

Rapid Adaptation of Established High-Throughput Molecular Testing Infrastructure for Monkeypox Virus Detection

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Beginning in May 2022, a rising number of monkeypox cases were reported in non-monkeypox-endemic countries in the Northern Hemisphere. We adapted 2 published quantitative PCRs for use as a dual-target monkeypox virus test on widely used automated high-throughput PCR systems. We determined analytic performance by serial dilutions of monkeypox virus reference material, which we quantified by digital PCR. We found the lower limit of detection for the combined assays was 4.795 (95% CI 3.6–8.6) copies/mL. We compared clinical performance against a commercial manual orthopoxvirus research use only PCR kit by using clinical remnant swab samples. Our assay showed 100% positive (n = 11) and 100% negative (n = 56) agreement. Timely and scalable PCR tests are crucial for limiting further spread of monkeypox. The assay we provide streamlines high-throughput molecular testing for monkeypox virus on existing broadly established platforms used for SARS-CoV-2 diagnostic testing.

In May 2022, an unusually high number of monkeypox cases were reported in countries in western Europe and North America; by May 29, 2022, 257 laboratory-confirmed infections were reported from Spain, Portugal, the United Kingdom, Canada, and the United States, sparking fear of another global outbreak on the heels of the continuing SARS-CoV-2 pandemic (1–4). Endemic transmission of the monkeypox virus (MPXV), a species of the *Orthopoxvirus* genus, is thought to be limited to central and

western Africa, where both zoonotic ($\approx 22\%$ – 72% of cases) and person-to-person transmission contribute to disease burden (5). Previous clusters outside Africa have usually been traceable to animal sources rather than to human-to-human transmission (6). In contrast, the 2022 cases seem to have occurred without any links to animal sources and have been concentrated in, but not exclusive to, men who have sex with men (7). The sudden appearance of infections in several non-monkeypox-endemic countries suggested that undetected transmission might have taken place for some time but that recent events could have served as a catalyst for spread (1).

The ongoing SARS-CoV-2 pandemic has demonstrated the potential and value of highly automated high-throughput molecular testing in outbreak scenarios. We aimed to rapidly adapt existing automated molecular testing infrastructure for SARS-CoV-2 in a large tertiary-care hospital in Hamburg, Germany, for detection of MPXV from clinical samples, thereby creating the capacity for high-throughput testing and quick turnaround times, if needed.

Materials and Methods

Multiplex Assay Setup

On the basis of diagnostic testing during the SARS-CoV-2 pandemic (8; C. Manohar et al., unpub. data, <https://doi.org/10.1101/2021.10.13.21264919>), we chose a dual-target approach, in which 1 assay targets a conserved sequence of the *Orthopoxvirus* genus, not including variola major or minor viruses (9), and the other targets a MPXV-specific sequence (10) (Table 1). The cobas 5800, 6800, and 8800 systems (Roche Diagnostics, <https://diagnostics.roche.com>) use a spike-in RNA full process control that is added automatically during extraction. The corresponding

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Table 1. Primer and probe sequences for a dual-target MPXV assay rapidly adapted from established high-throughput SARS-CoV-2 molecular testing infrastructure*

| Oligotype | Oligo name | Sequence, 5' → 3' | Final concentration, nM |
|-----------|------------|---|-------------------------|
| Primers | NVAR fwd | TCA ACT GAA AAG GCC ATC TAT (2'OMe-G)A | 400 |
| | NVAR rev | GAG TAT AGA GCA CTA TTT CTA AAT CC(2'OMe-C) A | 400 |
| | MPOX fwd | ACG TGT TAA ACA ATG GGT GA(2'OMe-U) G | 400 |
| | MPOX rev | AAC ATT TCC ATG AAT CGT AGT (2'OMe-C)C | 400 |
| Probes | NVAR P-YAK | YakYellow-CCA TGC AAT (BHQ1)ATA CGT ACA AGA TAG TAG CCA AC-BHQ1 | 75 |
| | MPOX P-FAM | FAM-TGA ATG AAT (BHQ1)GCG ATA CTG TAT GTG TGG G-BHQ1 | 75 |

*Sequences are derived from previously published NVAR assay by Li et al. (9) and MPXV assay by Shchelkunov et al. (10) and adapted for the cobas omni Utility Channel (Roche Diagnostics, <https://diagnostics.roche.com>). Oligos were custom made by Ella Biotech GmbH (<https://www.ellabiootech.com>). Indicated final concentration refers to the final oligo concentrations within the reaction mix. 2'-O-methyl-RNA bases are indicated as OMe-X. Internal abasic quenchers are indicated as (BHQ1). NVAR, nonvariola orthopoxvirus; MPXV, monkeypox virus.

internal control assay is preloaded in the open channel reagent for use with cobas omni Utility Channel (Roche Diagnostics) (Table 2). We modified and optimized all assays for use on cobas 5800, 6800, and 8800 systems, including 2'-O-methyl-RNA-modified primers and internal quenchers for TaqMan probes, as previously described (11).

