B cell immune responses were chosen for the panel and include the receptor binding site for MPXV (A29L) and VACV (A27L), outer envelope proteins (MPXV: B6R, A35R and VACV: B5R, A33R) and inner membrane proteins (MPXV: M1R, E8L and VACV: L1R, D8L). The assay uses a 10-spot 96-well plate coated with the 5 MPXV and 5 VACV antigens, along with an electrochemiluminescent (ECL) detection system. The assay simultaneously detects IgG antibodies to all 10 proteins. Specificity was assessed using purchased serum sample sets that were either collected pre-epidemic from aged smallpox-vaccinated individuals, or during the outbreak from convalescent individuals who recently recovered from MPXV infection. The multiplex MPXV ECL serology assay allows for sensitive, high throughput, and simultaneous measurement of IgG levels to multiple antigens in human sera, supporting its use in research, epidemiology, and vaccine testing.

2 Methods

MSD Technology

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.



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 V-PLEX® Orthopoxvirus Panel 1 (IgG) Kit includes a multiplex panel to detect antibodies to antigens from mpox and vaccinia viruses, including variants related to viral structures such as the receptor binding region, inner membrane and outer membrane regions of the viral core. Plates are provided with antigens on spots in the wells of a 96-well plate. Antibodies in the sample bind to the antigens on the spots and anti-human antibodies (IgG) conjugated with MSD SULFO-TAG are used for detection. The plate is read on an MSD® instrument, which measures the light emitted from the MSD SULFO-TAG.

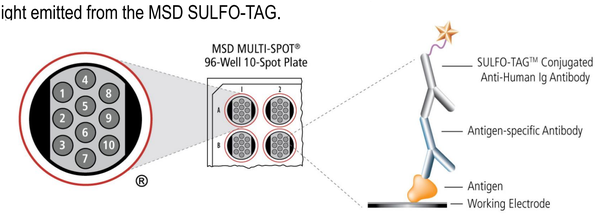


Figure 1. Schematic for Orthopoxvirus Serology Panels.

The kit includes a calibrator for quantitation, controls, plate(s), detection antibody (anti-human IgG), and all other reagents necessary to conduct the assay. Plates are coated with 5 mpox antigens (M1R, E8L, B6R, A35R, and A29L) and five analogous vaccinia viral antigens (A27L, A33R, B5R, D8L, and L1R). Including both mpox and vaccinia ortholog antigens in a single panel allows researchers to evaluate infection/vaccination status and potential cross-protection.

mpox Protein	Vaccinia Homolog
M1R	L1R
A29L	A27L
A35R	A33R
B6R	B5R
E8L	D8L

Spot #	Antigens
Spot 1	VACV A27L
Spot 2	VACV A33R
Spot 3	VACV B5R
Spot 4	VACV D8L
Spot 5	VACV L1R
Spot 6	MPXV M1R
Spot 7	MPXV E8L
Spot 8	MPXV B6R
Spot 9	MPXV A35R
Spot 10	MPXV A29L

3 Results

a. Signal Reproducibility

Orthopoxvirus plates tested for signal reproducibility across wells and plates were analyzed on a per-batch basis and passed a 20% CV intraplate average specification for all ten multiplexed assays. Representative data demonstrated consistent performance using a mid-level calibration standard (Cal-03) in a standard assay protocol.

Batch 1

Metric	Spec	Analyte									
		VACV A27L	VACV A33R	VACV B5R	VACV D8L	VACV L1R	MPXV M1R	MPXV E8L	MPXV B6R	MPXV A35R	MPXV A29L
CV of Intraplate Averages	≤ 18%	5.2%	5.1%	4.9%	3.4%	11.8%	5.5%	3.8%	2.8%	4.7%	5.2%
Ave Intra-plate CV	≤ 10%	4.3%	3.1%	4.3%	2.7%	5.2%	4.6%	4.7%	2.5%	2.8%	5.0%
Max Intra-plate CV	≤ 13%	5.0%	5.1%	5.1%	3.3%	8.4%	6.5%	5.8%	3.2%	3.4%	5.7%
Ave Signal by Batch	1,500-1,000,000	39,965	96,156	57,718	36,339	16,553	15,115	22,620	133,902	84,537	39,351
Result		PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS

