Analytical and Clinical Performance Evaluation Report

for Bioperfectus Monkeypox Virus Real Time PCR Kit (2T)

Prepared for Jiangsu Bioperfectus Technologies Co., Ltd.

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INTENDED USE

This kit is intended for qualitative detection of monkeypox virus nucleic acids in human specimens such as lesion exudate and scab. This kit is only used as an auxiliary method, rather than the sole basis for clinical diagnosis.

SPECIAL CONDITIONS FOR USE STATEMENTS

For Prescription Use Only
For In vitro Diagnostic Use Only

DEVICE DESCRIPTION AND TEST PRINCIPLE

The Monkeypox Virus (MPXV) Fast Real Time PCR Kit is a real-time PCR test intended for the qualitative detection of monkeypox virus DNA using lesion swab specimens collected from patients presenting an acute pustular or vesicular rash when suspected of monkeypox virus infection by their healthcare provider.

Lesion swab specimens are collected with synthetic swab and stored in viral transportation medium (VTM) (Lampire, Catalog # 7574001) or equivalent. A total of 200 µL of specimen is used for DNA extraction with the MagMAX™ Viral/Pathogen II (MVPII) Nucleic Acid Isolation Kit using KingFisher Flex Purification System, an automated nucleic acid extraction system from ThermoFisher Scientific following the manufacture's instruction. The program used is MVP_2Wash_400_Flex(1) and the DNA is eluted in 90 µL of the elution buffer (ThermoFisher Scientific, Catalog # A42364).

Amplification and detection are accomplished using TaqMan based chemistry on the real time PCR instruments such as ABI 7500 Fast Dx, QuantStudio 5 Dx from ThermoFisher Scientific, and CFX96 Touch from Bio-Rad. The assay detects two MPXV specific targets (MPXV-F3L and MPXV-B7R) and the human RNase P gene as the internal control (IC).

INSTRUMENTS USED WITH THE TEST

The Bioperfectus Monkeypox Virus (MPXV) Fast Real Time PCR Kit is to be used with the following instrumentation:

- Bio-Rad CFX96™Touch (Software CFX Maestro 2.2)
- Applied Biosystems 7500 Fast Dx Real-Time PCR System (Software v1.4.1)
- Applied Biosystems QuantStudio™ 5 Dx, 96-Well, 0.2 mL (Software v1.2.0)

INSTRUMENTS AND MATERIALS USED FOR THE STUDY

The instruments, reagents, and consumables are summarized in Table 1.

Table 1: Reagents/Materials Used to Perform the Bioperfectus Monkeypox Virus (MPXV) Fast Real Time PCR Kit

	Item	Utilization	Manufacture	Cat#	Lot or serial #
Instrument	KingFisher Flex, software: MVP_2Wash_400_Flex(1)	Automated instrument for monkeypox DNA extraction	ThermoFisher	5400630	711-84851
	Bio-Rad CFX96TM Touch 100. Software: CFX Maestro 2.2	Real-time PCR assay	Bio-Rad	NA	785BR26933
	Applied Biosystems™ 7500 Fast Dx. Software: 1.4.1	Real-time PCR assay	ThermoFisher	4406984	275032423
	Applied Biosystems™ QuantStudio™ 5 Dx, 96-Well, 0.2 mL. Software: 1.2.0	Real-time PCR assay	ThermoFisher	A28569	2888822080174
Reagent	MagMAX™ Viral/Pathogen II (MVPII) Nucleic Acid Isolation Kit	To be used with KingFisher to extract monkeypox DNA	ThermoFisher	A48383	Component Specific
	Monkeypox Virus (MPXV) Fast Real Time PCR Kit (2T)	Monkeypox viral DNA detection	BioPerfectus	JC(Z)70203N	Lot#1: 20220701; Lot#2: 20220702; Lot#3: 20220703; Lot#4: 20220601.
Consumables*	96 well Deep-well plate*	To be used on KingFisher to extract monkeypox DNA	ThermoFisher	A43075	Various
	Pharma KingFisher™ Flex 96 Deep-Well Tip Combs*	To be used on KingFisher to extract monkeypox DNA	ThermoFisher	A43074	Various
	0.1 mL low profile PCR plate*	Used on ABI7500 FAST DX and Bio-Rad CFX96 Touch	Stellar Scientific	P96-104	Various
	0.2 mL PCR Plate	Used on ABI Quant 5 DX	ThermoFisher	N8010560	I14U2
	Microseal 'B' PCR Plate Sealing Film, adhesive, optical. 100/pk	Seal the reaction plate for RT-PCR assay	Bio-Rad	MSB1001	Various
	Filtered tips*	setting up the RT-PCR assay reactions	Various	NA	NA
	Test tubes*	setting up the RT-PCR assay reactions	Various	NA	NA

*Different brand of consumables can be used for this assay if they are verified prior to using for the testing.

CONTROLS TO BE USED WITH THE BIOPERFECTUS TECHNOLOGY MONKEYPOX VIRUS QUALITATIVE REAL TIME PCR ASSAY

Each assay kit contains a positive and a negative control sample. These control samples will be carried throughout the entire process from DNA extraction to real-time PCR testing and results interpretation. Endogenous human RNase P gene is used as an internal control for sample quality.

INTERPRETATION OF RESULTS

All test controls must be examined prior to interpretation of patient results. If the control results are not acceptable, the patient results cannot be interpreted.

1) Assay Controls – Expected Results

Table 2. Expected control results

	MPXV-F3L (FAM)	MPXV-B7R (VIC or HEX)	Cy5
Blank Control	UNDET	UNDET	UNDET or Ct>40
Positive Control	Ct≤30	Ct≤30	Ct≤30

*A NCM control (Negative Matrix) shall be run during the assay verification study when it is used as matrix to generate contrived samples.

