

# Recombinase polymerase amplification assay for rapid detection of Monkeypox virus

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## 1. Abstract

In this study, a rapid method for the detection of Central and West Africa clades of Monkeypox virus (MPXV) using recombinase polymerase amplification (RPA) assay targeting the G2R gene was developed. MPXV, an *Orthopoxvirus*, is a zoonotic dsDNA virus, which is listed as a biothreat agent. RPA was operated at a single constant temperature of 42°C and produced results within 3 to 10 minutes. The MPXV-RPA-assay was highly sensitive with a limit of detection of 16 DNA molecules/μl. The clinical performance of the MPXV-RPA-assay was tested using 47 sera and whole blood samples from humans collected during the recent MPXV outbreak in Nigeria as well as 48 plasma samples from monkeys some of which were experimentally infected with MPXV. The specificity of the MPXV-RPA-assay was 100% (50/50), while the sensitivity was 95% (43/45). This new MPXV-RPA-assay is fast and can be easily utilised at low resource settings using a solar powered mobile suitcase laboratory.

**Keywords:** Recombinase polymerase amplification assay, Monkeypox Virus, mobile suitcase, point of need, rapid detection system

### Highlights:

1. Monkeypox virus infections can be detected in ten minutes
2. The assay based on an isothermal amplification technology named recombinase polymerase amplification
3. The whole procedure can be operated by a mobile suitcase laboratory

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## 54 **2. Introduction**

55 Monkeypox virus (MPXV) belongs to the genus *Orthopoxvirus* (OPXV, subfamily  
56 *Chordopoxvirinae*, family *Poxviridae*), which is an enveloped double stranded DNA virus  
57 [1]. It is subdivided into two clades: the West African and the Congo Basin clades. The  
58 latter is more pathogenic [2] and the clinical signs of MPXV infections are similar to that of  
59 smallpox but in a milder form and with lower mortality (1 to 10%). The majority of deaths  
60 occurs at a young age due to the lack of immunization [3]. Rodents (Squirrels and  
61 Gambian rats) are the primary hosts [4,5], which can transmit the virus to monkeys and  
62 humans through direct contact with blood and bodily fluids [6]. The handling and  
63 consumption of infected monkeys and squirrels were documented as major infection  
64 sources in Africa [7]. Furthermore, human-to-human transmission can occur through  
65 exposure to fomites and air droplets [8]. A specific vaccine for use in humans is not  
66 available, but cross protection in humans vaccinated against smallpox has been  
67 documented [9]. This protection however, has been waning because when smallpox was  
68 declared eradicated in 1980, nationwide vaccination against smallpox has stopped [10].  
69 The antiviral tecovirimat for treatment of accidental smallpox infections has been shown to  
70 reduce symptoms and to improve survival of MPXV infected macaques if applied up to 5  
71 days post infection [11].

72 Human MPXV infections are endemic in West and Central Africa [12]. The first MPXV  
73 outbreak outside Africa was reported in 2003 in the USA after the shipment of animals  
74 from Ghana [13]. The latest outbreak was in Nigeria with 113 laboratory confirmed cases  
75 and seven deaths from September 2017 until August 2018 [14]. Two recent zoonotic  
76 MPVX infections imported in the UK highlight ongoing MPXV activity in Nigeria [15].

77 Several diagnostic methods for the detection of MPXV are established with real-time PCR  
78 as the gold standard because of its high sensitivity and specificity [16]. To use this  
79 diagnostic tool, a highly equipped laboratory and specialized technicians are needed,  
80 which are not available in areas where MPXV infections occur. Therefore, an easy to  
81 handle simple molecular diagnostic method would improve the detection and surveillance  
82 of MPXV. Isothermal amplification methods have been proven to be an alternative to real-  
83 time PCR. Recombinase polymerase amplification (RPA) is one of these methods, in  
84 which an enzymatic based DNA amplification can be achieved at a temperature range of  
85 37 to 42°C within 15 minutes [17]. The amplification is initiated by a primer-recombinase-  
86 complex. This complex invades the DNA double strand at the homologues sequences of  
87 the primer, where single-strand-binding proteins stabilize the reaction. Then, a strand-  
88 displacing polymerase DNA conducts the extension step. For real-time detection, a  
89 fluorophore/quencher-probe is used. Since RPA reagents are freeze-dried, the RPA kit  
90 can be stored at room temperature for several months. This allows the use of the RPA  
91 assay at point of need making them even more versatile through a mobile suitcase  
92 laboratory [18].

93 In this study, we have developed a rapid detection method specific for both clades of  
94 MPXV using a recombinase polymerase amplification (RPA) assay targeting the tumor  
95 necrosis factor (TNF) binding protein gene, which is present in duplicate as ORF G2L and  
96 G2R in the inverted terminal repeats of the MPXV genome.

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### 106 **3. Materials and Methods**

#### 107 **3.1. Molecular MPXV DNA Standard and RPA Oligonucleotide**

108 For assay validation, a molecular DNA standard based on 300 bp of the TNF binding  
109 protein gene (ORF: G2R, Accession number: DQ011153, nucleotides: 195915 - 196964),  
110 was synthesized by GeneArt (Regensburg, Germany). Three forward primers (FP), three  
111 reverse primers (RP) and one exo-probe were designed (Figure S1). All oligos were  
112 synthesized by TIB MOLBIOL GmbH (Berlin, Germany).