In Silico Evaluation

As part of a support request for Utility Channel applications, we submitted all sequences of the duplex assay to Roche Diagnostics for evaluation of inclusivity and potential primer-probe interactions. The submitted sequences were aligned to currently available MPXV and orthopoxvirus sequences available in public databases.

Analytical Performance Evaluation

We conducted technical performance evaluations for the assays according to new European Union regulations (Regulation 2017/746 EU IVDR, <https://euivdr.com>). For reference material, we used inactivated cell culture supernatant containing MPXV recovered from a clinical case in central Africa in 1987 (12) and inactivated modified vaccinia virus Ankara (MVA) SARS-CoV-2 vaccine. To obtain a quantitative MPXV standard, we purified nucleic acids by using a MagNA-pure96 extractor and Viral NA Small Volume Kit (Roche Diagnostics) and analyzed on a QIAcuity digital PCR instrument (QIAGEN, <https://www.qiagen.com>) in conjunction

with 3 different previously described quantitative PCRs (qPCRs): 1 for nonvariola (NVAR) orthopoxviruses (9); 1 for MPXV (10); and the research-use only (RUO) LightMix Modular Orthopoxvirus Assay (TIB MOLBIOL, <https://www.tib-molbiol.de>).

We determined the lower limit of detection by serial 2-fold dilution of MPXV standard in universal transport medium from 100 to 0.78 copies/mL and 21 repeats per dilution step. Using MedCalc statistical software (<https://www.medcalc.org>), we determined the limit for 95% probability of detection. We assessed linearity by 10-fold serial dilution of MPXV standard (5 repeats per dilution step) at concentrations of $\approx 10^1$ – 10^7 copies/mL. We determined linearity and intra-assay variability by using Validation Manager software (Finbiosoft, <https://finbiosoft.com>). Concentrations represent copies per mL of specimen. For empirical inclusivity and exclusivity testing, we used the assay to test a set of 53 samples, including clinical samples, reference material, and external quality controls from a range of blood-borne and respiratory pathogens (Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/28/9/22-0917-App1.pdf>). We used an experimental MVA vector-based SARS-CoV-2 vaccine as reference material for a non-MPXV orthopoxvirus.

Clinical Evaluation and Follow-Up Samples

For clinical validation, we used the RUO LightMix Modular Orthopoxvirus assay as reference test,

Table 2. Software settings for run protocol for a dual-target MPXV assay rapidly adapted from established high-throughput molecular testing infrastructure*

| Protocol setting | Channel setting, use | | | | |
|--------------------------------|----------------------|---------------------|-------------------|-------------------|---------------------|
| | 1, Not used | 2, Monkeypox | 3, Nonvariola | 4, Not used | 5, Internal control |
| Relative fluorescence increase | NA | 2 | 2 | NA | 2 |
| PCR cycling conditions | UNG incubation | Pre-PCR step | 1st measurement | 2nd measurement | Cooling |
| No. cycles | Predefined | 1 | 5 | 45 | Predefined |
| No. steps | Predefined | 3 | 2 | 2 | Predefined |
| Temperature | Predefined | 55°C; 60°C; 65°C | 95°C; 55°C | 91°C; 58°C | Predefined |
| Hold time | Predefined | 120 s; 360 s; 240 s | 5 s; 30 s | 5 s; 25 s | Predefined |
| Data acquisition | Predefined | None | End of each cycle | End of each cycle | Predefined |

*Protocol was run on 400 μ L swab samples. Protocol adapted for cobas omni Utility Channel (Roche Diagnostics, <https://diagnostics.roche.com>). Relative fluorescence increase thresholds were used for automated result calls. NA, not applicable; UNG, uracyl-N-glycosylase.

which we performed according to manufacturer's recommendation by using the MagNA-pure96 system with 200- μ L extraction volume. In total, we tested 67 clinical samples consisting of respiratory, skin, and genital swab samples with both assays. Of those samples, 11 were positive for MPXV DNA, which we obtained from 2 confirmed clinical cases in Hamburg, Germany. We analyzed 33 consecutive clinical samples from the same 2 patients and 2 additional cases by using the duplex assay (Appendix Table 2). The clinic provided globalized patient characteristics.