2) Examination and Interpretation of Specimen Results:

Interpretation of clinical specimen test results can be conducted after the positive and negative controls have been examined and determined to be valid. If the controls are not valid, results for clinical specimens cannot be interpreted. The test results interpretation criteria are presented in Table 3.

Table 3. The test results interpretation criteria for the assay

FAM (F3L)	VIC or HEX (B7R)	Cy5 IC (RP)	Results	Action
Ct<40 with a valid amplification curve	Ct≤40 with a valid amplification curve	Any Ct values or undetected	Monkeypox Virus Detected	Report monkeypox virus positive Notify appropriate Federal, State, or local public health agencies of the test results in accordance with applicable laws
Undetected	Undetected	Ct≤37	Monkeypox Virus Not Detected	Report monkeypox virus negative
Ct≤40	Undetected	Ct≤37	Inconclusive	Re-extracted and retested, if the result remains the same, report positive. If there is not adequate specimen to re-extract, then the first extraction may be used for the re-test.
Undetected	Ct≤40	Ct≤37		
Ct>40 or undetected	Ct>40 or undetected	Ct>37 or undetected	Invalid	Re-extracted and retested, if the result remains the same, recollect the specimen. If there is not adequate specimen to re-extract, then the first extraction may be used for the re-test.

PERFORMANCE EVALUATION

1. Quantification of the MPXV positive clinical remnant sample

This experiment was done using the two-target assay (JC(Z)20203N) by Bioperfectus Technologies. A MPXV positive clinical sample was obtained from a private biobank. The sample came in two separate vials (one labeled as CMPXV-1 and the other as CMPXV-2). The viral concentration of these samples was quantified with a standard curve using a standard quantitative synthetic Monkeypox DNA sample purchased from ATCC (VR-3270SD). The standard was diluted to 1000000, 100000, 10000, 1000, and 100 copies/ mL and extracted with the testing samples at the same time with 5 replicates for each diluted sample. The testing samples were diluted by 100, 500, and 1000-fold and tested in triplicates. The results of the standard curve are summarized in Table 4, and Figure 1. No signals were detected at 100 copies/mL and was thus not included in Table 4.

Table 4. The results of the standard curve

Target	Reference Sample	R1	R2	R3	R4	R5	Mean Ct	STDEV	Con (copies/mL)
MPXV-F3L	VR-D1	24.02	23.57	23.73	24.02	23.82	23.83	0.19	10^6
	VR-D2	26.67	26.89	27.03	26.75	26.82	26.83	0.14	10^5
	VR-D3	30.67	30.65	30.42	30.16	30.31	30.44	0.22	10^4
	VR-D4	34.10	34.03	34.67	33.94	33.97	34.14	0.30	10^3
MPXV-B7R	VR-D1	23.89	23.65	23.71	24.02	23.72	23.80	0.15	10^6
	VR-D2	26.73	26.95	27.08	26.95	27.00	26.94	0.13	10^5
	VR-D3	30.65	30.82	30.53	30.21	30.38	30.52	0.24	10^4
	VR-D4	33.95	33.59	35.05	34.07	34.35	34.20	0.55	10^3

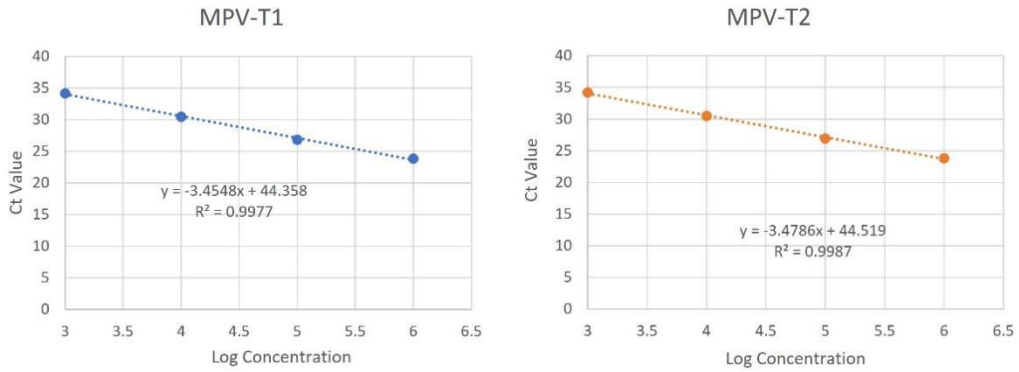

Figure 1. The standard curve of MPXV-F3L and MPXV-T . X-axis is Log_{10} (copy number/mL), Y-axis is Ct value

The results of the quantification test of the CMPXV-1 and CMPXV-2 clinical sample are summarized in Table 5.

Table 5. The Quantification Test results of MPXV sample

MPXV-F3L	R1	R2	R3	Mean	X	Detected	Dilution Fold	Sample Con (cps/mL)

CMPXV-1:1:0100	27.54	27.08	27.28	27.30	4.94	86693	100	8669348
CMPXV-1:1:0500	29.80	29.26	29.66	29.57	4.28	19063	500	9531488
CMPXV-1:1:1000	30.54	30.31	30.26	30.37	4.05	11195	1000	11194707
CMPXV-2:1:0100	26.99	26.76	26.87	26.88	5.06	114905	100	11490549
CMPXV-2:1:0500	29.45	29.51	29.33	29.43	4.32	20964	500	10482065
CMPXV-2:1:1000	30.34	30.50	30.38	30.40	4.04	10940	1000	10939866
MPXV-F3L								
CMPXV-1:1:0100	27.51	27.31	27.44	27.42	4.92	82238	100	8223812
CMPXV-1:1:0500	29.84	29.25	29.77	29.62	4.28	19175	500	9587749
CMPXV-1:1:1000	30.82	30.67	30.55	30.68	3.98	9516	1000	9516451
CMPXV-2:1:0100	27.16	27.08	27.04	27.09	5.01	102144	100	10214409
CMPXV-2:1:0500	29.43	29.91	29.60	29.65	4.28	18845	500	9422552
CMPXV-2:1:1000	30.19	30.61	30.47	30.42	4.05	11278	1000	11278221
Average								**10045935**

The result shown that the viral concentration of this MPXV sample is 10^7 copies/mL. This quantified MPXV sample is used for the entire study.