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#### 114 **3.3. RPA Assay Conditions**

115 The TwistAmp exo kit (TwistDx Ltd, Cambridge, UK) was used. Per reaction, 29.5 µl  
116 rehydration buffer, 10.7 µl H<sub>2</sub>O, 2.1 µl of each primer (10 µM) and 0.6 µl of 10 µM exo-  
117 probe were added into the lid of the reaction tube containing the freeze-dried pellet. After  
118 adding 2.5 µl of 280 mM magnesium acetate and 1 µl template, the reaction mixture was  
119 centrifuged, mixed, centrifuged and placed immediately into the tube scanner ESEQuant  
120 (QIAGEN Lake Constance GmbH, Stockach, Germany). The reaction was incubated at  
121 42°C for 15 minutes. To increase the sensitivity, a mixing and centrifugation step was  
122 performed after 230 seconds of starting the measurement. A positive result was measured  
123 by the FAM channel of the ESEQuant tube scanner and analysed with the Tubescanner  
124 studio software (version 2.07.06, QIAGEN Lake Constance GmbH, Stockach, Germany).

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#### 126 **3.4. MPXV RPA Assay Analytical Sensitivity**

127 In total, nine primer combinations were tested with the MPXV DNA standard with  
 128 concentration of  $10^5$  DNA molecules/ $\mu$ l. The best combination, which produced the earliest  
 129 and highest fluorescence signal, was selected for further assay validation. The ability of  
 130 the selected primer combination to amplify  $10^4$  to 1 DNA molecules/ $\mu$ l of the MPXV  
 131 standard DNA was checked in order to test the analytical sensitivity and to determine the  
 132 limit of detection.

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### 134 3.5. MPXV RPA assay cross reactivity

135 The specificity of the MPXV-RPA-assay was tested with DNA of viruses of the two MPXV  
 136 clades, six other *pox* viruses and other pathogens of clinical importance, see table 1.

137

138 **Table 1: Reactivity of the MPXV\_RPA assay to the genome of poxviruses and other**  
 139 **pathogens.** MPXV\_RPA assay detected both clades of MPXV, but not other poxviruses  
 140 and pathogens.

Pathogen	Clade/ Source	Concentration [ng/ $\mu$ l]	RPA	Real-time PCR
<b>Monkeypox</b>	Central Africa		+	+
<b>Monkeypox</b>	West Africa		+	+
<b>Vaccinia</b>	Elstree	7.6	-	-
<b>Cowpox</b>	2	3.6	-	-
<b>Camelpox</b>	-	18	-	-
<b>Sheeppox</b>	Russia	4.6	-	-
<b>Goatpox</b>	India	3.1	-	-
<b>Orf</b>	Burghessler	3	-	-
<b>Calpox virus</b>	-	6.1	-	-
<b>Herpes-simplex-Virus 1</b>	Quality Control for Molecular Diagnostics (QCMD)	1.7	-	-
<b>Herpes-simplex-Virus 2</b>		3.6	-	-
<b>Varicella-zoster Virus</b>		3.1	-	-
<b><i>Staphylococcus aureus</i></b>	DSMZ ID: 1104	4.2	-	-
<b><i>Clostridium perfringes</i></b>	DSMZ ID: 756	40.2	-	-

<b><i>Enterococcus faecialis</i></b>	DSMZ ID: 20478	35.2	-	-
<b><i>Plasmodium falciparum</i></b>	University of Ibadan, Nigeria	2.8	-	-
<b><i>Rickettsia rickettsia</i></b>	BNITM Hamburg, Germany	4.7	-	-
<b><i>Rickettsia africae</i></b>		4.3	-	-

### 3.6. Clinical samples

The MPXV-RPA-assay performance was validated with plasma samples of infected (n=25) and uninfected (n=23) monkeys. The animals were looked after by experienced personnel from the German Primate Center and kept according to the German Animal Welfare Act, which is in compliance with the European Union Guidelines on the use of non-human primates for biological research and the Weatherall report. Sampling from MPXV-infected monkeys was approved by the Lower Saxony State Office of Consumer Production and Food Safety with the project license 33.9.42502-04/019/07, that from uninfected animals with the project license 33.9.42502-04-15/1769. In addition, 20 positive (4 whole blood, 16 serum) and 27 negative (8 whole blood, 19 serum) human samples from the recent MPXV outbreak in Nigeria (November 2017) were tested with the RPA-MPXV-assay. The samples were collected for diagnostics purposes and handled anonymously. The DNA from these samples was isolated using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer instructions.

### 3.7 Real-time PCR

For comparison, the molecular DNA standards as well as clinical samples were tested with a reference MPXV real-time PCR assay targeting the same gene region of the developed RPA assay [19]. The G2R-G real-time PCR assay detects both MPXV clades and the real-time PCR reaction was performed as described previously [20] using the LightCycler DNA-Master HybProbe kit and the LightCycler 480 (Roche Mannheim, Germany).

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166 **3.8. Statistical Analysis**

167 The limit of detection of the MPXV-RPA-assay was calculated by performing a probit  
168 regression analysis on the data set of eight RPA assay using STATISTICA software  
169 (StatSoft, Hamburg, Germany) in order to determine the number of DNA molecules/μl,  
170 which were detected in 95% of the cases. Furthermore, the detection time was calculated  
171 by performing a semi-logarithmic regression on the same data set with GraphPad PRISM  
172 7 software (GraphPad Software Inc., San Diego, California).

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174 **4. Result**

175 **4.1 Selection of RPA Primers and Probe**

176 In order to select sensitive RPA oligonucleotides, all possible primer combinations were  
177 tested using a MPXV DNA molecular standard at a concentration of 10<sup>5</sup> DNA molecules/μl.  
178 As a result, the primer combination FP3 + RP3 (Table 2) produced the best amplification  
179 curves (Figure S1) and was selected for further assay validation.

