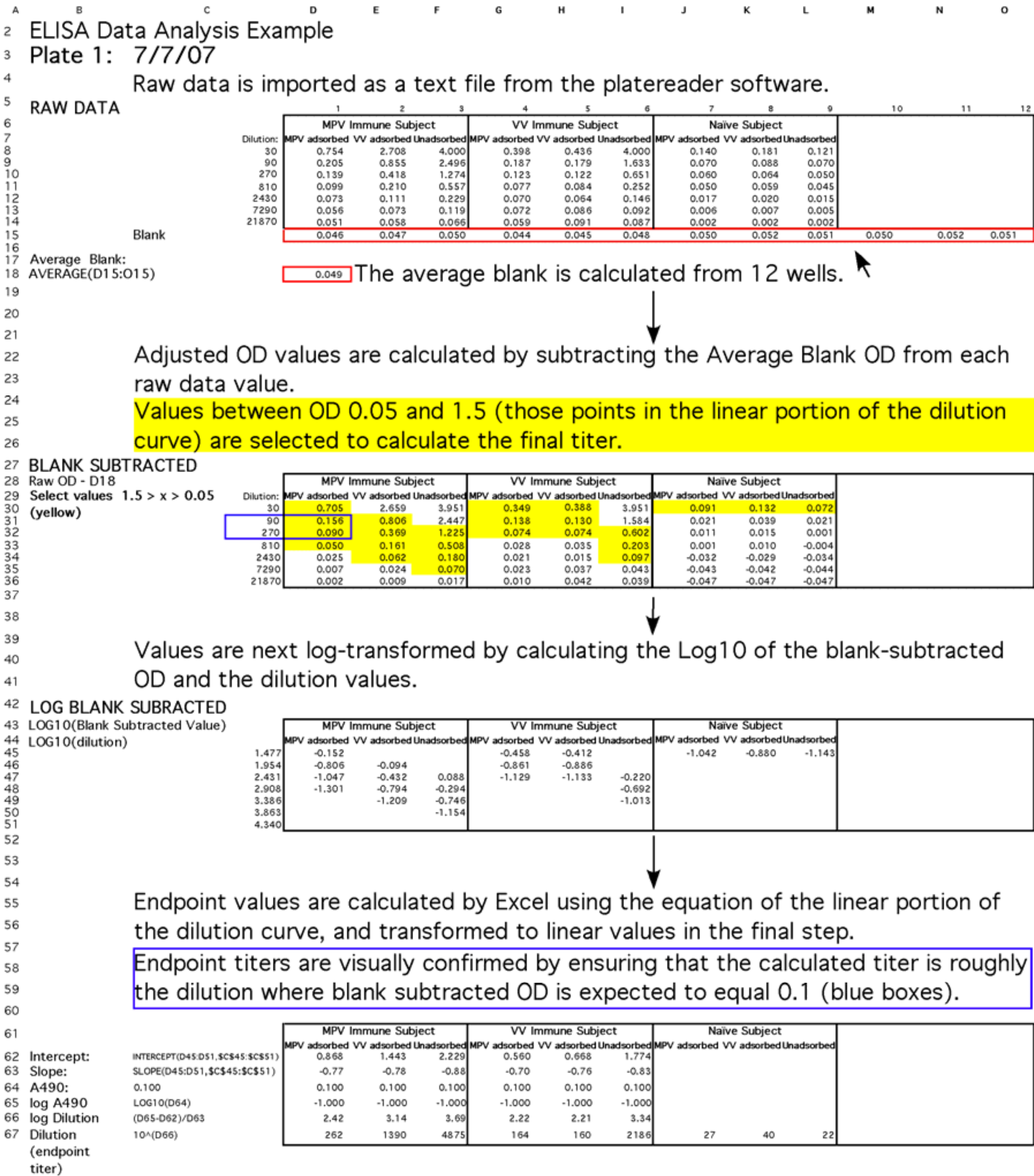
Technical Appendix



ELISA Data Analysis Example

Plate 1: 7/7/07

Raw data is imported as a text file from the platereader software.