2. Inclusivity (Analytical Reactivity):

An *in silico* inclusivity analysis was conducted by aligning the MPXV-F3L(F3L) and MPXV-B7R target primers and probe sequence against available monkeypox virus sequences as of October 11, 2022 in the NCBI complete genome database and GSIAD database using basic local alignment search tool (BLAST). The evaluation included a total of 3207 monkeypox virus sequences, including 3129 sequences of monkeypox virus (clade II) and 78 sequences of monkeypox virus (clade I Central African clade). Genome sequences with longer than 150,000 nucleotides were included.

For MPXV-F3L primers/probe sets, of the 3129 sequence entries analyzed for monkeypox virus (clade II), 3121 had 100% homology with both primers and the probe. The mismatches of other 8 sequences occurred only once at a non-critical position in the probe region. Of the 78 sequence entries analyzed for monkeypox virus (clade I), 77 had 100% homology with both primers and the probe, and 1 sequence had a mismatch at a non-critical position in the reverse primer region (Table 6). Therefore, MPXV-F3L can detect both monkeypox virus clade I and clade II.

For MPXV-B7R primers/probe sets, analyzed for monkeypox virus (clade II), 3099 had 95% homology with both primers and the probe. The mismatches occurred only once at a non-critical position in the probe. The other 22 sequences had 100% homology with the primers and probe. Of the 78 sequence entries analyzed for monkeypox virus (clade I), 78 has 100% homology with both primers and the probe (Table 6). Therefore, MPXV-B7R can detect both monkeypox virus clade I and clade II as well.

Table 6. Sequence homology between MPXV primers/probes and monkeypox virus by *in silico* analysis

Species	Clade	Number of sequences evaluated	Sequences with 100% match to both MPXV-F3L primers and probe	Sequences with 100% match to both MPXV-B7R primers and probe
Monkeypox virus	West African (clade II)	3129	3121 8 had only one mismatch at a non-critical position in the probe region	22 3099 had only one mismatch at a non-critical position in the probe
Monkeypox virus	Central African (clade I)	78	77 1 had only one mismatch at a non-critical position in the reverse primer region	78

3. Exclusivity/Cross-Reactivity (*In-silico* analysis):
Basic local alignment search tool (BLAST) searches were performed using the MPXV-F3L and MPXV-B7R primers and probe sets against the NCBI Complete genomes on October 11, 2022. Sequences were hit when higher than 80% homology was found with the primers and probe sets. The microorganisms analyzed were those recommended by the FDA and listed in Tables 7 and 8.

For MPXV-F3L: Its primers had an ≥80% homology with sequences in Camelpox virus, Cowpox virus, Ectromelia virus, and Vaccinia virus genomes, but the homologies happened on the same sense strand, at least 40,000 base pairs away, and multiple mismatches at 3'-end of the forward primer. Cross-reaction and interference are unlikely. For all other microorganisms some had sequences with ≥80% homology with one of primers or probe, or similar sequences were at very different genome locations. As a result, there would be no chance to form an amplicon. Therefore, those microorganisms would not cross-react or interfere with the MPXV-F3L assay (Table 7).

Table 7. Sequence homology between MPXVF3L primers/probe and other microorganisms by *in silico* analysis

Organism	TAXID	Number of sequences evaluated	Sequences with ≥80% homology with at least one primer	Sequences with ≥80% homology with both primers	Sequences with ≥80% homology with probe	Sequences with ≥80% homology with both primers and the probe
Camelpox virus	28873	10	10	10 (both on the same strand, and >40,000 away, multiple mismatches at 3'-end)	0	0
Cowpox virus	10243	92	92	77 (both on the same strand, and >40,000 away, multiple mismatches at 3'-end)	7	7
Ectromelia virus	12643	6	6	6 (both on the same strand, and >40,000 away, multiple mismatches at 3'-end)	0	0
Vaccinia virus	245	117	117	116 (both on the same strand, and >40,000 away, multiple mismatches at 3'-end)	0	0
Acinetobacter calcoaceticus	471	27	1	0	0	0
Bacteroides fragilis	817	247	4	0	0	0
Candida albicans	5476	4613	3	0	0	0
Chlamydia trachomatis	813	884	0	0	0	0

Corynebacterium diphtheriae	1717	144	0	0	0	0
Corynebacterium jeikeium	38289	27	0	0	0	0
Enterococcus faecalis	1351	997	0	0	0	0
Escherichia coli	562	23674	150	0	0	0
Homo sapiens	9606	244442	240	59 (two primers at different locations)	12	7
Human alphaherpesvirus 1	10298	127	0	0	0	0
Human alphaherpesvirus 2	10310	94	0	0	0	0
Human alphaherpesvirus 3	10335	159	0	0	0	0
Human papillomavirus	10566	250	6	0	0	0
Lactobacillus	1578	808	82	0	0	0
Molluscum contagiosum virus	10279	31	0	0	0	0
Mycoplasma genitalium	2097	38	0	0	0	0
Mycoplasma pneumoniae	2104	169	0	0	0	0
Neisseria gonorrhoeae	485	473	0	0	0	0
Pseudomonas aeruginosa	287	1785	53	0	0	0
Staphylococcus aureus	1280	4909	0	0	0	0
Staphylococcus	1282	926	4	0	0	0

epidermidis						
Streptococcus agalactiae	1311	382	83	2 (>1000000 away from each other)	0	0
Streptococcus mitis	28037	45	1	0	0	0
Streptococcus pyogenes	1314	568	1	0	0	0
Streptococcus sp. 'group C'	33972	13	0	0	0	0
Streptococcus sp. 'group G'	1320	78	0	0	0	0
Treponema pallidum	160	173	0	0	0	0
Trichomonas vaginalis	5722	63	0	0	0	0
Trichophyton rubrum	5551	1397	0	0	0	0