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181 **Table 2. RPA primers and exo-probe combination, yielding the earliest and highest**  
182 **signal in the MPXV RPA assay. QTF are sites of the quencher and fluorophore in the**  
183 **following order BHQ1-dt (Q), Tetrahydrofuran (T) and Fam-dT (F).**

Name	Sequence (5' to 3')
MPXV RPA P1	ACAGAAGCCGTAATCTATGTTGTCTATCGQTFCCTCCGGGA ACTTA

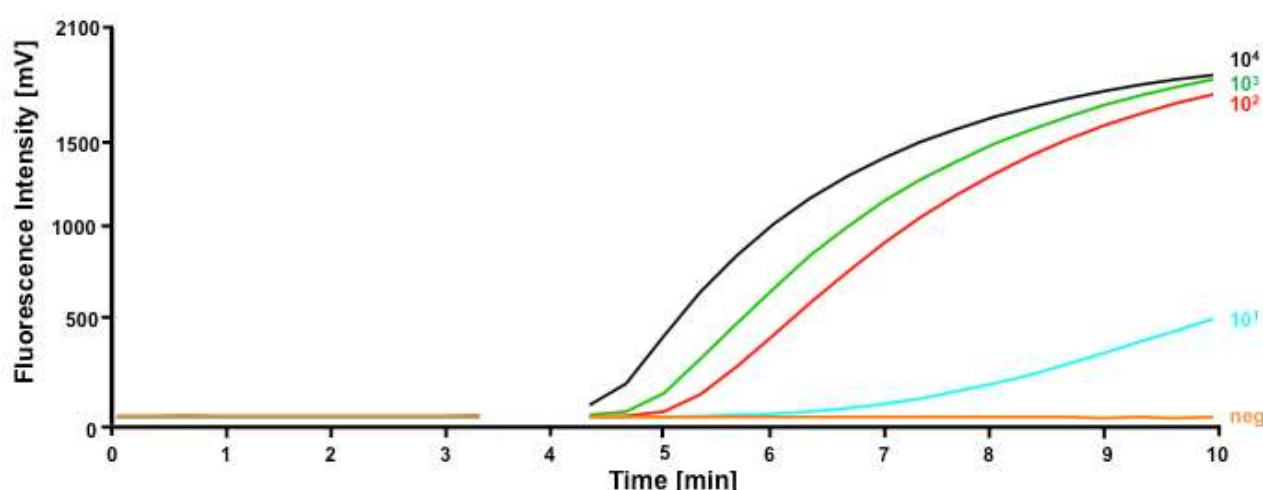


<b>MPXV RPA FP3</b>	AATAAACGGAAGAGATATAGCACCCACATGCAC
<b>MPXV RPA RP3</b>	GTGAGATGTAAAGGTATCCGAACCACACG

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## 185 4.2 Analytical Sensitivity and Specificity

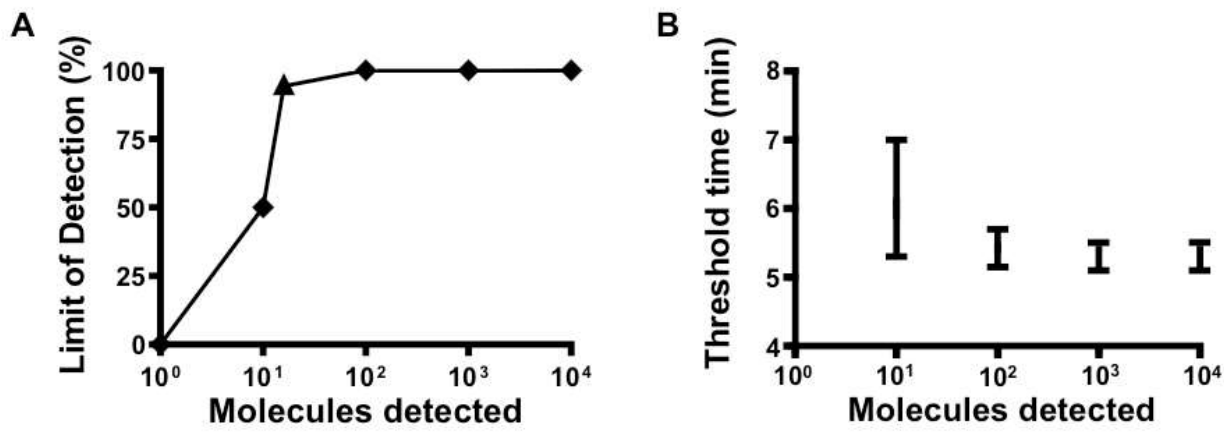
186 To determine the analytical sensitivity, the performance of the best primer combination  
187 FP3 and RP3 was evaluated with a tenfold dilution range of the MPXV DNA standard ( $10^4$   
188 to 1 DNA molecules/ $\mu$ l, Figure 1) in eight replicates. The MPXV-RPA-assay detected the  
189 molecular MPXV DNA standard with the concentration from  $10^4$  to  $10^2$  molecules/ $\mu$ l in all  
190 eight RPA runs and the concentration of  $10^1$  molecules/ $\mu$ l in four runs, while no  
191 amplification was observed in the tube containing one molecule/ $\mu$ l. With this data set, a  
192 probit regression analysis was performed and revealed a detection limit of 16 DNA  
193 molecules/ $\mu$ l in 95% of the cases (Figure 2). Seven minutes is the maximum time needed  
194 to amplify as low as 10 DNA molecules by the MPXV RPA assay (Figure 3). FP3 and RP3  
195 primers were able to amplify the two clades of MPXV but did not detect high concentration  
196 DNA of related poxviruses or other pathogens (Table 1).