Results

In Silico Analysis

We did not detect any concerning oligo interactions (Appendix Figure 1). Target-1: NVAR was still a 100% match for all but 1 MPXV sequence, which had 1 low-risk mismatch. NVAR also had high sequence similarity with many other orthopoxviruses but might not be optimal for reliable detection of camelpox or cowpox (Appendix Figure 2). Target-2: MPOX is a perfect match for almost all Congo Basin strain MPXV sequences but has a known mismatch for West Africa strain sequences in the probe region. This mismatch is expected to slightly reduce relative fluorescence increase signals, as demonstrated in the clinical sample set. Other orthopoxviruses have extensive sequence mismatches with this assay and are not expected to produce detectable signals (Appendix Figure 3).

Analytical performance

We determined LoD was 9.697 (95% CI 7.424–15.327) copies/mL for the NVAR assay and 6.359 (95% CI 4.908–10.110) copies/mL for the MPOX assay by probability of detection analysis. Overall LoD for both targets combined was 4.795 (95% CI 3.598–8.633) copies/mL. We compiled hit rates (Table 3) and probability of detection plots (Appendix Figure 4) for the assay. The assay showed excellent linearity. Cycle threshold (Ct) values were 37–18, $\approx 10^1$ – 10^7 copies/mL, and pooled SD and 95% CI were within linear range: Ct 0.194, SD 0.0662% for NVAR; Ct 0.175, SD 0.618% for MPXV (Figure 1).

No false positives occurred within the inclusivity-exclusivity set. The MVA vector vaccine was correctly detected by the NVAR assay, and not by the MPXV assay (Appendix Table 1).

Clinical Evaluation

In total, we tested 67 clinical samples, consisting of respiratory, skin, and genital swab samples, with both

Table 3. Hit rates during limit of detection studies of dual-target MPXV assay rapidly adapted from established high-throughput molecular testing infrastructure*

| Concentration, copies/mL | NVAR | MPXV | Overall |
|--------------------------|-------|-------|---------|
| 100 | 21/21 | 21/21 | 21/21 |
| 50 | 21/21 | 21/21 | 21/21 |
| 25 | 21/21 | 21/21 | 21/21 |
| 12.5 | 20/21 | 21/21 | 21/21 |
| 6.25 | 19/21 | 20/21 | 21/21 |
| 3.125 | 11/21 | 13/21 | 15/21 |
| 1.56 | 11/21 | 11/21 | 17/21 |
| 0.78 | 3/21 | 6/21 | 7/21 |

*Results represent no. positive/no. tested. Limits of detection were determined by serial dilution of a quantified MPXV standard (quantified by digital PCR) as a reference. Concentrations represent copies/mL of specimen. Dilution series were generated automatically using a STARlet Liquid Handler (Hamilton, <https://www.hamiltoncompany.com>). We calculated 95% probability of detection by using MedCalc statistical software (<https://www.medcalc.org>). MPXV, monkeypox virus; NVAR, nonvariola orthopoxvirus.

assays. Of those, 11 samples obtained from 2 confirmed clinical monkeypox case-patients in Hamburg, Germany, were positive for MPXV DNA. We noted 100% positive (11/11) and 100% negative agreement (56/56) for the 2 assays (Figure 2).

Results from Different Sample Types and Timepoints

Another 33 clinical samples were longitudinally collected from 4 patients, all of whom were male, 20–40 years of age, and had 6–50 skin lesions; 1 patient had known HIV infection under treatment. Lesion swabs generated Ct values of 13.3–16.1, oropharyngeal swabs Ct values of 13.1–33.3, and blood samples Ct values of 30.3–38.4. A small sample set of urine had only low concentrations of viral DNA, Ct 31.1–37.8. A single patient provided seminal fluid, which had Ct values of 32.9 for the NVAR assay and 33.9 for the MPXV assay when diluted in guanidine hydrochloride solution (Appendix Table 2).

Discussion

The trajectory of the ongoing MPXV outbreak in Europe and North America has many uncertainties. However, the World Health Organization acknowledges that known clusters represent a change in transmission pattern and emphasizes the need to limit further spread (1). Broad availability of molecular testing with short turnaround times is a crucial prerequisite for reducing monkeypox spread.