For MPXV-B7R: Its primers had an ≥80% homology with sequences in Camelpox virus, Cowpox virus, Ectromelia virus, and Vaccinia virus genomes. All had mismatch at 3'-end of the forward primer and multiple mismatches in the reverse primer. Most did not have a significant homology with the probe (Table 8), a cross reaction and interference are not expected. For all other microorganisms some had sequences with ≥80% homology with one of primers or probe, or similar sequences were at very different genome locations. As a result, there would be no chance to form an amplicon. Therefore, those microorganisms would not cross-react or interfere with the MPXV-B7R assay (Table 8).

Table 8. Sequence homology between MPXVB7R primers/probe and other microorganisms by *in silico* analysis

Organism	TAXID	Number of sequences evaluated	Sequences with ≥80% homology with atleast one primer	Sequences with ≥80% homology with both primers	Sequences with ≥80% homology with probe	Sequences with ≥80% homology with both primers and the probe
Camelpox virus	28873	10	10	10 (mismatch at 3'-end of the forward primer and multiple	0	0

				mismatches in the reverse primer)		
Cowpox virus	10243	92	92	65 (mismatch at 3'-end of the forward primer and at least 2 mismatches in the reverse primer)	2 (multiple mismatches)	2
Ectromelia virus	12643	6	6	0	0	0
Vaccinia virus	245	117	117	47 (mismatch at 3'-end of the forward primer and multiple mismatches in the reverse primer	37 (multiple mismatches)	37
Acinetobacter calcoaceticus	471	27	9	8 (multiple mismatches, primers at different locations)	1	0
Bacteroides fragilis	817	247	84	70 (multiple mismatches, forward and reverse primers are at least 10000 bp away)	80 (multiple mismatches, and at different locations fromthe primers	60
Candida albicans	5476	4613	13	3 (Primer are >100000 bp away)	2	0
Chlamydia trachomatis	813	884	1	0	0	0
Corynebacterium diphtheriae	1717	144	117	0	0	0
Corynebacterium jeikeium	38289	27	6	4 (Primer are >100000bp away)	0	0
Enterococcus faecalis	1351	997	285	15 (multiple mismatches, and >10000away)	109 (multiple mismatches at 5-end)	4

Escherichia coli	562	23674	7332	2954(multiple mismatches, and at different locations)	3230 multiple mismatches, and at different locations	1546
Homo sapiens	9606	244442	1035	134 (multiple mismatches and on the same strand or faraway apart)	422 (multiple mismatches)	124
Human alphaherpesvirus 1	10298	127	81	0	0	0
Human alphaherpesvirus 2	10310	94	0	0	0	0
Human alphaherpesvirus 3	10335	159	0	0	0	0
Human papillomavirus	10566	250	3	0	0	0
Lactobacillus	1578	808	249	86 (multiple mismatches or >10000 bp away)	118	28
Molluscum contagiosum virus	10279	31	0	0	0	0
Mycoplasma genitalium	2097	38	10	0	0	0
Mycoplasma pneumoniae	2104	169	156	0	0	0
Neisseria gonorrhoeae	485	473	0	0	2	0
Pseudomonas aeruginosa	287	1785	943	31 (multiple mismatches and f >10000 bp away)	9	0
Staphylococcus aureus	1280	4909	2034	1793 (multiple mismatches and f >10000 bp away)	2020 (multiple mismatches and at different locations)	1793

Staphylococcus epidermidis	1282	926	220	16 (multiple mismatches and >10000 bp away)	190(multiple mismatches and at different locations)	14
Streptococcus agalactiae	1311	382	5	0	87	0
Streptococcus mitis	28037	45	19	3(multiple mismatches and >100000 bp away)	0	0
Streptococcus pyogenes	1314	568	427	166 (multiple mismatches and >10000 bp away)	4	0
Streptococcus sp. 'group C'	33972	13	0	0	0	0
Streptococcus sp. 'group G'	1320	78	0	0	0	0
Treponema pallidum	160	173	47	0	0	0
Trichomonas vaginalis	5722	63	0	0	0	0
Trichophyton rubrum	5551	1397	0	0	0	0

4. Microbial Interference Study

The results of in-silico analysis indicate both of the primers and probe sets for the F3L and B7R gene targets are Monkeypox virus specific, no cross-reactivity and interference were predicted for all other microorganisms including the other non-variola orthopoxvirus.

To confirm the in-silico analysis predication, we tested the contrived vaccinia virus and cowpox virus, two close relatives of the Monkeypox virus, with concentration higher than 10^5TCID50/with or without the presence of 3X LoD MPXV. The results are summarized in Table 9.

Table 9. The results of microbial cross reactivity and interference study.

		Without MPXV				**With MPXV (1200 copies/mL)**			
Virus	Concentration	Sample	MPXV-F3L	MPXV-B7R	IC	Sample	MPXV-F3L	MPXV-B7R	IC
Vaccinia virus	1 X 10^5 TCID50/mL	MV031	NaN	NaN	28.38	MV031+	33.43	33.31	29.17
		MV031	NaN	NaN	28.90	MV031+	33.63	32.89	29.19
		MV031	NaN	NaN	28.82	MV031+	34.10	33.07	29.21
Cowpox		MV032	NaN	NaN	28.37	MV032+	35.34	33.01	29.28

	2.5 X 10^5 TCID50/mL	MV032	NaN	NaN	28.09	MV032+	34.11	32.84	29.10
		MV032	NaN	NaN	26.32	MV032+	32.88	32.79	28.65
	Controls	NM	NaN	NaN	29.09	NM+	33.15	32.06	29.25
		NTC	NaN	NaN	NaN	NM+	34.30	32.81	29.38
						NM+	34.59	32.09	29.56

As the table shown, all contrived vaccina and cowpox virus samples tested negative without addition of MPXV, whereases all samples with 3XLoD MPXV added were tested positive. Additionally, the Ct value of the MPXV-F3L and MPXV-B7R of the contrived samples are equivalent to that the control samples that lack of either vaccina or cowpox viruses. These results confirm that vaccina and cowpox viruses don't interact with the primer and prob sets of the assay. Therefore, this assay is highly specific for the Monkeypox virus as expected.