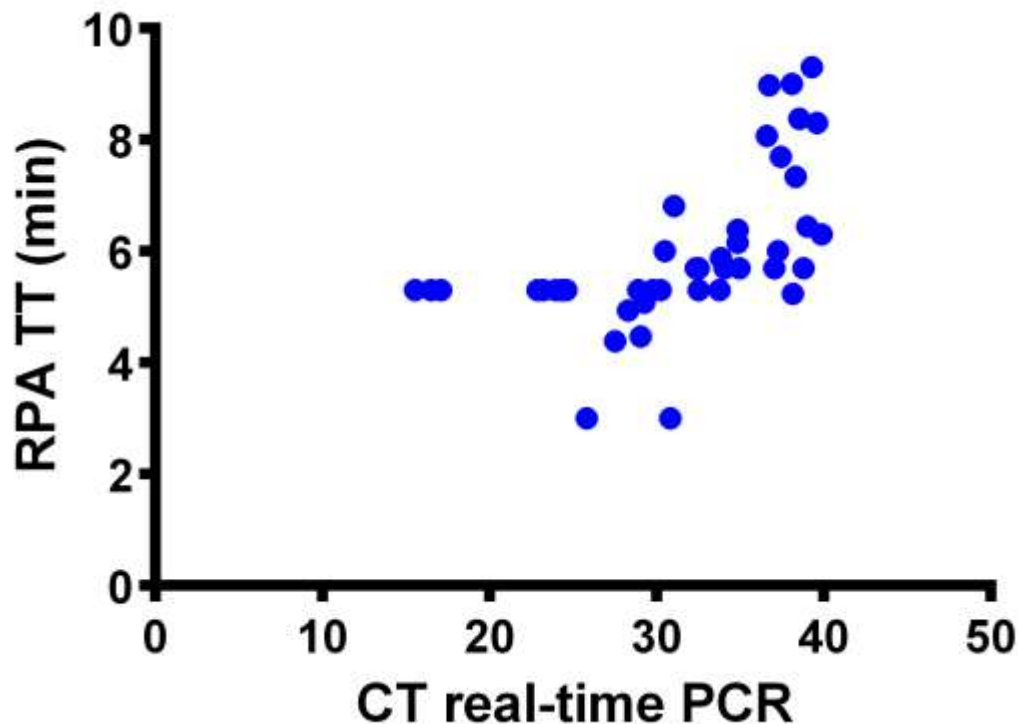
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198 **Figure 1. Analytical sensitivity of the MPXV-RPA-assay tested with a tenfold dilution**  
199 **of the molecular DNA standard ( $10^4$  –  $10^0$  DNA molecules/ $\mu$ l). The primer combination**

200 FP3 + RP3 detected the concentration  $10^4 - 10^1$  DNA molecules/ $\mu$ l. After 230 seconds a  
201 mixing step was performed.



202  
203 **Figure 2. Probit regression analysis of the dataset of the eight repetitions of the**  
204 **analytical sensitivity test of the MPXV-RPA-assay for the determination of the**  
205 **detection limit (A) and semi-logarithmic regression of the detection time (B).**  
206 Performing the probit regression analysis on the dataset revealed a detection limit of 16  
207 DNA molecules/ $\mu$ l in 95% of the cases (A). Using Prism Software, a semi-logarithmic  
208 regression of the data from the eight runs on a dilution range of the molecular DNA  
209 standard ( $10^4$ - $10^0$  DNA molecules/reaction) were performed. The lowest concentration of  
210  $10^1$  DNA molecules/ $\mu$ l was detected after a maximum of seven minutes (B).



**Figure 3. Screening of 45 blood, plasma or serum samples from MPXV infected macaques and humans by real-time PCR and RPA assays.** Linear regression analysis of real-time RT-PCR cycle threshold values (Ct) and RPA threshold time in minutes (TT) were determined. No correlation was found between TT and Ct values since the RPA is much faster than the real-time PCR. Diagnostic sensitivity of real-time PCR assay was 100%, while that of MPXV-RPA-assay was 95 % (43/45).

### 221 4.3. Clinical Samples

## 228 5. Discussion

229 Infection with MPXV occurs in West Africa and the Congo Basin [12]. The most affected  
230 regions suffer from limited resources, infrastructure and diagnostic capacities, beside  
231 insufficient accessibility to remote and conflict areas. Thus, identification of MPXV infected  
232 cases is difficult [21]. Therefore, a simple point of need diagnostic test is crucial in order to  
233 limit the spread of MPXV and control the outbreaks.

234 Applying the MPXV-RPA-assay both the West Africa and the Congo Basin clade were  
235 detected within seven minutes with a detection limit of 16 DNA molecules/ $\mu$ l. The RPA  
236 oligonucleotides target the TNF receptor gene as no mismatch between both MPXV  
237 clades was identified and thus cover the currently known diversity of MPXV, while between  
238 13-31 mismatches were identified when this sequence was compared to those of other  
239 poxviruses (Figure S3). The number of mismatches between the targeted MPXV gene  
240 sequence and the sequences of closely related poxviruses was the key to a specific RPA  
241 assay. Two samples were negative in the RPA assay but weakly positive in real-time PCR  
242 (CT: 38.8 and 39.97). Eight samples with CT values around 38-39 and eight samples with  
243 CT values 35-37 were scored positive in the RPA. All these samples had low DNA levels  
244 and lack of positive scoring of two samples in the RPA lay within the probability of missing  
245 weak positives as shown by the probit analysis.

246 Real-time PCR assays for MPXV detection need at least 90 minutes and highly  
247 sophisticated thermal cycler [19]. Although freeze-dried PCR reagents are slowly  
248 becoming available [22], they are as yet not in widespread use, whereas the RPA kits per  
249 se are freeze-dried and stable under different environmental conditions including  
250 temperatures above 30°C [23]. This is a huge advantage in areas where highly equipped  
251 laboratories are not available. When comparing the performance of the MPXV-RPA-assay  
252 with the real-time PCR assay on clinical samples with linear regression analysis, no  
253 correlation was found between TT and Ct values since the RPA is much faster than the

254 real-time PCR (Figure 3). One reason for this observation for several RPA assays [23-25]  
255 is that the RPA reaction is optimized for maximal enzymatic activity at one temperature  
256 leading to very dynamic non linear amplification [17], whereas the real-time PCR reaction  
257 depends on different temperature steps for denaturation, annealing and amplification  
258 yielding a close to exponential amplification [26].