We adapted 2 established nonvariola orthopoxvirus and MPXV qPCR assays (9,10) as a duplex test for the cobas 5800, 6800, and 8800 fully automated sample-to-result platforms, which are widely used for high-throughput SARS-CoV-2 diagnostic testing (13). Both assays have been validated extensively against other orthopoxvirus species in previous studies (9,10) and remain inclusive and highly

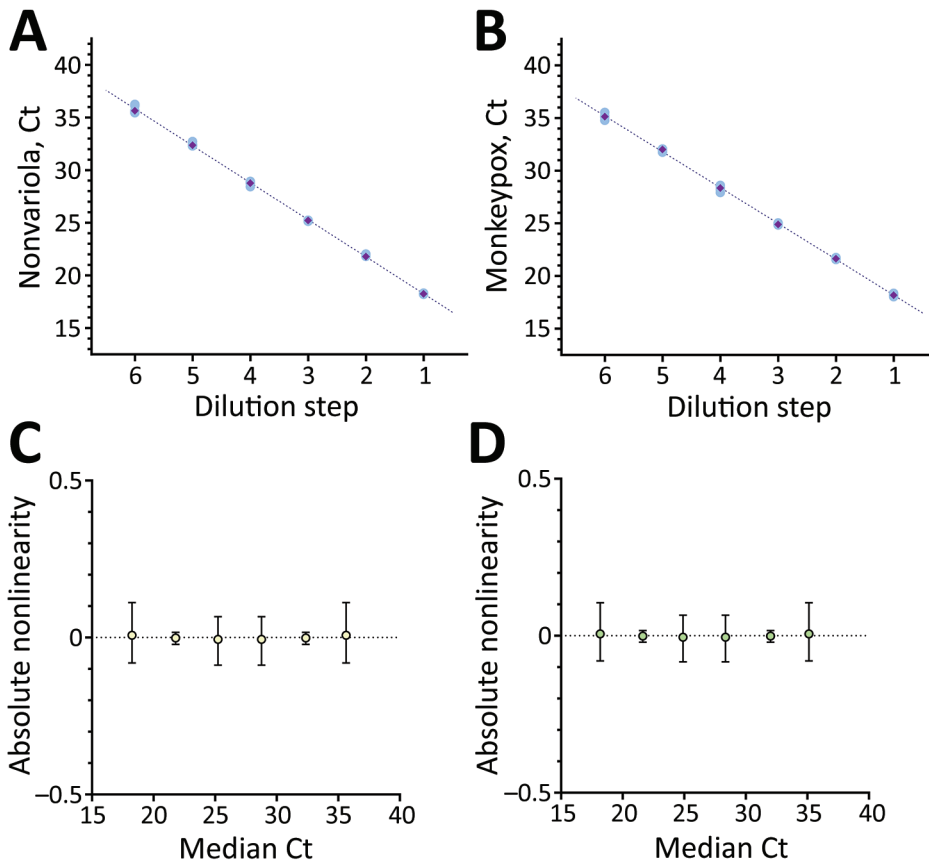


Figure 1. Linearity data for the dual-target monkeypox virus assay rapidly adapted from established high-throughput molecular testing infrastructure. A) Nonvariola orthopoxvirus target; B) monkeypox virus target; C) absolute Ct for nonvariola orthopox virus target; D) absolute Ct for monkeypox virus target. Linearity was determined by serial dilution of monkeypox virus reference material from cell culture supernatant of Congo Basin monkeypox strain collected in 1987. Analysis was performed on Validation Manager software (Finbiosoft, <https://finbiosoft.com>). Nonvariola orthopoxvirus slope was -3.52 , $r^2 = 0.999$; monkeypox virus slope was -3.40 , $r^2 = 0.999$. Ct, cycle threshold.

specific for in silico analysis with currently available monkeypox sequences. We demonstrated excellent analytical performance of the duplex assay, showing single-digit detection limits and near-perfect PCR efficiency. A spike-in full-process control assay, similar to commercial in vitro diagnostic assays, already is included in the open channel reagents we used.

Our institution confirmed 4 clinical cases of monkeypox, and we used the initial clinical samples as our clinical positive set. Although the assay was only validated on swab samples, we also detected MPXV DNA in EDTA plasma, urine, and seminal fluid diluted in guanidine hydrochloride solution without any method adaptations. Among all tested clinical samples positive for MPXV DNA, swabs of skin