5. Interfering Substances:

The substances that could be found on skin lesions and the surroundings and might be swabbed with the lesion specimens together could potentially interfere with the assay. Based on the FDA's recommendation, the following 20 potential interfering substances were studied. The substance info and 2X solution preparation procedure are summarized in Table 10.

Table 10. The potential interfering substance and their 2X solution preparation

Item #	Potential Interfering Substances	Source/Cat#	2x Preparation	Test Concentration
1	Abreva	GSK/Lot 22N2940957	To 0.75 mL NCM, add 105 mg, and stir to mix	7% w/v (0.07g/mL)
				Milky
2	Acyclovir	Prescription Drug	75 uL 10X stock (70 mg/ mL PBS) + 680 uL NCM	3.5 mg/mL
				Milky
3	Albumin	Sigma/A9731-1G	33 uL (10 mg/100uL) to 0.717 mL NCM, see note	2.2 mg/mL
				Clear
4	Blood/EDTA	In-house	75 uL blood/45 uL 10mM EDTA in 630 uL NCM, see note	5% v/v
				Bloody
5	Mucin	Sigma/	Add 3.6 uL stock (25 mg/mL in DMSO) to 747 uL NCM	60ug/mL
6	Hydrocortisone cream*	HEB	See notes below	~7% w/v
7	Benadryl cream/ointment	Johnson & Johnson Consumer	To 0.75 mL NCM, add 105 mg directly, stir to mix	7% w/v
				Turbulent

8	Carmex*	CARMA Laboratories	See notes below	~7% w/v
9	Casein	Bulk Supplements.com	To 0.75 mL NCM, add 105 mg, and stir to mix	7mg/mL
				Milky
10	Lanacane	Reckitt Benckiser Healthcare	To 0.75 mL NCM, add 52.5 mg directly, stir to mix	3.5% w/v
				Milky
11	KY Jelly	RB Health	To 645 uL NCM, add 105 uL	7% v/v
				Clear
12	Douche	Summer's Eve	To 645 uL NCM, add 105 uL	7%
				Clear
13	Neosporin*	Johnson & Johnson Consumer	See notes below	7% w/v
14	Female urine	In-house	To 645 uL NCM, add 105 uL	7-10% v/v (7%)
				Clear
15	Male urine	In-house	To 645 uL NCM, add 105 uL	7-10% v/v (7%)
				Clear
16	Feces	In-house	To 0.75 mL NCM, add 3.3 mg, mix well	0.22% w/v (2.2 mg/mL)
				Muddy
17	Seminal fluid	In-house	To 645 uL NCM, add 105 uL	2-7% (7%)
				A little turbulent
18	Zinc Oxide ointment	CVS Health	To 0.75 mL NCM, add 105 mg, and stir to mix	7%
				Milky
19	Vagisil Cream	Combe Incorporated	To 0.75 mL NCM, add 15 mg, and stir to mix	1%
				A little turbulent
20	Cornstarch	Baby powder	To 0.75 mL NCM, add 3.75 mg, and stir to mix	2.5mg/mL
				Milky

*Note: These 3 substances are not water soluble. We tried several different solvents and methods but failed to get them into solution. The interference study aims to evaluate the potential interference substances introduced into the specimen during the sample collection. These creams are often used to stop itching or infection of the skin. Therefore, we mimicked the usage of these creams on the skin surface and used the swab to collect the samples. About 300 mg of each cream was put onto the arm of a volunteer covering a 2x3 cm area. Using two swabs to collect most of the creams and some skin cells by swabbing the cream covered skin area backing and forth until the swabs were covered with the cream. Put one swab into a sample collection tube with 3 mL VTM. In the bio-safety hood, pipette 3.6 µL of the 1000 copies/uL MPXV stock on to the other swab covered with the oily creams and then drop the swab into the sample collection tube with 3 mL VTM so that the final concentration is equivalent to 3X LoD. These sample were then be processed and tested together with other interference study samples.

The prepared 2X interfering substance solutions were split into two tubes with 375 µL each. In one set, 375 µL of the negative matrix were add into each tube to make the 1X solution. In the 2nd set, 375 µL of the contrived MPXV sample with a concentration of 6X LoD were added to make the sample with the final concentration of 3X LoD of MPXV. The samples were processed and tested. The results are summarized in Table 11.