RAW DATA

Dilution:	MPV Immune Subject: MPV adsorbed	MPV Immune Subject: VV adsorbed	MPV Immune Subject: Unadsorbed	VV Immune Subject: MPV adsorbed	VV Immune Subject: VV adsorbed	VV Immune Subject: Unadsorbed	Naïve Subject: MPV adsorbed	Naïve Subject: VV adsorbed	Naïve Subject: Unadsorbed	10	11	12
30	0.754	2.708	4.000	0.398	0.436	4.000	0.140	0.181	0.121			
90	0.205	0.855	2.496	0.187	0.179	1.633	0.070	0.088	0.070			
270	0.139	0.418	1.274	0.123	0.122	0.651	0.060	0.064	0.050			
810	0.099	0.210	0.557	0.077	0.084	0.252	0.050	0.059	0.045			
2430	0.073	0.111	0.229	0.070	0.064	0.146	0.017	0.020	0.015			
7290	0.056	0.073	0.119	0.072	0.086	0.092	0.006	0.007	0.005			
21870	0.051	0.058	0.066	0.059	0.091	0.087	0.002	0.002	0.002			
Blank	0.046	0.047	0.050	0.044	0.045	0.048	0.050	0.052	0.051	0.050	0.052	0.051

Average Blank:
AVERAGE(D15:O15)

0.049 The average blank is calculated from 12 wells.

Adjusted OD values are calculated by subtracting the Average Blank OD from each raw data value.

Values between OD 0.05 and 1.5 (those points in the linear portion of the dilution curve) are selected to calculate the final titer.

BLANK SUBTRACTED
Raw OD - D18
Select values 1.5 > x > 0.05 (yellow)

Dilution:	MPV Immune Subject: MPV adsorbed	MPV Immune Subject: VV adsorbed	MPV Immune Subject: Unadsorbed	VV Immune Subject: MPV adsorbed	VV Immune Subject: VV adsorbed	VV Immune Subject: Unadsorbed	Naïve Subject: MPV adsorbed	Naïve Subject: VV adsorbed	Naïve Subject: Unadsorbed
30	0.705	2.659	3.951	0.349	0.388	3.951	0.091	0.132	0.072
90	0.156	0.806	2.447	0.138	0.130	1.584	0.021	0.039	0.021
270	0.090	0.369	1.225	0.074	0.074	0.602	0.011	0.015	0.001
810	0.050	0.161	0.508	0.028	0.035	0.203	0.001	0.010	-0.004
2430	0.025	0.062	0.180	0.021	0.015	0.097	-0.032	-0.029	-0.034
7290	0.007	0.024	0.070	0.023	0.037	0.043	-0.043	-0.042	-0.044
21870	0.002	0.009	0.017	0.010	0.042	0.039	-0.047	-0.047	-0.047

Values are next log-transformed by calculating the Log10 of the blank-subtracted OD and the dilution values.

LOG BLANK SUBRACTED
LOG10(Blank Subtracted Value)
LOG10(dilution)

	MPV Immune Subject: MPV adsorbed	MPV Immune Subject: VV adsorbed	MPV Immune Subject: Unadsorbed	VV Immune Subject: MPV adsorbed	VV Immune Subject: VV adsorbed	VV Immune Subject: Unadsorbed	Naïve Subject: MPV adsorbed	Naïve Subject: VV adsorbed	Naïve Subject: Unadsorbed
1.477	-0.152			-0.458	-0.412		-1.042	-0.880	-1.143
1.954	-0.806	-0.094		-0.861	-0.886				
2.431	-1.047	-0.432	0.088	-1.129	-1.133	-0.220			
2.908	-1.301	-0.794	-0.294			-0.692			
3.386		-1.209	-0.746			-1.013			
3.863			-1.154						
4.340									

Endpoint values are calculated by Excel using the equation of the linear portion of the dilution curve, and transformed to linear values in the final step.