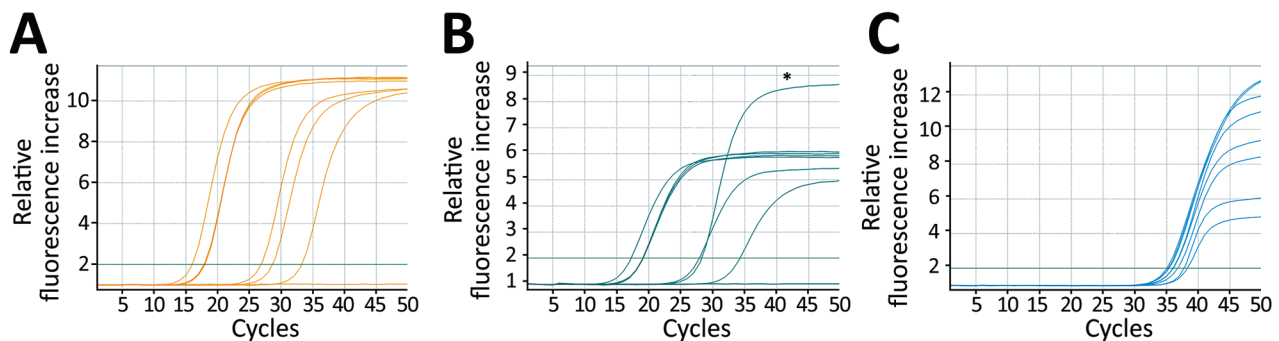


Figure 2. Amplification curves of clinical samples, including internal controls for dual-target monkeypox virus assay rapidly adapted from established high-throughput molecular testing infrastructure. A) Nonvariola orthopoxvirus; B) monkeypox virus; C) internal control. Samples included clinical swab specimens of monkeypox lesions, oropharyngeal swab samples, and EDTA plasma from patients with confirmed monkeypox, Hamburg, Germany. Asterisk (*) in panel B indicates the positive control curve in channel 2, which was the cell culture supernatant of Congo Basin monkeypox strain collected in 1987. West Africa strain samples exhibit a reduction of approximately one third in relative fluorescence increase for monkeypox virus, due to a known mismatch in the probe region (Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/28/9/22-0917-App1.pdf>).

lesions consistently yielded early Ct values in the low-to mid-teens, indicating exceedingly high viral DNA loads, which might be a concern for both personnel safety and contamination risks. Viral DNA was readily detectable in oropharyngeal swab samples, as previously reported (2,14). Likewise, EDTA plasma samples were consistently positive but had later Ct values, mostly around 30. Further studies could evaluate the practical usefulness of plasma or urine for monkeypox diagnostic purposes or longitudinal viral load monitoring. Our data regarding MPXV DNA in different clinical specimen types are well in line with other published studies (2,14); overall, skin lesion swab samples appeared to be best suited for diagnostic purposes based on our sample set.

In conclusion, we provided technical performance evaluation for a laboratory-developed duplex qPCR assay for MPXV detection for use on the cobas 5800, 6800, and 8800 high-throughput systems. The assay we describe enables laboratories to adapt existing automated SARS-CoV-2 molecular testing infrastructure for a potential large-scale monkeypox outbreak.

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M.L., S.P., and D.N. conceptualized and supervised the study. H.T.T., D.N., and P.E. performed the experiments. K.G. performed data analysis. D.N., M.L., S.P., N.F., S.S., M.M.A. and M.A. wrote and edited the manuscript. All authors agreed to the publication of the final manuscript.

About the Author

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Rapid Adaptation of Established High-Throughput Molecular Testing Infrastructure for Monkeypox Virus Detection

Appendix

Appendix Table 1. Clinical samples, reference material, and external control panels used for the inclusivity and exclusivity set for rapid adaptation of established high-throughput molecular testing infrastructure for monkeypox virus detection*

| Species | No. tested | Target | |
|--|------------|--------------------------|-----------------|
| | | Nonvariola orthopoxvirus | Monkeypox virus |
| External reference material, quality control panel | | | |
| Monkeypox virus (Congo Basin, cell culture, 1987) | 3 | Positive | Positive |
| Vaccinia virus Ankara strain SARS-2-ST_HD | 1 | Positive | Negative |
| Influenza-B (INSTAND e.V.) | 1 | Negative | Negative |
| Parainfluenzavirus-1 (Zeptomatrix) | 1 | Negative | Negative |
| Parainfluenzavirus-4 (Zeptomatrix) | 1 | Negative | Negative |
| Human coronavirus OC43 (Zeptomatrix) | 1 | Negative | Negative |
| Clinical samples | | | |
| Herpes simplex virus 1 | 5 | Negative | Negative |
| Varicella-zoster virus | 4 | Negative | Negative |
| Cytomegalovirus | 5 | Negative | Negative |
| Ebola virus | 5 | Negative | Negative |
| Human herpesvirus 6 | 5 | Negative | Negative |
| BK virus | 5 | Negative | Negative |
| John Cunningham virus | 4 | Negative | Negative |
| SARS-CoV-2 | 1 | Negative | Negative |
| Influenza A | 1 | Negative | Negative |
| Respiratory syncytial virus | 1 | Negative | Negative |
| Parainfluenzavirus-3 | 1 | Negative | Negative |
| Rhinovirus/enterovirus | 3 | Negative | Negative |
| Adenovirus | 2 | Negative | Negative |
| Bocavirus | 2 | Negative | Negative |
| Human coronavirus HKU1 | 1 | Negative | Negative |

*No false positives occurred. The MVA-SARS-2 vacciniavirus-based vaccine (Ankara strain) was correctly detected by the nonvariola orthopoxvirus assay and not by the monkeypox virus assay.

