



Elsevier has created a [Monkeypox Information Center](#) in response to the declared public health emergency of international concern, with free information in English on the monkeypox virus. The Monkeypox Information Center is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its monkeypox related research that is available on the Monkeypox Information Center - including this research content - immediately available in publicly funded repositories, with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the Monkeypox Information Center remains active.



Validation Study of a Direct Real-Time PCR Protocol for Detection of Monkeypox Virus

Zachary L. Chelsky, David Dittmann, Timothy Blanke, Michael Chang, Erica Vormittag-Nocito, and Lawrence J. Jennings

From the Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois

Accepted for publication
September 2, 2022.

Address correspondence to
Lawrence J. Jennings, M.D.,
Ph.D., Department of Pathol-
ogy, Feinberg School of Medi-
cine, Northwestern University,
710 N. Fairbanks Ct., Chicago,
IL 60611.

E-mail: l-jennings@northwestern.edu

Monkeypox has recently been described as a public health emergency of international concern by the World Health Organization and a public health emergency by the United States. If the outbreak continues to grow, rapid scalability of laboratory testing will be imperative. During the early days of the coronavirus disease 2019 (COVID-19) pandemic, laboratories improved the scalability of testing by using a direct-to-PCR approach. To improve the scalability of monkeypox testing, a direct real-time PCR protocol for the detection of monkeypox virus was validated. The assay retains the sensitivity and accuracy of the indirect assay while eliminating the need for nucleic acid extraction kits, reducing laboratory technologist time per sample and decreasing exposure to an infectious agent. The direct method will make it easier for laboratories across the world to rapidly develop, validate, and scale testing for monkeypox virus. (*J Mol Diagn* 2022, 24: 1155–1159; <https://doi.org/10.1016/j.jmoldx.2022.09.001>)

Monkeypox virus, an encapsulated double-stranded DNA virus and member of the Poxviridae family, is responsible for the recent monkeypox outbreak that has been declared a public health emergency of international concern by the World Health Organization and a public health emergency by the United States. Prompt identification of infected individuals followed by contact tracing is important for stemming the spread of disease. The characteristic rash of monkeypox progresses through multiple stages, beginning with a macular phase, progressing through papular, vesicular, and pustular phases, and ending with a scab phase. Its appearance can be similar to rashes associated with other infections, including varicella zoster, herpes, and syphilis; thus, testing of the lesion is important for accurate diagnosis.¹ The CDC monkeypox diagnostic assay is in use in many laboratories across the United States. It is a real-time PCR-based assay utilizing a nucleic acid extraction step before amplification.² Other reported monkeypox diagnostic assays also utilize a nucleic acid extraction step.³

If the monkeypox outbreak continues to worsen, testing will need to be rapidly scaled to meet demand. As we have learned from the coronavirus disease 2019 (COVID-19) pandemic, supply chain shortages, costs, and time constraints can significantly impair the ability of laboratories to quickly scale testing. During the early days of the COVID-19

pandemic, some laboratories were able to mitigate these challenges by omitting the nucleic acid extraction step of the CDC assay for detection of RNA virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and replacing it with an alternative method using proteinase K and heat.^{4–6} The aim of this study is to show that a DNA virus, such as monkeypox, can also be identified by this approach. Herein, a nucleic acid extraction-free approach (direct method) for the detection of monkeypox is compared with the nucleic acid extraction method of the CDC monkeypox assay to show retention of the direct assay's diagnostic ability, thereby making it easier for laboratories to rapidly scale monkeypox virus testing.

Materials and Methods

Clinical Validation of a Direct Monkeypox PCR Assay

A modified multiplex version of the CDC monkeypox assay was performed for clinical validation purposes. Probe and primers targeting monkeypox have previously been

Supported by departmental funding from Feinberg School of Medicine, Northwestern University.

Disclosures: None declared.

described.² The *RPI* gene that is present in normal human skin epithelium was used as an internal control in all samples. In the nucleic acid extraction-free PCR method, 5 μ L of proteinase K (Qiagen, Hilden, Germany) was added to a 50- μ L aliquot of sample taken from 3 mL of M4 viral transport medium (VTM). The sample was then heated for 10 minutes at 65°C, followed by 5 minutes at 95°C. This was followed by real-time PCR on the Quant Studio 6 instrument (Thermo Fisher Scientific, Waltham, MA). Cycling conditions included a 20-second activation step at 95°C, followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C.