259 Another isothermal amplification assay based on loop-mediated isothermal amplification  
260 for the detection of MPXV is available [27]. This assay has a clinical sensitivity of 72%.  
261 However, our MPXV-RPA-assay proved to be more sensitive (95 % sensitivity). The LAMP  
262 MPXV assay requires 6 primers to amplify the MPXV DNA in around 60 minutes, while  
263 RPA uses two primers and one probe producing a result within 15 minutes.

264 The MPXV-RPA-assay appears an appropriate assay for the point of need detection of  
265 active MPXV cases as RPA is fast, highly sensitive and specific as well as utilizing cold-  
266 chain independent reagents.

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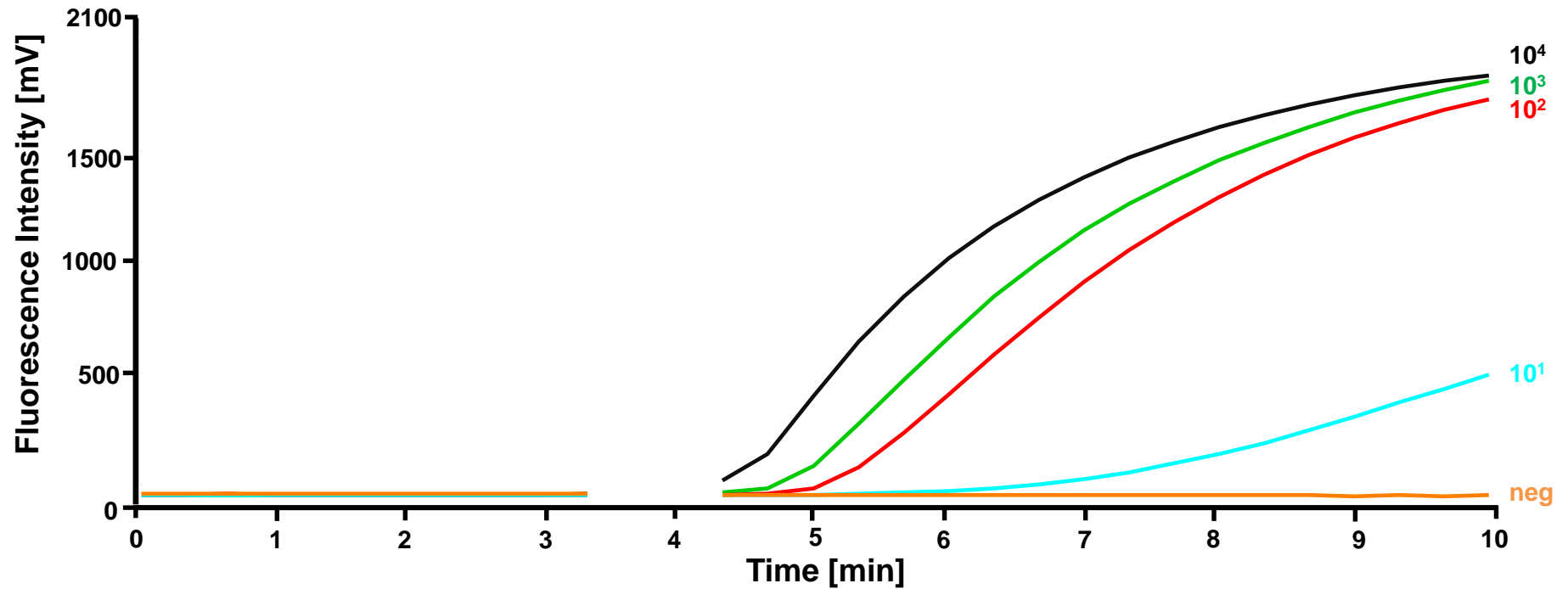
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## 279 7. References