Appendix Table 2. Clinical samples tested with the dual-target monkeypox virus assay rapidly adapted from established high-throughput SARS-CoV-2 molecular testing infrastructure*

| Sample type | Admission day | Target, cycle threshold | |
|--------------------|---------------|--------------------------|-----------------|
| | | Nonvariola orthopoxvirus | Monkeypox virus |
| Lesion swab | 1 | 15 | 15.5 |
| Lesion swab | 1 | 14.9 | 15.8 |
| Lesion swab | 1 | 13.3 | 14.7 |
| Lesion swab | 2 | 14.3 | 13.9 |
| Lesion swab | 2 | 13.5 | 14.7 |
| Lesion swab | 3 | 13.9 | 15.3 |
| Lesion swab | 4 | 15.3 | 15.7 |
| Lesion swab | 5 | 14.3 | 15.4 |
| Lesion swab | 6 | 15.2 | 16.1 |
| Lesion swab | 7 | 14.4 | 15.6 |
| Lesion swab | 9 | 13.9 | 15.2 |
| Oropharyngeal swab | 1 | 23.8 | 24.2 |
| Oropharyngeal swab | 1 | 13.1 | 13.8 |
| Oropharyngeal swab | 1 | 17.1 | 18.4 |
| Oropharyngeal swab | 2 | 24.1 | 24.6 |
| Oropharyngeal swab | 2 | 21.9 | 23 |
| Oropharyngeal swab | 3 | 22.4 | 23.6 |

| Sample type | Admission day | Target, cycle threshold | |
|--------------------|---------------|--------------------------|-----------------|
| | | Nonvariola orthopoxvirus | Monkeypox virus |
| Oropharyngeal swab | 4 | 20.5 | 20.9 |
| Oropharyngeal swab | 5 | 26.9 | 28.08 |
| Oropharyngeal swab | 6 | 29.3 | 30.6 |
| Oropharyngeal swab | 7 | 27.9 | 28.9 |
| Oropharyngeal swab | 9 | 32.1 | 33.3 |
| EDTA plasma | 1 | 30.5 | 30.4 |
| EDTA plasma | 2 | 31.3 | 31.4 |
| EDTA plasma | 2 | 30.3 | 31.1 |
| EDTA plasma | 3 | 33.4 | 33.6 |
| EDTA plasma | 4 | 38.4 | Not detected |
| EDTA plasma | 4 | 30.7 | 31.5 |
| EDTA plasma | 6 | 36.1 | 37.2 |
| EDTA plasma | 8 | Not detected | 38 |
| Urine | 2 | 37.6 | 37.8 |
| Urine | 5 | 31.32 | 32.58 |
| Seminal fluid | 4 | 32.9 | 33.9 |

*Follow-up patient samples were analyzed with the dual-target assay. Specimens were collected from 4 different patients with previously confirmed monkeypox virus infection in Hamburg, Germany.

1:

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GAGTATAGAGCACTATTTCTAAATCCCACACATACAGTATCGCATTCA
CDC-NVAR rev .....
..................................................................... VEC-MPOX P-FAM

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Appendix Figure 1. Potential oligo-oligo interaction of the dual-target monkeypox virus assay rapidly adapted from established high-throughput molecular testing infrastructure. The NVAR reverse primer (1) and MPOX probe (2) show a 3' overlap of 4 bases. However, the interaction cannot create dimers on its own. Similar interactions can, in rare cases, create a viable dimer in a secondary unspecific amplification event, thereby also leading to unspecific signals. The risk for such an event is further mitigated by 2'Omethyl-RNA modified primers and internal quenchers. No unspecific signals were observed in wetlab experiments. Unspecific signals of the MPOX assay would yield nonsensical results (i.e., NVAR negative, MPOX positive), prompting further investigation. Based on these factors, the interaction was deemed not significant. MPOX, monkeypox virus; NVAR, nonvariola orthopox virus.