Batch 2

Metric	Spec	Analyte									
		VACV A27L	VACV A33R	VACV B5R	VACV D8L	VACV L1R	MPXV M1R	MPXV E8L	MPXV B6R	MPXV A35R	MPXV A29L
CV of Intraplate Averages	≤ 18%	5.2%	5.1%	4.9%	3.4%	11.8%	5.5%	3.8%	2.8%	4.7%	5.2%
Ave Intra-plate CV	≤ 10%	4.2%	2.5%	4.0%	2.7%	4.8%	4.5%	4.2%	2.4%	2.5%	4.3%
Max Intra-plate CV	≤ 13%	6.4%	3.2%	4.7%	2.8%	6.7%	5.9%	5.1%	2.7%	2.9%	4.9%
Ave Signal by Batch	1,500-1,000,000	41,772	103,198	61,958	37,634	17,907	15,974	23,559	138,489	90,473	41,823
Result		PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS	PASS

b. Specificity and Sample Panel

Screening was performed to identify potential antibodies capable of distinguishing between mpox antigens, to be used to evaluate plate coating specificity. Most antibodies recognized their mpox target as well as the target's vaccinia ortholog. Selected antibodies (monoclonals and polyclonals) were used to build specificity reagents to confirm plates were coated correctly, based on a specification of ≤ 1% ECL signal of nonspecific binding (NSB). Each antigen's identity was confirmed prior to coating via sequencing and other analytical methods.

	Antibody Target				
	MPXV M1R	MPXV E8L	MPXV B6R	MPXV A35R	MPXV A29L
VACV A27L	0.03%	0.01%	0.01%	0.10%	73.20%
VACV A33R	0.04%	0.01%	0.00%	198.60%	0.00%
VACV B5R	0.02%	0.01%	84.20%	0.02%	0.01%
VACV D8L	0.07%	43.40%	0.03%	0.02%	0.00%
VACV L1R	99.20%	0.01%	0.02%	0.09%	0.00%
MPXV M1R	100%	0.01%	0.01%	0.07%	0.01%
MPXV E8L	0.04%	100%	0.00%	0.02%	0.03%
MPXV B6R	0.07%	0.00%	100%	0.02%	0.00%
MPXV A35R	0.04%	0.00%	0.01%	100%	0.00%
MPXV A29L	0.02%	0.01%	0.00%	0.01%	100%

To further distinguish between antigens, we also screened commercially-sourced human serum samples that were collected pre-epidemic (smallpox antibody positive, smallpox/mpox negative) and convalescent for mpox. Results calculated from two plate lots and highlighted in green showed at least 20% difference in ECL signal between ortholog pairs.

	% Difference in ECL Signal				
Sample	VACV A27L/ MPXV A29L	VACV A33R/ MPXV A35R	VACV B5R/ MPXV B6R	VACV D8L/ MPXV E8L	VACV L1R/ MPXV M1R
Serum-01	3.50%	18.9%	0.80%	152.4%	4.40%
Serum-02	23.6%	7.90%	11.4%	67.3%	9.30%
Serum-03	116%	75.6%	11.8%	85.0%	0.70%
Serum-04	9.80%	13.8%	7.50%	5.10%	10.9%
Serum-05	29.3%	61.7%	5.30%	35.8%	9.50%
Serum-06	2.70%	14.5%	1.90%	103%	13.6%
Serum-07	13.7%	9.60%	0.50%	102%	1.10%
Serum-08	0.80%	13.0%	4.10%	29.2%	8.60%
Serum-09	2.60%	4.30%	8.90%	105%	2.80%
Serum-10	20.5%	84.6%	16.7%	60.5%	62.7%
Serum-11	27.0%	130%	66.5%	139%	6.70%
Serum-12	23.3%	137%	67.9%	137%	5.00%
Serum-13	59.4%	2.30%	33.5%	30.2%	9.90%
Serum-14	21.6%	8.60%	139%	56.9%	53.0%
Serum-15	1.00%	37.0%	107%	98.5%	27.5%

c. V-PLEX Orthopoxvirus Panel 1 Representative Data (Calibrator, Controls and Sample Testing)

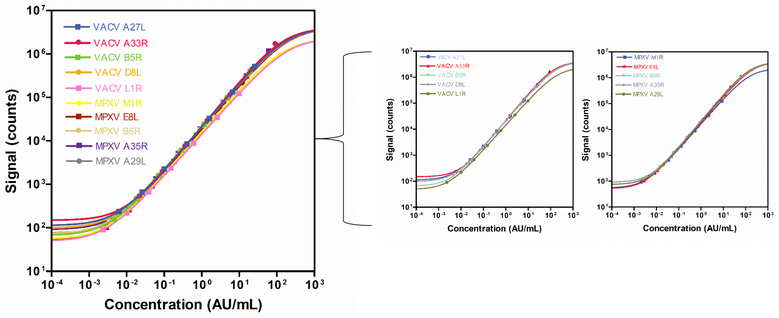
Human serum was used to build Orthopoxvirus Serology Calibrator 1 and Orthopoxvirus Serology Controls 1, 2, and 3 for use in the V-PLEX Orthopoxvirus Serology Kit. Selectivity was assessed using a set of purchased sera (as described in Section b), to confirm reproducible signal measurements for calibrator, controls and samples.

Using MSD assigned concentrations for the Orthopoxvirus Serology Calibrator 1, signal concentration curves for two lots confirmed that lot-to-lot variability was <20% CV. Functional IgG measurements were performed for each assay, and Hill slopes for the lots were tracked closely.