A standard curve for copy number quantitation was generated by diluting plasmid control DNA to seven concentrations between 1 and 1,000,000 copies per mL and determining the crossing threshold cycle (C_T) associated with each concentration. This was done in triplicate, and the mean C_T values were plotted against plasmid concentration to generate a standard curve. The equation of the standard curve and the R^2 value were determined by Excel version 2108 (Microsoft, Redmond, WA). The estimated number of detected copies of monkeypox virus was extrapolated from the equation for the standard curve. The limit of detection was validated by replicate determinations of C_T values ($n = 20$) of varying concentrations in negative VTM.

Analytical specificity was determined by spiking VTM with varying control materials and determining if signal was detected within the limit of detection. The infectious disease control materials used included Zika virus, Epstein-Barr virus, herpes simplex virus 1, herpes simplex virus 2, influenza A virus H3N2, influenza A virus H1N1, influenza B, respiratory syncytial virus A, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, adenovirus 3, metapneumovirus, rhinovirus, coronavirus OCA3, coronavirus 228E, coronavirus NL63, coronavirus HKL1-1, *Bordetella pertussis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Candida albicans*, *Neisseria meningitis*, and SARS-CoV-2.

A carryover study was performed by alternating 10 samples containing a high positive monkeypox control with 10 blank control samples in a 96-well plate. A second 96-well plate was also used, which contained 10 samples of a human control gene alternating with 10 blank control samples.

Prospective Correlation of Direct versus Indirect PCR Methods

Clinical specimens were collected from patients at locations within the Northwestern Medicine (Chicago, IL) health system. Lesions were swabbed with sterile synthetic swabs, and the swabs were submitted to the laboratory dry or in 3 mL of viral transport media (M4 VTM). Dry swabs received by the laboratory were immediately added to 3 mL of M4 VTM. At the start of the monkeypox outbreak, a total of 20 samples identified as positive by the direct assay

and 20 samples identified as negative by the direct assay were sequentially chosen for confirmation by indirect method. DNA extraction for the indirect method was performed using the Qiagen manual DNA extraction kit utilizing spin-column-based nucleic acid purification following manufacturer's instructions (Qiagen). The C_T values between the direct and indirect methods were compared by linear regression analysis, Deming regression analysis, and Bland-Altman analysis.

Results

Clinical Validation of a Direct Monkeypox PCR Assay

A standard curve was generated by diluting plasmid monkeypox control DNA to concentrations ranging from 1 to 1,000,000 copies/mL and determining the corresponding C_T value. The assay displayed excellent linearity ($R^2 = 0.9994$) (Figure 1). The limit of detection was determined by replicate determinations of C_T values ($n = 20$) of 5, 50, and 1000 copies/mL samples. The mean C_T values of 5 copies/mL were determined to be 36 on both the direct and indirect assay, with an SD of 0.75 (range, 34.61 to 37.39). Samples run with 50 copies/mL showed a mean C_T of 35 and an SD of 0.62 (range, 33.93 to 36.55). Signal was detected in 4 of 19 negative samples (range, 36.99 to 37.38); thus, to avoid signal associated with background (false positives), the limit of detection for the clinical qualitative assay was set at two SDs from the mean C_T value for 50 copies/mL (36.24).

The analytical specificity was determined by running the assay with control materials for 23 different viruses, bacteria, and fungi. No signal within the limit of detection was detected by the assay in any of the control materials (Table 1). Positive and negative samples were also spiked with varying concentrations of blood (5% to 20%). Blood did have an inhibitory effect on the assay, with increasing concentration of blood leading to greater inhibition. Samples with 20% blood had complete inhibition.

A carryover study was performed by alternating 10 samples containing a high positive monkeypox control with 10 blank control samples in a 96-well plate. Virus was not detected in any of the blank control wells. A second carryover study was performed by alternating 10 samples containing *RPI* control gene with 10 blank control samples in a 96-well plate. No signal was detected within our limit of detection in the wells containing a blank control (Table 2).

Three positive patient samples were tested in duplicate at dilution factors of 1, 10, and 100 by both direct and indirect protocols. Table 3 lists the mean C_T value of each sample under direct and indirect conditions obtained from the dilutions. The number of viral copies detected by the direct method was extrapolated from the corresponding indirect C_T value and the plasmid standard curve. The direct protocol was able to detect as few as 16 viral copies/mL VTM.