Target-1: NVAR

| group | n(Sequences) | hits | forward-1(f) | probe-1(p) | reverse-1(r) |
|-------------------|--------------|------|-------------------------|----------------------------------|-----------------------------|
| Monkeypox | n = 105 | 1 | TCAACTGAAAAGGCCATCTATGA | CCATGCAATATACGTACAAGATAGTAGCCAAC | GAGTATAGAGCACTATTCTAAATCCCA |
| Monkeypox | n = 1 | 1 |T..... | | |
| Abatino_macacapox | n = 2 | 1 | | |G..... |
| Akhmeta | n = 5 | 1 | ..C..... | |G..... |
| Akhmeta | n = 2 | 1 | ..C.....A..... |A.....A..C..... | |
| Camelpox | n = 10 | 1 | |G..... |C..... |
| Cowpox | n = 29 | 1 | | | |
| Cowpox | n = 2 | 1 | |C..... |C..G..... |
| Cowpox | n = 5 | 1 | | |C..G..... |
| Cowpox | n = 1 | 1 | |G.....C..... |G..... |
| Cowpox | n = 1 | 1 | |G.....C..G..... | |
| Cowpox | n = 5 | 1 | | |G..... |
| Cowpox | n = 1 | 1 | |C..... |G..... |
| Cowpox | n = 2 | 1 |T..... |G.....C..... | |
| Cowpox | n = 5 | 1 | | |C..G..... |
| Ectromelia | n = 13 | 1 | | |G..... |
| Raccoonpox | n = 5 | 1 | ..T..... |A.....A.....T..G..T |G..... |
| Skunkpox | n = 4 | 1 | ..T.....A..... |T..T..A.....G..T |G..... |
| Taterapox | n = 3 | 1 | |G.....C..... | |
| Vaccinia | n = 31 | 1 |T..... | | |
| Vaccinia | n = 1 | 1 |A..... |G.....A..... | A..... |
| Vaccinia | n = 72 | 1 | |G..... | |
| Vaccinia | n = 11 | 1 |T..... |A..... | |
| Vaccinia | n = 11 | 1 | |A..... | |
| Variola | n = 57 | 1 | |G.....CA..... | |
| Volepox | n = 4 | 1 | ..T.....A.....A..... | ..A.....T..T..A.....G..T |G..... |

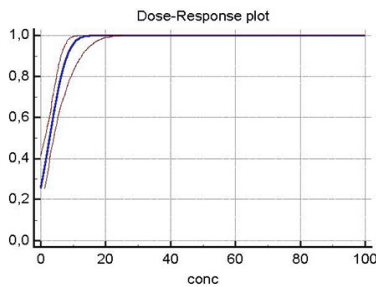
Appendix Figure 2. Primer and probe alignments for the NVAR target of the dual-target assay rapidly adapted from established high-throughput SARS-CoV-2 molecular testing infrastructure. Alignments were provided by Roche Diagnostics (<https://diagnostics.roche.com>) as part of a support request. Currently available orthopoxvirus sequences were checked for mismatches against the NVAR assay. The number of sequences represented by each line is indicated on the left. A single monkeypox virus sequence with 1 primer mismatch is noted, with a low risk for relevant effect. NVAR, nonvariola orthopoxvirus