Table 11: The interfering substance test results

Without MPXV				With 3X LoD MPXV			
Sample	*MPXV-F3L	*MPXV-B7R	IC	Sample	MPXV-F3L	MPXV-B7R	IC
2T-IF1-1	NaN	NaN	29.56	2T-IF1+MPV1	34.52	34.23	29.23
2T-IF1-2	NaN	NaN	30.24	2T-IF1+MPV2	34.43	35.05	29.99
2T-IF1-3	NaN	NaN	29.51	2T-IF1+MPV3	34.15	34.58	29.41
2T-IF2-1	NaN	NaN	27.38	2T-IF2+MPV1	32.59	32.48	27.28
2T-IF2-2	NaN	NaN	27.57	2T-IF2+MPV2	33.66	32.28	27.00
2T-IF2-3	NaN	NaN	27.43	2T-IF2+MPV3	33.56	32.79	27.14
2T-IF3-1	NaN	NaN	27.72	2T-IF3+MPV1	33.38	33.05	28.02
2T-IF3-2	NaN	NaN	28.07	2T-IF3+MPV2	32.58	33.23	28.03
2T-IF3-3	NaN	NaN	27.95	2T-IF3+MPV3	30.97	30.84	27.41
2T-IF4-1	NaN	NaN	27.82	2T-IF4+MPV1	32.35	32.39	27.93
2T-IF4-2	NaN	NaN	28.57	2T-IF4+MPV2	32.58	32.61	27.81
2T-IF4-3	NaN	NaN	27.88	2T-IF4+MPV3	32.44	32.73	27.41
2T-IF5-1	NaN	NaN	28.27	2T-IF5+MPV1	33.61	32.61	28.08
2T-IF5-2	NaN	NaN	27.87	2T-IF5+MPV2	33.55	33.23	28.05
2T-IF5-3	NaN	NaN	28.13	2T-IF5+MPV3	32.28	32.61	28.04
2T-IF6-1	NaN	NaN	28.38	2T-IF6+MPV1	33.88	35.44	34.82
2T-IF6-2	NaN	NaN	28.21	2T-IF6+MPV2	36.00	34.88	33.67
2T-IF6-3	NaN	NaN	28.15	2T-IF6+MPV3	35.75	34.40	NaN
2T-IF7-1	NaN	NaN	28.08	2T-IF7+MPV1	34.27	32.30	27.82
2T-IF7-2	NaN	NaN	28.15	2T-IF7+MPV2	33.58	34.44	28.22
2T-IF7-3	NaN	NaN	28.20	2T-IF7+MPV3	33.52	33.38	27.93
2T-IF8-1	NaN	NaN	28.90	2T-IF8+MPV1	34.03	32.45	36.32
2T-IF8-2	NaN	NaN	29.18	2T-IF8+MPV2	33.07	33.20	35.42
2T-IF8-3	NaN	NaN	29.18	2T-IF8+MPV3	35.03	33.99	33.05
2T-IF9-1	NaN	NaN	27.59	2T-IF9+MPV1	30.98	31.63	27.81
2T-IF9-2	NaN	NaN	28.09	2T-IF9+MPV2	31.24	32.12	27.21
2T-IF9-3	NaN	NaN	27.59	2T-IF9+MPV3	32.56	32.77	27.12
2T-IF10-1	NaN	NaN	28.24	2T-IF10+MPV1	31.87	32.04	28.00
2T-IF10-2	NaN	NaN	28.05	2T-IF10+MPV2	34.06	33.58	28.11
2T-IF10-3	NaN	NaN	28.39	2T-IF10+MPV3	32.78	33.31	28.05
2T-IF11-1	NaN	NaN	26.86	2T-IF11+MPV1	30.33	30.56	26.57
2T-IF11-2	NaN	NaN	27.40	2T-IF11+MPV2	32.29	32.03	27.10
2T-IF11-3	NaN	NaN	26.63	2T-IF11+MPV3	26.34	25.83	26.70
2T-IF12-1	NaN	NaN	28.15	2T-IF12+MPV1	32.86	33.01	28.21
2T-IF12-2	NaN	NaN	28.24	2T-IF12+MPV2	33.59	33.05	28.14
2T-IF12-3	NaN	NaN	27.81	2T-IF12+MPV3	32.87	32.69	28.12

2T-IF13-1	NaN	NaN	28.35	2T-IF13 +MPV1	33.11	33.15	33.76
2T-IF13-2	NaN	NaN	28.07	2T-IF13 +MPV2	35.75	35.90	34.21
2T-IF13-3	NaN	NaN	27.95	2T-IF13 +MPV3	34.84	35.59	32.86
2T-IF14-1	NaN	NaN	28.04	2T-IF14+MPV1	33.32	33.20	28.16
2T-IF14-2	NaN	NaN	28.12	2T-IF14+MPV2	32.64	33.10	28.15
2T-IF14-3	NaN	NaN	28.43	2T-IF14+MPV3	31.89	32.43	28.47
2T-IF15-1	NaN	NaN	27.61	2T-IF15+MPV1	32.92	33.18	28.11
2T-IF15-2	NaN	NaN	27.93	2T-IF15+MPV2	33.66	32.97	27.90
2T-IF15-3	NaN	NaN	27.83	2T-IF15+MPV3	33.63	33.77	28.23
2T-IF16-1	NaN	NaN	26.19	2T-IF16+MPV1	33.46	32.64	26.17
2T-IF16-2	NaN	NaN	26.24	2T-IF16+MPV2	33.56	33.64	26.36
2T-IF16-3	NaN	NaN	26.60	2T-IF16+MPV3	34.07	33.37	26.52
2T-IF17-1	NaN	NaN	23.80	2T-IF17+MPV1	32.92	32.03	23.93
2T-IF17-2	NaN	NaN	24.01	2T-IF17+MPV2	32.64	32.51	23.96
2T-IF17-3	NaN	NaN	23.69	2T-IF17+MPV3	33.09	32.62	24.13
2T-IF18-1	NaN	NaN	28.95	2T-IF18+MPV1	33.63	34.43	28.55
2T-IF18-2	NaN	NaN	28.52	2T-IF18+MPV2	33.02	33.90	28.72
2T-IF18-3	NaN	NaN	28.47	2T-IF18+MPV3	34.21	34.50	28.32
2T-IF19-1	NaN	NaN	27.18	2T-IF19+MPV1	32.31	32.43	27.34
2T-IF19-2	NaN	NaN	27.49	2T-IF19+MPV2	33.44	33.28	27.66
2T-IF19-3	NaN	NaN	27.67	2T-IF19+MPV3	33.32	32.27	27.90
2T-IF20-1	NaN	NaN	28.04	2T-IF20+MPV1	33.16	34.30	28.62
2T-IF20-2	NaN	NaN	28.28	2T-IF20+MPV2	33.42	35.04	28.95
2T-IF20-3	NaN	NaN	28.13	2T-IF20+MPV3	34.27	34.20	28.59
2T-NM	NaN	NaN	28.68	**2T-SWAB+MPV1	36.42	35.20	32.17
2T-NTC	NaN	NaN	35.70	**2T-SWAB+MPV2	36.24	35.48	32.34
2T-POS	22.92	22.99	23.24	**2T-SWAB+MPV3	34.36	35.92	33.50

*NaN: not detected

**3.6 µL 10^6copies/mL MPXV stock was added onto a swab of skin sample without interference substances and the swab was then dropped into a sample collection tube with 3mL VTM to make 3XLoD of MPXV equivalent skin swab sample.