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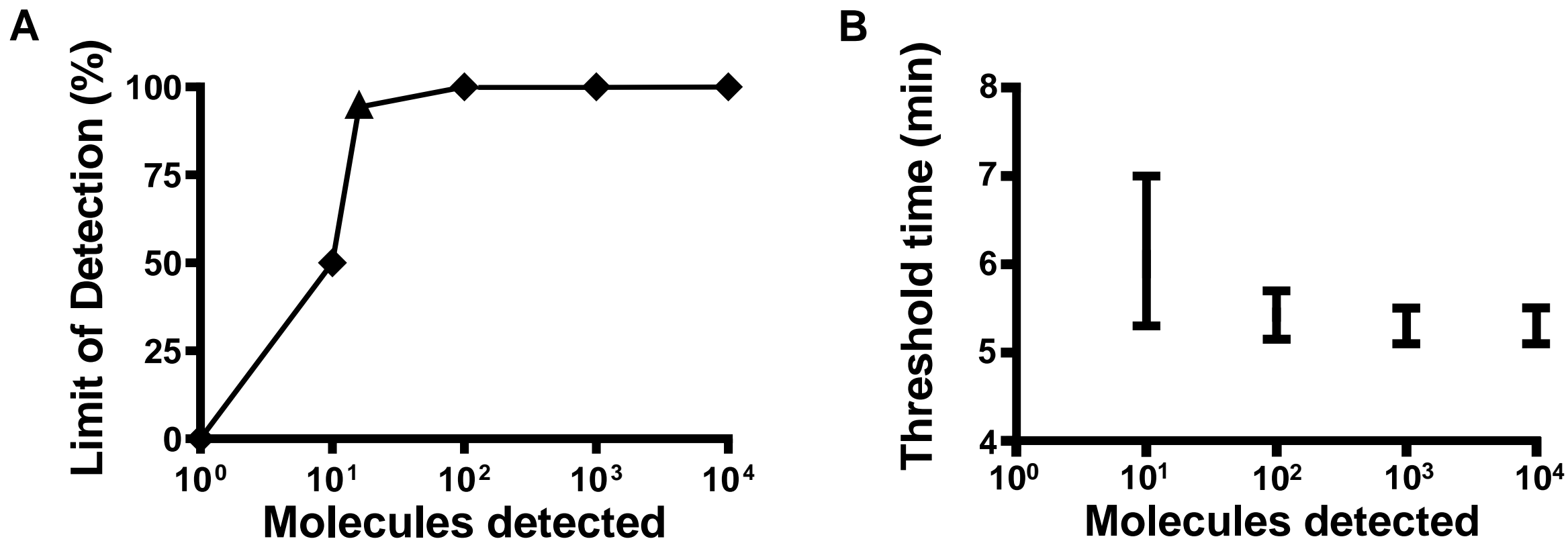
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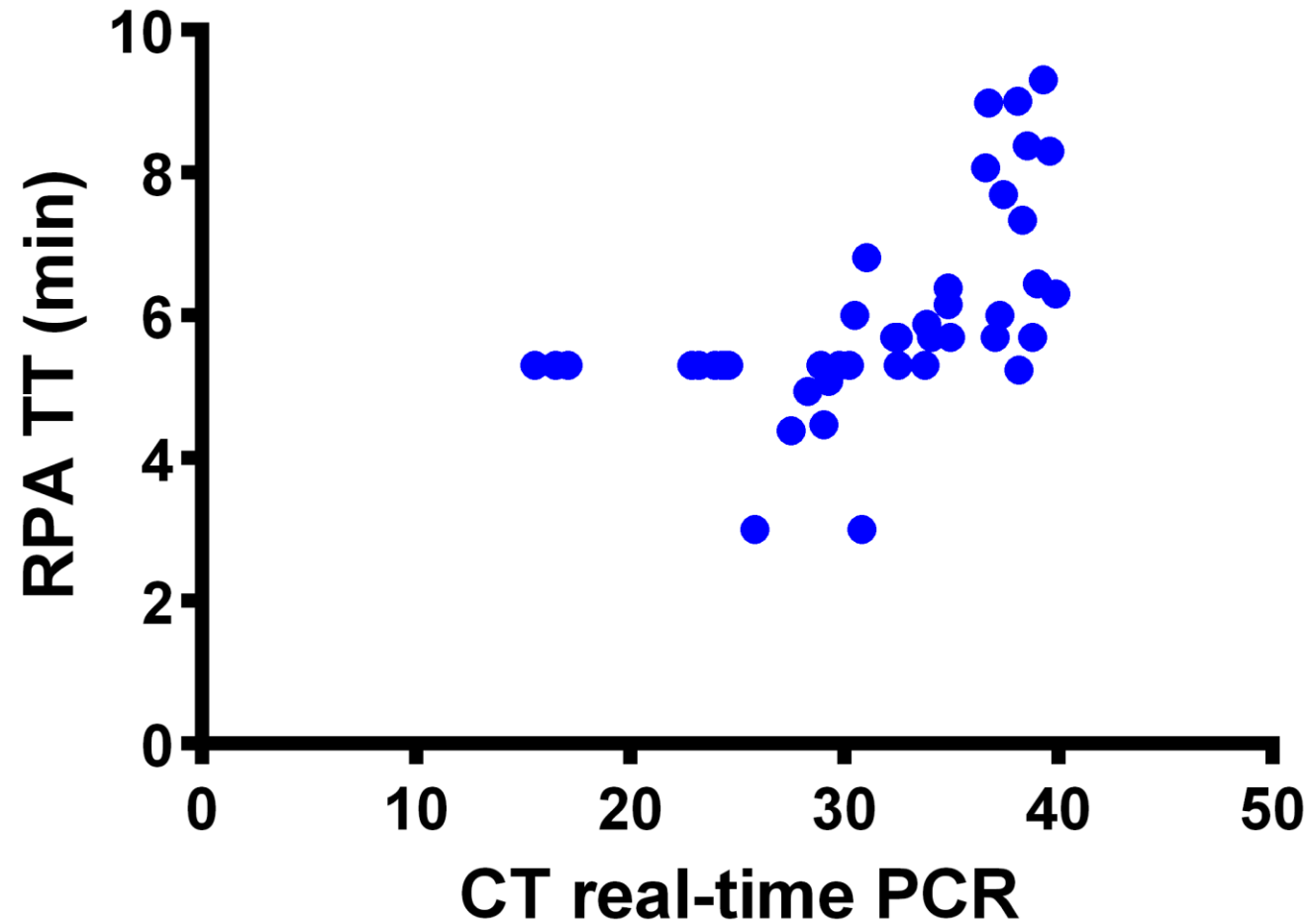


**Figure 1. Analytical sensitivity of the MPXV-RPA-assay tested with a tenfold dilution of the molecular DNA standard ( $10^4$  –  $10^0$  DNA molecules/ $\mu$ l).** The primer combination FP3 + RP3 detected the concentration  $10^4$  –  $10^1$  DNA molecules/ $\mu$ l. After 230 seconds a mixing step was performed.





**Figure 2. Probit regression analysis of the dataset of the eight repetitions of the analytical sensitivity test of the MPXV-RPA-assay for the determination of the detection limit (A) and semi-logarithmic regression of the detection time (B).** Performing the probit regression analysis on the dataset revealed a detection limit of 16 DNA molecules/ $\mu$ l in 95% of the cases (A). Using Prism Software, a semi-logarithmic regression of the data from the eight runs on a dilution range of the molecular DNA standard ( $10^4$ - $10^0$  DNA molecules/reaction) were performed. The lowest concentration of  $10^1$  DNA molecules/ $\mu$ l was detected after a maximum of seven minutes (B).