Endpoint titers are visually confirmed by ensuring that the calculated titer is roughly the dilution where blank subtracted OD is expected to equal 0.1 (blue boxes).

		MPV Immune Subject: MPV adsorbed	MPV Immune Subject: VV adsorbed	MPV Immune Subject: Unadsorbed	VV Immune Subject: MPV adsorbed	VV Immune Subject: VV adsorbed	VV Immune Subject: Unadsorbed	Naïve Subject: MPV adsorbed	Naïve Subject: VV adsorbed	Naïve Subject: Unadsorbed
Intercept:	INTERCEPT(D45:D51,$C$45:$C$51)	0.868	1.443	2.229	0.560	0.668	1.774			
Slope:	SLOPE(D45:D51,$C$45:$C$51)	-0.77	-0.78	-0.88	-0.70	-0.76	-0.83			
A490:	0.100	0.100	0.100	0.100	0.100	0.100	0.100			
log A490	LOG10(D64)	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000			
log Dilution	(D65-D62)/D63	2.42	3.14	3.69	2.22	2.21	3.34			
Dilution (endpoint titer)	10^(D66)	262	1390	4875	164	160	2186	27	40	22

Figure 1. Illustration of ELISA data analysis. Optical density values at 490 nm (OD_{490}) values were imported directly into Excel (Microsoft, Redmond, WA) from the ELISA microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA). The average OD_{490} of 12 blank wells (Line 18) was then subtracted from the raw OD_{490} obtained for each sample well. These values were transformed to logarithmic scale to create a log-log curve. We use linear OD_{490} values between 1.5 and 0.05 because these typically fall within the linear portion of the curve and are used to determine the endpoint titer. The slope and intercept of the linear portion of the curve were determined by a standard $y = mx + b$ calculation, and the dilution at which $OD_{490} = 0.1$ was transformed to a linear value to give a final endpoint titer.