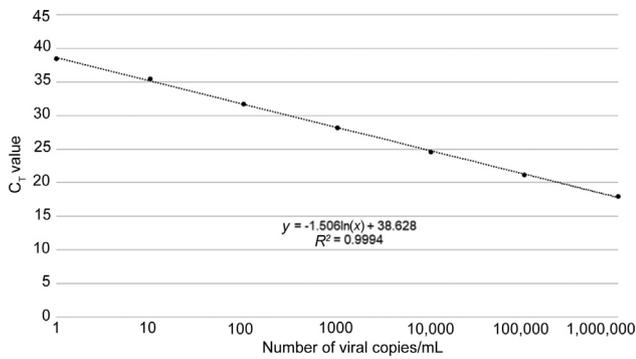


Figure 1 Overall standard curve demonstrating C_T values for plasmid DNA concentration from 1 to 1,000,000 copies/mL viral transport medium. The logarithmic equation for the trendline is shown as well as the R^2 value.

Prospective Correlation of the Direct versus Indirect PCR Methods

Comparison of the C_T values and qualitative results obtained by the direct method and the indirect method was undertaken. A total of 20 positive and 20 negative samples, as determined by the direct method, were sequentially chosen to undergo testing by the indirect method. Linear regression analysis of positive samples demonstrated an R^2 value of 0.88, and the Deming regression line had a slope of 0.9063 and an intercept of 2.8542 (Figure 2). Bland-Altman analysis of positive samples revealed a mean difference in

Table 1 Analytical Specificity

Pathogen	C_T value
Zika virus	0
EBV	0
HSV 1	0
Influenza A H3N2	0
Influenza A H1N1	0
Influenza B	0
RSV A	0
Parainfluenza 1	0
Parainfluenza 2	39.84
Parainfluenza 3	0
HSV 2	0
Adenovirus 3	0
Metapneumovirus	0
Rhinovirus	0
Coronavirus OCA3	0
Coronavirus 228E	0
Coronavirus NL63	0
Coronavirus HKL1-1	0
<i>Bordetella pertussis</i>	0
<i>Chlamydia pneumoniae</i>	0
<i>Mycoplasma pneumoniae</i>	0
<i>Candida albicans</i>	0
<i>Neisseria meningitis</i>	0
SARS-CoV-2	0

EBV, Epstein-Barr virus; HSV, herpes simplex virus; RSV, respiratory syncytial virus.

Table 2 Carryover Study

Monkeypox probe	C_T value	RP1 probe	C_T value
High Positive	27.6	Negative	27.32
BLANK	0	BLANK	0
High Positive	27.4	Negative	26.397
BLANK	0	BLANK	0
High Positive	27.49	Negative	27.25
BLANK	0	BLANK	0
High Positive	27.5	Negative	26.99
BLANK	0	BLANK	0
High Positive	27.38	Negative	26.93
BLANK	0	BLANK	0
High Positive	27.34	Negative	26.72
BLANK	0	BLANK	37.22
High Positive	27.38	Negative	26.83
BLANK	0	BLANK	0
High Positive	27.37	Negative	26.99
BLANK	0	BLANK	0
High Positive	27.37	Negative	26.9
BLANK	0	BLANK	0
High Positive	27.37	Negative	26.9
BLANK	0	BLANK	0
High Positive	27.37	Negative	26.99
BLANK	0	BLANK	0

C_T values between the direct and indirect methods of 0.499 and an SD of 1.52. The upper 95% CI for the difference in C_T values was determined to be 3.48, and the lower 95% CI was determined to be -2.48. No values fell outside the upper 95% CI, but one value fell outside of the lower 95% CI (-2.52) (Figure 3). All samples positive for monkeypox by the direct method were positive when tested with the indirect method. Similarly, all samples negative for monkeypox with the direct method were also negative when tested with the indirect method (Table 4).

Discussion

The need for rapid scalability of testing for emerging pathogens became important during the recent and ongoing COVID-19 pandemic. In the early steps of the pandemic, laboratories faced significant challenges in meeting testing demand, including staffing shortages, high costs, and supply

Table 3 Dilution Study

Sample	Dilution factor	Direct	Extracted	Calculated copies
100017515822	1	34.455	34.305	16
100017385420	1	25.28	25.02	7061
100018465412	1	26.45	25.14	3247
100017515822	10	ND	ND	N/A
100017385420	10	28.415	28.8	881
100018465412	10	29.805	29.245	350
100017515822	100	ND	ND	N/A
100017385420	100	31.3	32.19	130
100018465412	100	32.74	32.675	50

N/A, not applicable; ND, not detected.