No interference was observed for any of these 20 tested substances.

6. Specimen Stability:

To assess the specimen stability, 4 sets of 10 negative matrix samples (NCM), 30 2X LoD samples and 10 4X LoD samples were prepared. One set was tested on the same day of the sample preparation as the baseline. One set was stored at room temperature for 3 days, one set stored at -80 °C for 3 days and one set stored at 2-8 °C for 5 days. These samples were than tested. The results are summarized in Table 12.

Table 12. The specimen stability test results with the 2T reagent kit.

Sample storage Condition		MPXV-F3L		MPXV-B7R	
	Sample	Mean Ct	% Detected	Mean Ct	% Detected
Day 0 Baseline	2XLOD	34.93	97 (29/30)	35.21	97 (29/30)
	4XLOD	33.66	100 (10/10)	34.17	100 (10/10)

	NCM	NA	0	NA	0
Day 3 at Room Temperature	2XLOD	35.01	100 (30/30)	35.17	97 (29/30)
	4XLOD	33.75	100 (10/10)	34.07	100 (10/10)
	NCM	NA	0	NA	0
Day 3 at -80 °C	2XLOD	34.51	100 (30/30)	34.95	100 (30/30)
	4XLOD	33.23	100 (10/10)	33.23	100 (10/10)
	NCM	NA	0	NA	0
Day 5 at 2-8 °C	2XLOD	35.03	100 (30/30)	35.46	100 (30/30)
	4XLOD	33.80	100 (10/10)	34.14	100 (10/10)
	NCM	NA	0	NA	0

As the table shown, all contrived positive samples tested positive, and the mean Ct value are like that of day 0 after storage. The results indicate that MPXV samples are stable in the VTM under the tested conditions.

7. Clinical evaluations

Clinical evaluation of the assay was conducted using contrived positive samples made of unique clinical remnant skin lesion samples. A total of 40 individual remnant samples were used. Each sample was split to two portions, one was used as the negative sample and the other one was used to make the contrived positive sample. Different amount of MPXV positive sample was spiked into the individual lesion sample to create contrived positive samples composed of 5 5X LoD, 5 2.5X LoD , 10 1.5X LoD and 20 1X LoD samples. A total of 80 samples were randomized using the "=RAND ()" function of the excel file. Based on the randomized list, each sample was given a testing ID. The randomized 80 samples were then processed and tested blindly. The teste results are summarized in Table 13.

Table 13. The clinical evaluation results

Testing ID	**MPXV Con. (Cps/mL)**	**Expected**	Sample	MPXV-F3L	MPXV-B7R	IC	Matched
CT01	0	Negative	CT-01	NaN	NaN	30.45	Y
CT02	0	Negative	CT-02	NaN	NaN	32.94	Y
CT03	0	Negative	CT-03	NaN	NaN	33.04	Y
CT04	400	Positive	CT-04	35.48	35.76	34.30	Y
CT05	0	Negative	CT-05	NaN	NaN	38.51	Invalid
CT06	400	Positive	CT-06	35.59	35.66	31.65	Y
CT07	400	Positive	CT-07	36.94	34.67	27.96	Y
CT08	400	Positive	CT-08	36.49	35.16	34.27	Y
CT09	0	Negative	CT-09	NaN	NaN	NaN	Invalid
CT10	400	Positive	CT-10	35.03	35.62	34.82	Y
CT11	400	Positive	CT-11	36.14	35.59	36.64	Y
CT12	0	Negative	CT-12	NaN	NaN	32.42	Y
CT13	400	Positive	CT-13	35.81	35.50	22.81	Y