**Figure 3.** Screening of 45 blood, plasma or serum samples from MXPV infected macaques and humans by real-time PCR and RPA assays. Linear regression analysis of real-time RT-PCR cycle threshold values (Ct) and RPA threshold time in minutes (TT) were determined. No correlation was found between TT and Ct values since the RPA is much faster than the real-time PCR. Diagnostic sensitivity of real-time PCR assay was 100%, while that of MPXV-RPA-assay was 95 % (43/45).

**Table 1: Reactivity of the MPXV\_RPA assay to the genome of poxviruses and other pathogens.** MPXV\_RPA assay detected both clades of MPXV, but not other poxviruses and pathogens.

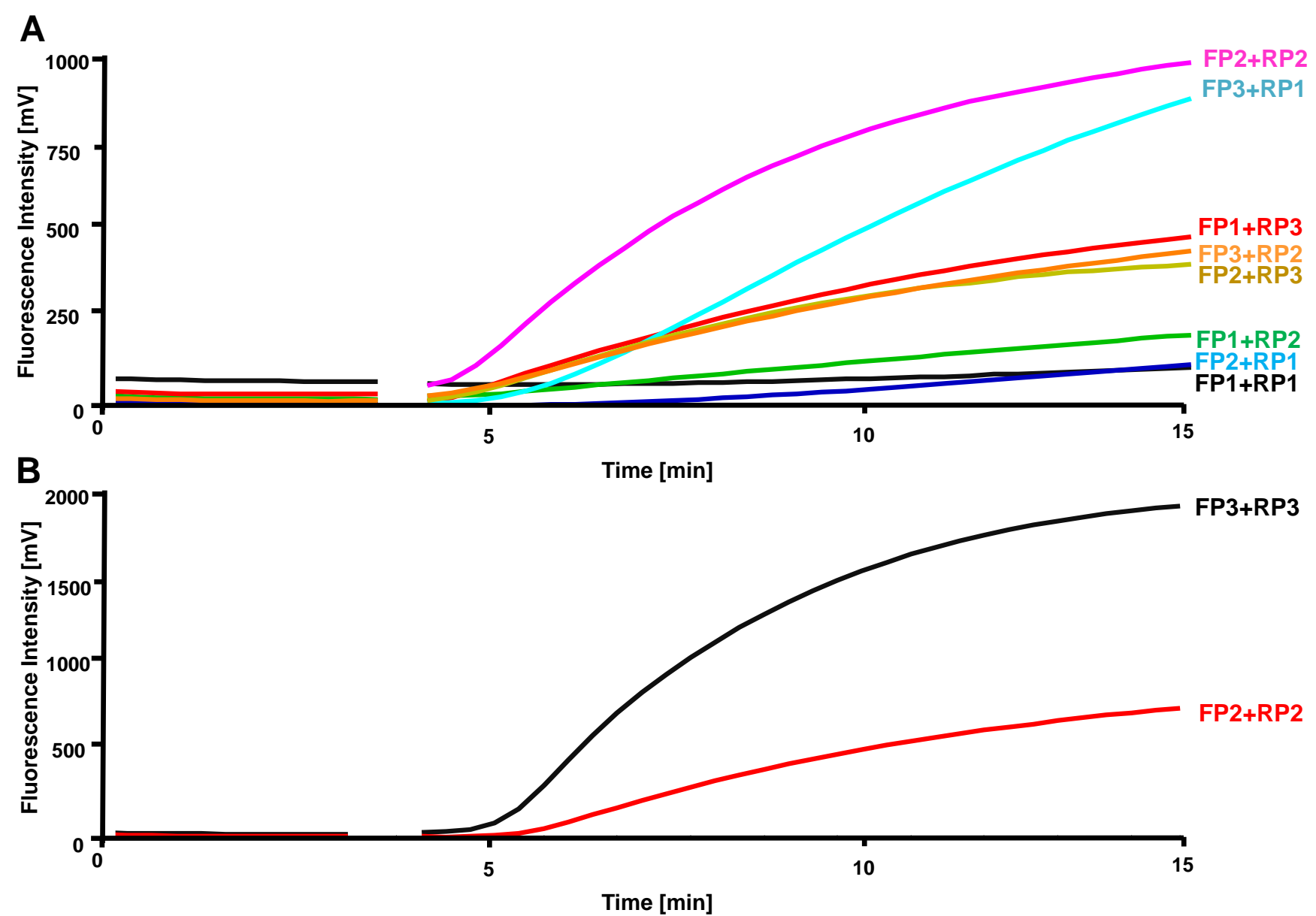
Pathogen	Clade/ Source	Concentration [ng/μl]	RPA	Real-time PCR
<b>Monkeypox</b>	Central Africa		+	+
<b>Monkeypox</b>	West Africa		+	+
<b>Vaccinia</b>	Elstree	7.6	-	-
<b>Cowpox</b>	2	3.6	-	-
<b>Camelpox</b>	-	18	-	-
<b>Sheeppox</b>	Russia	4.6	-	-
<b>Goatpox</b>	India	3.1	-	-
<b>Orf</b>	Burghessler	3	-	-
<b>Calpox virus</b>	-	6.1	-	-
<b>Herpes-simplex-Virus 1</b>	Quality Control for Molecular Diagnostics (QCMD)	1.7	-	-
<b>Herpes-simplex-Virus 2</b>		3.6	-	-
<b>Varicella-zoster Virus</b>		3.1	-	-
<b><i>Staphylococcus aureus</i></b>	DSMZ ID: 1104	4.2	-	-
<b><i>Clostridium perfringes</i></b>	DSMZ ID: 756	40.2	-	-
<b><i>Enterococcus faecialis</i></b>	DSMZ ID: 20478	35.2	-	-
<b><i>Plasmodium falciparum</i></b>	University of Ibadan, Nigeria	2.8	-	-
<b><i>Rickettsia rickettsia</i></b>	BNITM Hamburg, Germany	4.7	-	-
<b><i>Rickettsia africae</i></b>		4.3	-	-