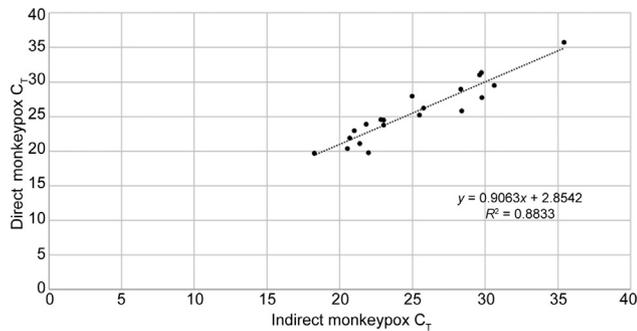


Figure 2 Linear regression analysis of positive samples. The equation of the trendline and the R^2 value are shown.

chain problems, such as a shortage of nucleic acid extraction kits.⁷ During this time, it was shown that the shortage of nucleic acid extraction kits could be bypassed by generation of nucleic acid extraction-free protocols, and nucleic acid extraction-free protocols were generated for COVID-19 PCR testing.^{4–6,8} Instead of a nucleic acid extraction step, these protocols used proteinase K and heat to destroy PCR inhibitors and release the nucleic acid from the viral envelope. This extraction-free approach to COVID-19 testing helped laboratories to avoid supply constraints, decrease turnaround time, reduce expenses, and improve laboratory safety for technologists.^{9–12}

Given the rapid spread of the current monkeypox outbreak, rapid scalability of testing for monkeypox testing may also be needed. Considering the difference in virus structure between SARS-CoV-2 and monkeypox (RNA versus DNA virus), the CDC DNA extraction indirect method was compared with a direct PCR method employed by many for RNA coronavirus testing. Although the current testing volume for monkeypox remains low, the CDC recommends testing two to three lesions per patient, so the number of samples tested in a laboratory will increase at a much faster rate than during the COVID-19 pandemic, when the recommended specimen was a single nasal swab.

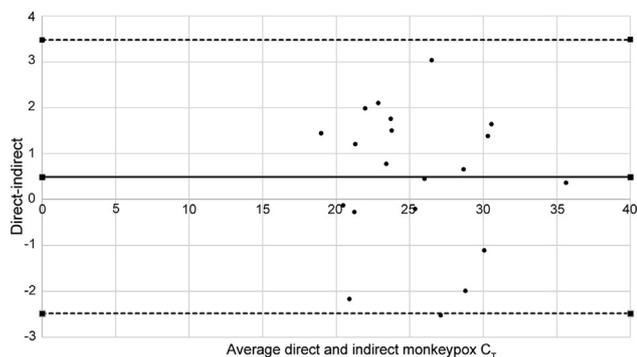


Figure 3 Bland-Altman analysis of positive samples. The mean difference in C_T values is 0.499 (solid line), and the upper (3.48) and lower (–2.48) 95% CIs are shown (dashed lines).

Table 4 Accuracy

Variable	Positive indirect	Negative indirect
Positive direct	20	0
Negative indirect	0	20

The results of the nucleic acid extraction-free approach to monkeypox PCR shows that a direct method can also be employed to improve the scalability of testing for monkeypox. By avoiding the requirement for nucleic acid extraction, the direct method avoids potential shortages in extraction kits. In addition, utilization of the direct approach decreased technician hands-on time by 30 to 60 minutes per run, depending on the number of samples being tested. This not only reduced technician exposure to potentially infectious specimens but increased the number of samples that could be run in a single day. The direct method also decreases the direct costs an estimated \$16 per sample in reagents, disposables, and technologist time, which can be costly given the recommendation that two to three lesions per patient be tested.

The direct method described herein demonstrates 100% concordance when comparing results from the direct and indirect methods of both the positive and negative results. However, one potential concern with direct testing is that it may be less sensitive than an indirect/extraction-based method because of the target nucleic acid concentration potentially being less than with an indirect/extraction-based method. In a direct method, there is no lysis or nucleic acid concentration step, and these direct methods use proteinase K, so the nucleic acid is diluted further by this addition. In the direct protocol, 5 μ L of proteinase K is added to 50 μ L of patient sample, and this addition can be seen as a negligible dilution given that the assay was able to detect as few as 16 copies/ μ L. In addition, the mean difference in C_T values for positive samples when comparing direct and indirect methods was 0.499, suggesting that there may have been a small dilution effect present; however, sensitivity of the assay did not appear to be affected, even at low viral concentrations through the dilution studies performed. Furthermore, the Bland-Altman analysis of positive samples demonstrates a wide distribution of differences across the 0 line without a positive trend in difference with increasing C_T values. A limitation to the study, however, may be that only four positive samples had a mean C_T value (C_T direct and indirect) of >30 , which may not be enough samples with viral loads near the limit of detection to see an effect of dilution. In addition, for samples with visible blood contamination, nucleic acid extraction is advisable to avoid inhibition.