Each run consisted of calibrator curves, controls, Upper Limit of Quantification (ULOQ) samples, and Lower Limit of Quantification (LLOQ) samples (sample panel, serum-01-12 from Section b).

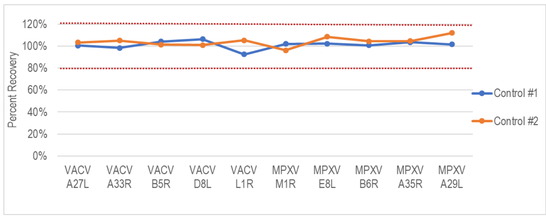
d. V-PLEX Orthopoxvirus Panel 1 Representative Data (Calibrator, Controls and Sample Testing) con't

Representative calibrator curves show a dynamic range of 3-4 logs for all assays. Orthopoxvirus Panel 1 MPXV and VACV antigens demonstrated good precision with specification of ≤ 20%.



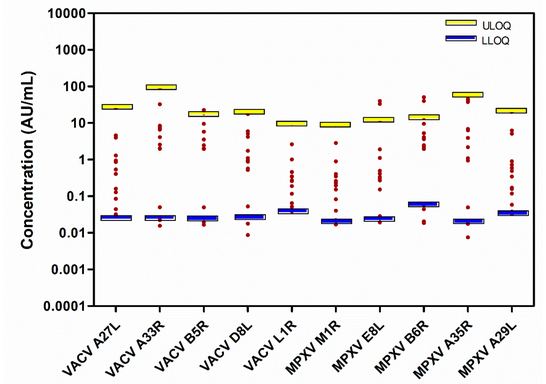
Calculated percent recovery using each control's concentration was within specification as indicated below. The precision met the specification of ≤ 20%, indicating control recoveries were very similar across two plate lots.

Orthopoxvirus Serology Control 3 is a "negative" control with concentration values <0.1 AU/mL (Arbitrary Units/mL) and are less than LLOQ (data not shown).



IgG functional testing using a set of human serum samples (as described in Sections b and c) demonstrated precision that met specification (≤ 20%).

MPXV and VACV specific IgG were measured at higher concentrations in all human serum samples compared to the negatives which have concentrations ~0.01 – 0.10 AU/mL.



4 Conclusions

Mpox infection has been a global public health concern since the multi-country outbreak in 2022. Vaccination against smallpox was protective; however, due to the global cessation of smallpox vaccination campaigns following the disease's eradication, certain populations may now be more susceptible to mpox. Studies aimed at better understanding the epidemiology, sources of infection, and transmission patterns in regards to mpox would benefit from a multiplex serological method for researching at-risk populations. This method would help distinguish between vaccinated and/or mpox-infected samples versus negative samples, and provide researchers with an alternative to PCR.

MSD was already positioned to provide tools for COVID outbreaks, and with the launch of the V-PLEX Orthopoxvirus Panel 1 in 2023, MSD was able to offer the first multiplex serology kit for mpox research applications. This multiplex panel is based on mpox and vaccinia viral antigens coated on MSD 96-well MULTI-SPOT plates. The kit includes a calibrator for quantitation (Orthopoxvirus Serology Calibrator 1), controls (Orthopoxvirus Serology Control Pack), antigen-coated plate(s), detection antibody (SULFO-TAG Anti-Human IgG), and all other reagents necessary to conduct a quantitative research use only (RUO) assay. The kit has various applications, including correlation of protection studies for vaccine development, study of long-term humoral immunity to mpox infection following natural infection or vaccination, and orthopoxvirus vaccination or surveillance (Hicks, et al., 2024; Macedo Cincotta, et al., 2024).

The V-PLEX Orthopoxvirus Serology Kit is a validated, quantitative, multiplexed RUO serology kit that accurately and precisely quantifies IgG antibodies against the MPXV and VACV proteins (VACV A27L, VACV A33R, VACV B5R, VACV D8L, VACV L1R, MPXV M1R, MPXV E8L, MPXV B6R, MPXV A35R and MPXV A29L).

- Evaluation of a Multiplexed Immunoassay for Assessing Long-Term Humoral Immunity to Monkeypox infection and Orthopoxvirus Vaccination. B Hicks, S Jones, H Callaby, E Linley, S Tonge, C Oeser, R Jones, M Pond, R Mehta, D Wright, B Hallis, C Rowe, A Otter. medRxiv 2024.05.30.24308119; doi: https://doi.org/10.1101/2024.05.30.24308119.
- Analytical sensitivity of MSD discovery mpox E8L antigen on the V-PLEX Orthopoxvirus Panel 1 IgG binding plates. C Macedo Cincotta, D Coleman, Jr Enoch, S Padilla, S Mansouri, K Guy, A Mozeyko, JA. Malia, JM Darden, SA Peel and KK Peachman. 2024. Keystone Conference, Germany.

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