**Table 2. RPA primers and exo-probe combination, yielding the earliest and highest signal in the MPXV RPA assay. QTF are sites of the quencher and fluorophore in the following order BHQ1-dt (Q), Tetrahydrofuran (T) and Fam-dT (F).**

<b>Name</b>	<b>Sequence (5' to 3')</b>
<b>MPXV RPA P1</b>	ACAGAAGCCGTAATCTATGTTGTCTATCG <b>QTF</b> CCTCCGGGAACCTTA
<b>MPXV RPA FP3</b>	AATAAACGGAAGAGATATAGCACCACATGCAC
<b>MPXV RPA RP3</b>	GTGAGATGTAAAGGTATCCGAACCACACG

Name	Sequence (5' to 3')
<b>Amplicon</b>	AATAAACGGAAGAGATATAGCACCACATGCACCATCCAATGGAAAGTGTAAAGACAACGAATACAGAAGCCGTAATCTATGTTGTCTATCGTGTCTCCGGGAACCTACGCTTCCAGATTATGTGATAGCAAGACTAATACACAATGTACACCGTGTGGTTCGGATACCTTTACATCTCACAAT
<b>MPXV Probe</b>	-----ACAGAAGCCGTAATCTATGTTGTCTATCGTGTCTCCGGGAACCTA-----
<b>MPXV FP1</b>	-----AAGACAACGAATACAGAAGCCGTAATCTATG-----
<b>MPXV FP2</b>	-----ATAGCACCACATGCACCATCCAATGGAAAGT-----
<b>MPXV FP3</b>	AATAAACGGAAGAGATATAGCACCACATGCAC-----
<b>MPXV RP1 rc</b>	-----CGCTTCCAGATTATGTGATAGCAAGACTAAT-----
<b>MPXV RP2 rc</b>	-----CTAATACACAATGTACACCGTGTGGTTCGGAT-----
<b>MPXV RP3 rc</b>	-----CGTGTGGTTCGGATACCTTTACATCTCAC---

**Figure S1. MPXV\_RPA \_assays amplicon as well as primer and probe sequences.** MPXV-RPA-assay oligonucleotides were placed at nucleotides 195962-196146; Genbank accession number: DQ011153. Three forward and three reverse primers as well as one exo-probe were screened to select the combination with higher RPA analytical sensitivity. RC: reverse complementary sequence.



**Figure S2. Testing of all possible primer combination of the MPXV-RPA-assay.** All nine primer combination were tested with the molecular DNA standard with a concentration of  $10^5$  DNA molecules/ $\mu$ l. A mixing step was conducted after 230 sec. The combination FP3 + RP3 showed the earliest and highest fluorescence signal and was therefore chosen for further assay validation.

Virus	Forward Primer →	exo-Probe →	Reverse Primer ←
MPX West African	AATAAACGGAAGAGATAT-AGCACCACAT---GCACCATCCAA--TGGAAAGTGT-AAAGACAA--CG----AATACAGAAGCCGTAATCTATGTT-GTCTATCGTGTCTCCG---GGAAGTTACGCTTCCAGATTATG-TGATAGCAAGACTA-----ATACACAATGTACA---CCGTGTGGTTCGGATACCTTTACATCTCAC		
MPX Congo Basin	.....GC.....GT.....A.....C.....C.....A.C.....A.....G.....T.....A.....C.....G.....G.....		
Variola	.....G.....G.....A.C.....A.....T.G.....A.....C.....G.....G.....C.....G.....C.....G.....		
Vaccinia	.....G.....A.....GT.....A.C.....A.....G.....T.....A.....C.....G.....G.....C.....G.....C.....G.....		
Camelpox	.....C.....A.....G.....A.....C.....A.C.....T.....A.....C.....ACACAA.C.....G.....CG.....C.....G.....G.....		
Cowpox	.....T.....T.....A.A.....TA.....A.....ATCGAG.....A.....TA.....AC.T.T.....C.A.....A.A.....ATA.....TTACA.....A.AG.A.TT.....AA.....A.....TG.....AT.....AAAA.AGT.....G.....AA.....A.....A.....T.....T.A.T.....T.....G.....TCG.....GGTT.....ACTATA.A.....TGG.....TA.AA.....A		
Sheeppox	.....T.....T.....A.A.....CA.....A.....ATCGAG.....A.....TA.....AC.T.T.....C.A.....A.A.....ATA.....TTACA.....A.AG.A.TT.....AA.....A.....TG.....AAG.AGT.....G.....AA.....A.....A.....T.....T.A.T.....T.....GC.....TC.....GGTT.....ACTATA.A.....TGG.....TA.AA.....A		
Goatpox			

**Figure S3. Alignment of the MPXV-RPA-assays amplicon with the Congo Basin clade and other *Chordopoxvirinae* of interest.** Using Geneious (Version: 11.1.2, Biomatters Limited, New Zealand) the target sequence of the G2R gene of the monkeypox West African virus (Genebank accession number: DQ011153, nucleotides: 195962 – 1969143) was compared with monkeypox Congo Basin virus (accession number: NC\_003310, nt: 194120 – 194301), variola virus (accession number: NC\_001611, nt: 182618 - 182749), vaccinia virus (accession number: NC\_006998, nt: 189299 – 189472), camelpox virus (accession number: NC\_003391, nt: 201497 – 201678) ,cowpox virus (accession number: NC\_003663, nt: 219885 – 220071), sheeppox virus (accession number: NC\_004002, nt: 112967 – 113171) and goatpox virus (accession number: NC\_004003, nt: 112695 – 112892). Between 1 to 31 mismatches could be identified in the primers and probe sequences.