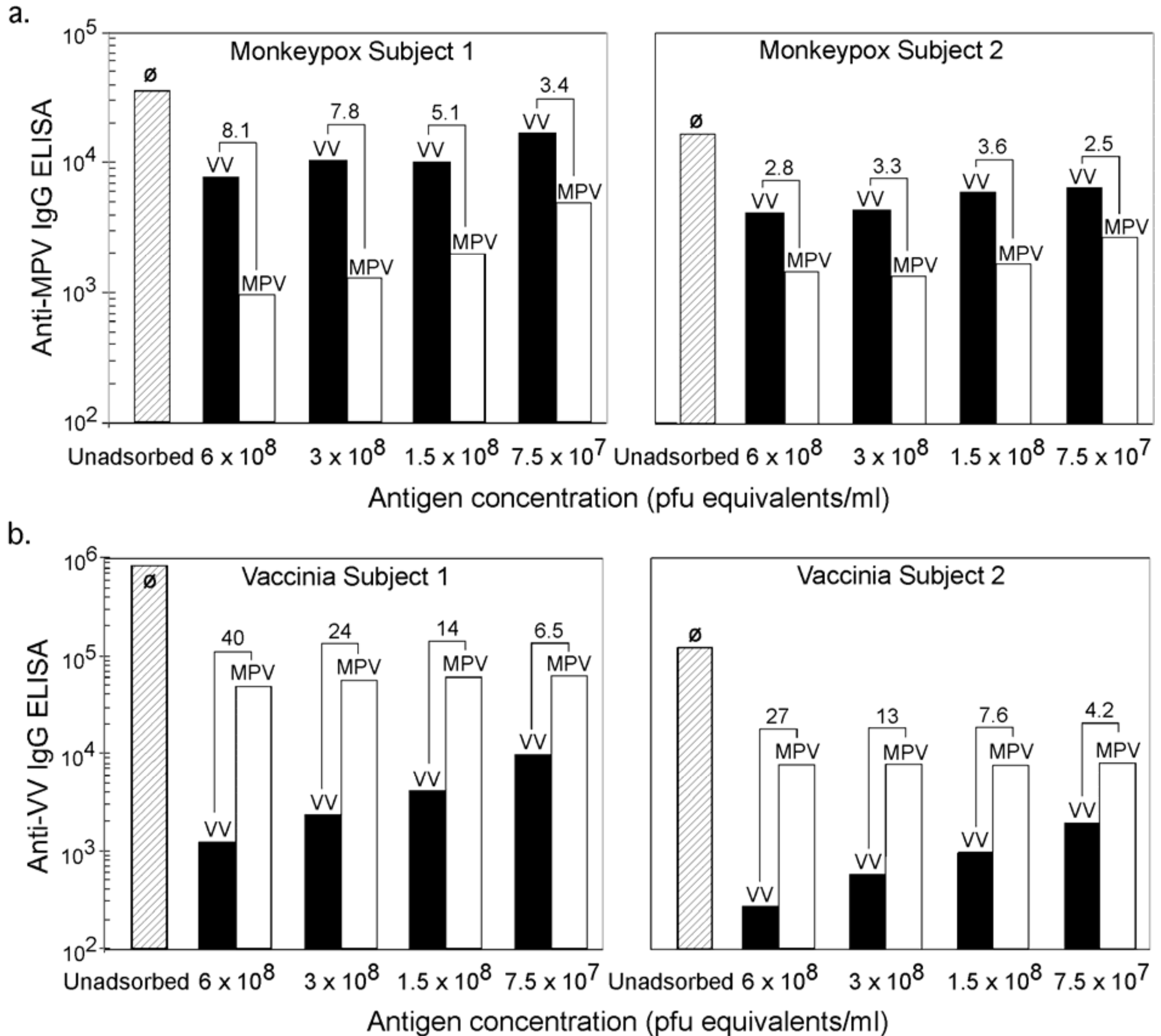


Figure 2. Titration of antigen for postadsorption ELISA. Preliminary experiments were performed to determine the optimal concentration of antigen at which orthopoxvirus infections could be differentiated by postadsorption ELISA. Plasma samples were obtained from a) 2 monkeypox-immune subjects (2–4 months postinfection), or b) 2 vaccinia-immune subjects (21 days postbooster vaccination) and tested on ELISA plates coated with a) inactivated monkeypox antigen or b) inactivated vaccinia antigen. Plasma from each subject was not preadsorbed (hatched bars), preadsorbed with inactivated vaccinia antigen (black bars), or preadsorbed with inactivated monkeypox antigen (white bars) at the indicated antigen concentrations. Values above brackets refer to the fold difference in postadsorption ELISA titers. Postadsorption ELISA may be used for diagnosing monkeypox infection by testing the plasma samples from an unvaccinated monkeypox-infected person (MPV)- or VV-adsorbed plasma on a MPV-coated plate. If the infecting virus is monkeypox (panel a), then MPV antigen is better at reducing MPV immunoglobulin (Ig) G titers than VV antigen. Alternatively, vaccinia infection may be distinguished from monkeypox by testing the adsorbed plasma on a VV-coated plate (panel b). If the infection was caused by vaccinia, then vaccinia antigen is better at reducing anti-VV IgG (i.e., tested on a VV-coated plate) than monkeypox antigen.