CT14	0	Negative	CT-14	NaN	NaN	35.44	Y
CT15	0	Negative	CT-15	NaN	NaN	37.10	Invalid
CT16	0	Negative	CT-16	NaN	NaN	NaN	Invalid
CT17	0	Negative	CT-17	NaN	NaN	32.20	Y
CT18	2000	Positive	CT-18	34.47	33.05	36.07	Y
CT19	400	Positive	CT-19	36.63	35.17	32.53	Y
CT20	2000	Positive	CT-20	33.53	32.98	33.09	Y
CT21	400	Positive	CT-21	36.55	35.56	33.11	Y
CT22	0	Negative	CT-22	NaN	NaN	38.67	Invalid
CT23	0	Negative	CT-23	NaN	NaN	26.13	Y
CT24	0	Negative	CT-24	NaN	NaN	34.00	Y
CT25	400	Positive	CT-25	36.50	36.10	33.17	Y
CT26	0	Negative	CT-26	NaN	NaN	NaN	Invalid
CT27	0	Negative	CT-27	NaN	NaN	23.22	Y
CT28	400	Positive	CT-28	36.88	34.67	26.91	Y
CT29	0	Negative	CT-29	NaN	NaN	39.11	Invalid
CT30	0	Negative	CT-30	NaN	NaN	33.35	Y
CT31	0	Negative	CT-31	NaN	NaN	35.17	Y
CT32	400	Positive	CT-32	36.10	36.01	29.49	Y
CT33	400	Positive	CT-33	37.01	36.02	35.32	Y
CT34	0	Negative	CT-34	NaN	NaN	34.86	Y
CT35	1000	Positive	CT-35	38.10	36.49	39.38	Y
CT36	0	Negative	CT-36	NaN	NaN	36.91	Y
CT37	400	Positive	CT-37	NaN	34.70	36.56	Inconclusive
CT38	0	negative	CT-38	NaN	NaN	31.51	Y
CT39	0	negative	CT-39	NaN	NaN	35.27	Y
CT40	0	negative	CT-40	NaN	NaN	NaN	Invalid
CT41	0	negative	CT-41	NaN	NaN	28.10	Y
CT42	0	negative	CT-42	NaN	NaN	26.60	Y
CT43	1000	Positive	CT-43	34.15	34.00	33.11	Y
CT44	0	Negative	CT-44	NaN	NaN	26.14	Y
CT45	400	Positive	CT-45	36.37	36.49	26.05	Y
CT46	400	Positive	CT-46	36.64	37.27	39.09	Y
CT47	400	Positive	CT-47	36.78	35.16	26.68	Y
CT48	1000	Positive	CT-48	35.73	34.73	37.22	Y
CT49	0	Negative	CT-49	NaN	NaN	34.22	Y
CT50	2000	Positive	CT-50	NaN	NaN	NaN	Invalid
CT51	400	Positive	CT-51	35.96	35.32	35.62	Y
CT52	2000	Positive	CT-52	33.76	33.21	36.94	Y
CT53	1000	Positive	CT-53	34.51	33.71	NaN	Y
CT54	400	Positive	CT-54	36.49	36.01	32.95	Y
CT55	2000	Positive	CT-55	33.45	33.43	38.02	Y
CT56	0	Negative	CT-56	NaN	NaN	33.08	Y
CT57	400	Positive	CT-57	36.56	35.31	30.28	Y
CT58	0	Negative	CT-58	NaN	NaN	35.74	Y

CT59	1000	Positive	CT-59	35.31	34.18	36.04	Y
CT60	0	Negative	CT-60	NaN	NaN	29.58	Y
CT61	600	Positive	CT-61	34.55	34.51	31.89	Y
CT62	0	Negative	CT-62	NaN	NaN	30.87	Y
CT63	0	Negative	CT-63	NaN	NaN	26.00	Y
CT64	0	Negative	CT-64	NaN	NaN	35.71	Y
CT65	0	Negative	CT-65	NaN	NaN	29.23	Y
CT66	0	Negative	CT-66	NaN	NaN	26.19	Y
CT67	600	Positive	CT-67	34.34	35.12	26.37	Y
CT68	600	Positive	CT-68	35.06	34.36	27.33	Y
CT69	600	Positive	CT-69	35.21	33.89	29.17	Y
CT70	0	Negative	CT-70	NaN	NaN	31.58	Y
CT71	600	Positive	CT-71	35.44	34.34	36.42	Y
CT72	600	Positive	CT-72	35.57	34.28	31.31	Y
CT73	600	Positive	CT-73	35.42	34.03	37.20	Y
CT74	600	Positive	CT-74	34.66	34.91	25.89	Y
CT75	0	Negative	CT-75	NaN	NaN	27.87	Y
CT76	600	Positive	CT-76	35.86	34.44	30.57	Y
CT77	0	Negative	CT-77	NaN	NaN	30.72	Y
CT78	0	Negative	CT-78	NaN	NaN	29.70	Y
CT79	0	Negative	CT-79	NaN	NaN	36.38	Y
CT80	600	Positive	CT-80	35.19	34.23	30.05	Y
NEG	0	Negative	NEG	NaN	NaN	30.19	Y
NTC	0	Negative	NTC	NaN	NaN	NaN	Y
POS	NA	Positive	POS	23.11	22.67	23.81	Y

The internal RNase P control was not detected in 8 negative samples (CT09, CT16, CT26, CT40, CT05, CT15, CT22, CT29) and 2 contrived positive samples (CT50 & CT53). CT50 failed to detect all three targets, therefore it is identified as invalid. CT53 was tested positive for both MPXV-F3L and MPXV-B7R targets, so it is identified positive. The overall results are summarized in table 14.

Table 14. The results of clinical evaluation of the assay using contrived individual samples.

Con. Cps/mL	# Sample tested	Number of Positives Expected	Number of Positives Detected
2000	5	4	4*
1000	5	5	5
600	10	10	10
400	20	20	19**
0	40***	0	0

*One sample CT-50 invalid.

**One of the contrived positive samples, CT-37, with its MPXV concentration at the LoD was tested as Inconclusive (MPXV-F3L: not detected; MPXV-B7R: detected).

***Eight were invalid samples with no internal control

The positive percentage of agreement (PPA) = 38/39 = 97.43% (95% CI: 86.82% – 99.95%)
The Negative percentage of agreement (NPA) = 32/32 = 100% (95% CI: 90.36% – 100%)

Summary:

In this study, we have performed a systematic evaluation of the analytical performance of the Bioperfectus Monkeypox Virus Real Time PCR Kit. The assay is highly sensitive, fast, and easy to use. This study has established the specification of the assay when used in a CLIA certificated high complexity Laboratory:

- Validated sample type: skin lesion swab samples in VTM medium
- The assay can be run on three instruments with comparable sensitivity: Bio-Rad CFX96 Touch (both standard and fast cycling programs) , ABI7500 Fast Dx (standard cycling program) or QuantStudio 5 Dx (fast cycling program).
- The clinical performance with contrived clinical samples: 97.43% PPA and 100%NPA.
- The sample is stable under the tested condition including 3 days at room temperature, 5 days at 2-8 °C

The end

Performed and Reported by XYZ Laboratory
CLIA#: 45D2193108
CAP#: 8911731

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