Target-2: MPOX

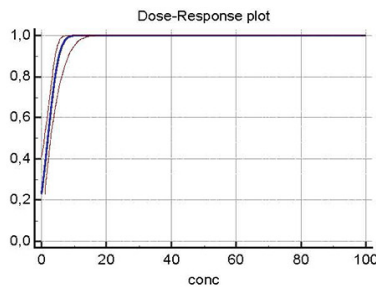
| group | id/number | hits | forward-1(f) | probe-1(p) | reverse-1(r) |
|------------------|-----------|------|------------------------|---|--------------|
| | | | ACGTGTTAAACAATGGGTGATG | TGAATGAATCGCA---T---A---CTGTATGTGTGGG AACATTTCCATGAATCGTAGTCC | |
| Monkeypox | n = 1 | 1 |T..... |T..... | |
| Monkeypox | n = 22 | 1 | |T..... | |
| Monkeypox | n = 77 | 1 | | | |
| Abatino_macacapo | n = 2 | 1 |A |CA.....GTTT.....C.....A..T.. | |
| Camelpox | n = 10 | 1 | T.....C.....A |CA.....GTTG.....A.....CA..... | |
| Cowpox | n = 8 | 1 |C.....A |C..A.....GTTG.....A.....C..... | |
| Cowpox | n = 24 | 1 |C.....A |CA.....GTTG.....A.....C..... | |
| Cowpox | n = 1 | 1 |A |CA.....GTTG..... | |
| Cowpox | n = 4 | 1 |C.....A |C..A.....GTTG.....T.....C..... | |
| Cowpox | n = 2 | 1 | G.....A |CA.....GTTG..... | |
| Cowpox | n = 1 | 1 | T.....C.....A |CA.....GTTG.....A.....C..... | |
| Cowpox | n = 2 | 1 | |C.....TTA..... | |
| Raccoonpox | n = 3 | 1 |G.....A..T | T..C.....A..CGT---C.....C..A.....C..A..... | |
| Skunkpox | n = 2 | 1 | AA...G...A..C | A.....A.....GTCG---G...A.....A..... | |
| Taterapox | n = 2 | 1 |C.....A |CA.....GTTG.....A.....C..... | |
| Vaccinia | n = 1 | 1 | |C..A.....GTTT.....A.C..T.....A..T.. | |
| Vaccinia | n = 1 | 1 | |C..A.....TTA..... | |
| Vaccinia | n = 8 | 1 | |C.....TTA..... | |
| Vaccinia | n = 10 | 1 | |C.....GTTT.....A.C..T..... | |
| Vaccinia | n = 73 | 1 | |C.....GTTT.....A.C..T.....A..T.. | |
| Vaccinia | n = 28 | 1 | |C.....TTA.....A..T.. | |
| Vaccinia | n = 1 | 1 |T...A..... | T...C..T.....TTA..... | |
| Vaccinia | n = 1 | 1 |A..... |C.....GTTT.....A.C..T.....A..T.. | |
| Vaccinia | n = 1 | 1 | |C.....GTTT.....A.C..T..... | |
| Variola | n = 76 | 1 | T.....A | CATCATT...C---TA---G---C.A.CAC.....CA..... | |
| Variola | n = 2 | 1 |C.....A |CA.....GTTG.....A.....C..... | |
| Variola | n = 2 | 1 |C.....A |CA.....GTTG.....A.....C..... | |
| Variola | n = 2 | 1 |C.....A |CA.....G---T..C.....CA..... | |
| Volepox | n = 2 | 1 | AA...G...C..C | T..C...A.....GTT---A.....C..A..A..... | |

Appendix Figure 3. Primer and probe alignments for the MPOX of the dual-target monkeypox virus assay rapidly adapted from established high-throughput SARS-CoV-2 molecular testing infrastructure. MPOX alignments were provided by Roche Diagnostics (<https://diagnostics.roche.com>) as part of a support request. Currently available orthopoxvirus sequences were checked for mismatches against the MPOX assay. The number of sequences represented by each line is indicated on the left. Monkeypox virus of the West Africa strain have a known mismatch in the probe region (G to T), leading to a reduction in RFI of $\approx 1/3$ (Figure 2). MPOX, monkeypox virus; RFI, relative fluorescence increase.

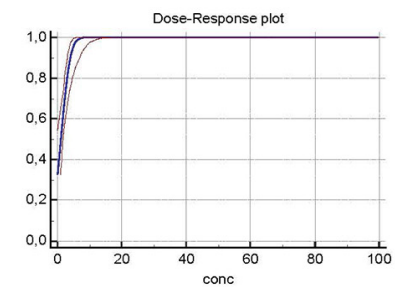
Target-1: NVAR



Target-2: MPOX



Target-1/2 combined



Appendix Figure 4. Probit curves of the lower limit of detection experiment for dual-target monkeypox virus assay rapidly adapted from established high-throughput SARS-CoV-2 molecular testing infrastructure. In brief, a 2-fold dilution series of quantified monkeypox virus standard (quantified by digital PCR) was used to determine the 95% probability of detection (21 repeats per dilution step). Confidence intervals are indicated in red. Hit-rates are displayed in Table 3 in the main text. Conc, concentration; MPOX, monkeypox virus; NVAR, nonvariola orthopoxvirus.

References

1. Li Y, Olson VA, Laue T, Laker MT, Damon IK. Detection of monkeypox virus with real-time PCR assays. *J Clin Virol.* 2006;36:194–203. [PubMed https://doi.org/10.1016/j.jcv.2006.03.012](https://doi.org/10.1016/j.jcv.2006.03.012)
2. Shchelkunov SN, Shcherbakov DN, Maksyutov RA, Gavrilova EV. Species-specific identification of variola, monkeypox, cowpox, and vaccinia viruses by multiplex real-time PCR assay. *J Virol Methods.* 2011;175:163–9. [PubMed https://doi.org/10.1016/j.jviromet.2011.05.002](https://doi.org/10.1016/j.jviromet.2011.05.002)