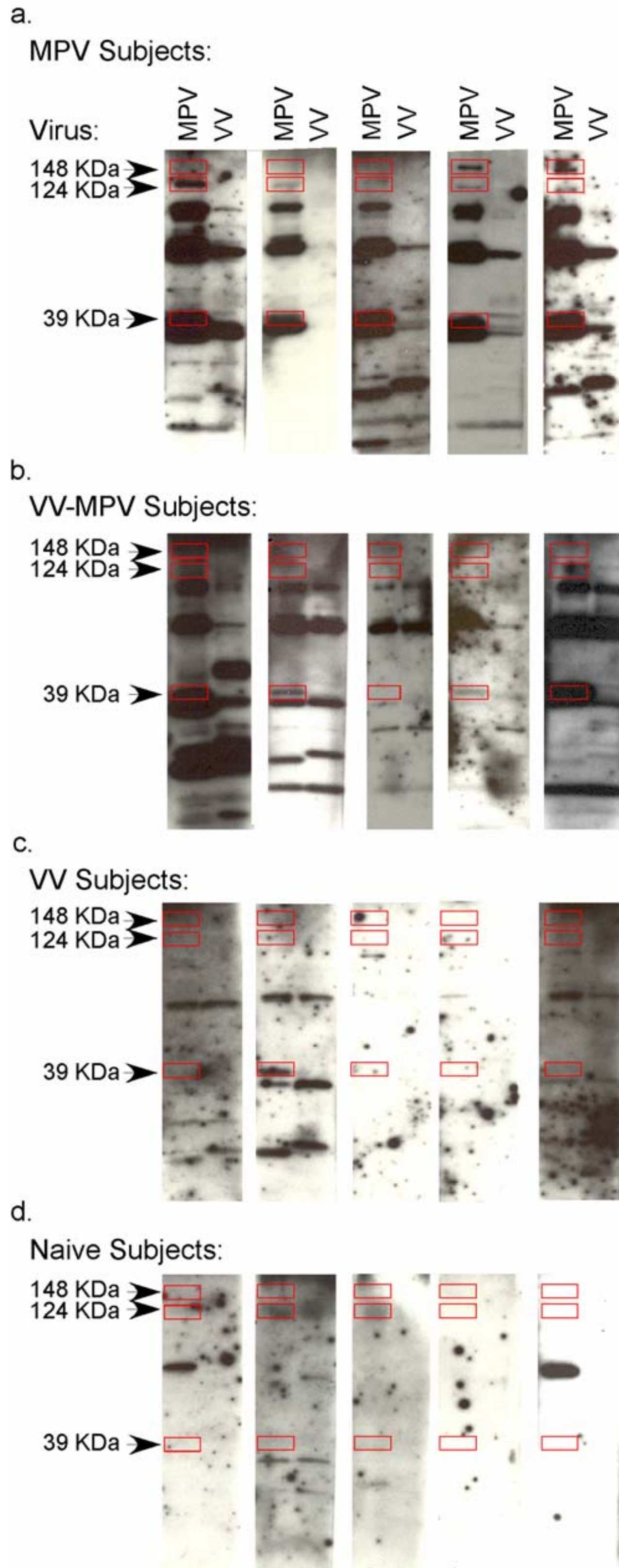


Figure 3. Examples of Western blot analysis of orthopoxvirus infections. Preadsorption of cross-reactive anti-orthopoxvirus antibodies with vaccinia antigen before Western blot analysis provided easier identification of monkeypox-specific bands. Purified monkeypox virus and vaccinia (2 μg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 4%–20% Tris-glycine gels. Proteins were electrophoretically transferred to polyvinylidene fluoride membranes, and subsequently probed with plasma from a) monkeypox-immune persons, b) vaccinia-monkeypox immune persons, c) vaccinia-immune persons, or d) orthopoxvirus-naive persons after preadsorption of plasma with vaccinia antigen (H_2O_2-inactived vaccinia lysate). Immunoreactive bands were detected with peroxidase-conjugated anti-human immunoglobulin G plus chemiluminescent substrate and exposed to x-ray film. Samples from monkeypox virus (MPV), VV (vaccina virus)–MPV, or VV persons were exposed to film for 15 s to 5 min. Samples from OPV-naive persons were exposed to film for 30 s to 1 h. Arrows and boxes indicate location of diagnostic bands with apparent molecular masses of 148 kDa, 124 kDa, and 39 kDa.