This validation of a direct method monkeypox assay will allow laboratories to lower costs, reduce dependence on the supply chain for nucleic acid extraction kits, and decrease exposure of laboratory scientists to potentially infectious specimens. In addition, it may be suitable for incorporation into automated and high-throughput testing. This direct

method will make it easier for laboratories across the world to rapidly develop, validate, and scale testing for monkeypox virus.

References

1. Titanji BK, Tegomoh B, Nematollahi S, Konomos M, Kulkarni PA: Monkeypox: a contemporary review for healthcare professionals. *Open Forum Infect Dis* 2022, 9:ofac310
2. 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
3. Wawina-Bokalanga T, Sklenovska N, Vanmechelen B, Bloemen M, Vergote V, L Laenen, André E, Van Ranst M, Muyembe-Tamfum JJ, Maes P: An accurate and rapid real-time PCR approach for human monkeypox virus diagnosis. *medRxiv* 2022, [Preprint] doi: 10.1101/2022.06.23.22276033
4. Vogels CBF, Watkins AE, Harden CA, Brackney DE, Shafer J, Wang J, Caraballo C, Kalinich CC, Ott IM, Fauver JR, Kudo E, Lu P, Venkataraman A, Tokuyama M, Moore AJ, Muenker MC, Casanovas-Massana A, Fournier J, Bermejo S, Campbell M, Datta R, Nelson A, Yale IMPACT Research Team, Dela Cruz CS, Ko AI, Iwasaki A, Krumholz HM, Matheus JD, Hui P, Liu C, Farhadian SF, Sikka R, Wyllie AL, Grubaugh ND: SalivaDirect: a simplified and flexible platform to enhance SARS-CoV-2 testing capacity. *Med N Y N* 2021, 2:263–280.e6
5. Mallmann L, Hermann BS, Schallenberg K, Demoliner M, Eisen AKA, Heldt FH, Gularte JS, Hansen AW, de Almeida PR, Weber MN, Spilki FR, Fleck JD: Proteinase K treatment in absence of RNA isolation classical procedures is a quick and cheaper alternative for SARS-CoV-2 molecular detection. *J Virol Methods* 2021, 293: 114131
6. Mio C, Cifù A, Marzinotto S, Bergamin N, Caldana C, Cattarossi S, Cmet S, Cussigh A, Martinella R, Zucco J, Verardo R, Schneider C, Marcon B, Zampieri S, Pipan C, Curcio F: A streamlined approach to rapidly detect SARS-CoV-2 infection avoiding RNA extraction: workflow validation. *Dis Markers* 2020, 2020:8869424
7. Esbin MN, Whitney ON, Chong S, Maurer A, Darzacq X, Tjian R: Overcoming the bottleneck to widespread testing: a rapid review of nucleic acid testing approaches for COVID-19 detection. *RNA N Y N* 2020, 26:771–783
8. Genoud V, Stortz M, Waisman A, Berardino BG, Veneri P, Dansey V, Salvatori M, Remes Lenicov F, Levi V: Extraction-free protocol combining proteinase K and heat inactivation for detection of SARS-CoV-2 by RT-qPCR. *PLoS One* 2021, 16:e0247792
9. Fomsgaard AS, Rosenstjerne MW: An alternative workflow for molecular detection of SARS-CoV-2 - escape from the NA extraction kit-shortage, Copenhagen, Denmark, March 2020. *Euro Surveill* 2020, 25: 2000398
10. Naranbat D, Schneider L, Kantor R, Beckwith CG, Bazerman L, Gillani F, Sahu S, Rapoza K, Sam S, Novitsky V, Shin J, Hipolito E, Diaz I, Carnevale D, Tripathi A: DirectDetect SARS-CoV-2 direct real-time RT-PCR study using patient samples. *ACS Omega* 2022, 7:4945–4955
11. Alaifan T, Altamimi A, Obeid D, Alshehri T, Almatrouk S, Albarrag A: SARS-CoV-2 direct real-time polymerase chain reaction testing in laboratories with shortage challenges. *Future Virol* 2021, 16: 133–139
12. Morehouse ZP, Proctor CM, Ryan GL, Nash RJ: A novel two-step, direct-to-PCR method for virus detection off swabs using human coronavirus 229E. *Virol J* 2020, 17:129