Bioanalytical Contributing Scientist Report

RT-qPCR Determination of SARS-CoV-2 for Protocol FY21-155, "SARS-CoV-2 Efficacy of Aerosolized Calcium Glycerophosphate (CGP) in a Syrian hamster model"

Study Number:	FY21-055
Sponsor:	AkPharma Inc P.O. Box 111 Pleasantville, NJ 08232
Test Facility:	Lovelace Biomedical 2425 Ridgecrest Drive SE Albuquerque, NM 87108
	Location of Laboratory: Bldg. 9217, Area Y Kirtland Air Force Base Albuquerque, NM 87115
Contributing Scientist	Adriana Rascon, M.S. Research Assistant Microbial Analysis

LOVELACE SIGNATURES

Name/Signature	**Date**
Adriana A. Rascon, M.S. **Research Associate, Microbial Analysis**	

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REVISION HISTORY **Page No.**

Current Version	Description of Change(s)	Supersedes
FY21-155 RT-qPCR	Initial	NA

LIST OF ABBREVIATIONS

AQL	Above the quantification limit
BQL	Below the quantification limit
Cq	Quantification Cycle
ºC	Degrees Celsius
CV	Coefficient of Variation
LLOQ	Lower Limit of Quantification
mL	Milliliter
NA	Not applicable
ng	nanogram
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-qPCR	Quantitative Reverse Transcription PCR
rxn	Reaction
R^2	coefficient of determination
SD	Standard Deviation
SOP	Standard Operating Procedure
µg	Microgram
µL	Microliter

PROJECT INFORMATION

Study Director:	Adam Werts, DVM, PhD, Dipl. ACLAM Lovelace Biomedical
Test Facility:	Lovelace Biomedical 2425 Ridgecrest Drive SE Albuquerque, NM 87108 Location of Laboratory: Bldg. 9217, Area Y Kirtland Air Force Base Albuquerque, NM 87115
Sponsor Details:	AkPharma
Contributing Scientist:	Adriana Rascon, M.S. Research Assistant Microbial Analysis
Protocol No:	FY21-155, original effective on 03Jun2021.

1 INTRODUCTION

Tissue samples were collected and analyzed to test whether aerosolized calcium glycerophosphate (CGP) has a therapeutic effect against SARS-CoV-2 in a Syrian hamster model. These samples were analyzed using test method MBM-1387. This report provides the sample results, calibration standards and positive control (PC) performance for the sample analysis.

2 EXPERIMENTAL

2.1 PCR Method

Test method MBM-1387 is a RT-qPCR method developed at Lovelace Biomedical for the Multiplex Quantitative Real-Time PCR Analysis SARS-CoV-2 Genomic and Subgenomic Content in Tissues, Fluids and Feces using Trizol Inactivation. These methods were verified for performance prior to sample analysis. Refer to Table 1 for a summary of the methods, Appendix A for method MBM-1387 details.

Table 1. Method Information Summary

Method No.	**MBM-1387**
Analytes	SARS-CoV-2 N2 gene/ E gene
Reference Gene	NA
Assay Range (copies/ Rxn)	2.5×10^8 to 2.5×10^1
Positive Control Concentrations (copies/Rxn)	5.0×10^4
Matrix	Hamster Tissue, Fluid, Feces

2.2 Study Samples

2.2.1 Sample Source and Date of Collection

Samples were collected at Lovelace Biomedical on 18 – 25Jun2021.

2.2.2 Sample Storage

Study samples were stored at Lovelace Biomedical in a freezer with a temperature of -70 to -90 °C.

3 RESULTS

3.1 Sample Assay Schedule

Details of the sample analysis runs are presented in Table 2.

3.2 Quantification Cycle Values of Calibration Standards

The results for the Cq values generated by the calibrators are provided in Table 3.

3.3 Standard Curve Performance

The curve parameters including the efficiency, coefficient of determination (R^2), slope, and intercept are provided in Table 4.

3.4 Concentrations of QC Samples

Results for the positive control samples are provided in Table 5.

3.5 Concentrations of Study Samples

The concentration of SARS-CoV-2 N2 genomic material and SARS-CoV-2 E gene subgenomic content in study samples are reported as the copy number per gram of tissue in Table 6 and copy number per swab in Table 7.

3.6 Deviations

There were no study deviations.

4 STUDY SAMPLE RESULTS

Table 2. Sample Assay Schedule

Run Number	Assay Date	Run Information	Run Accepted/Rejected
SARS-CoV 2 N2 Assay			
1	29-Jun-21	PCR01 – Tissues and Swabs	Accepted
2	29-Jun-21	PCR02 - Swabs	Accepted
SARS CoV-2 sgm RNA Assay			
1	29-Jun-21	PCR01 – Tissues and Swabs	Accepted
2	29-Jun-21	PCR02 - Swabs	Accepted

Table 3. Performance of Calibration Standards

Sample Name		STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8
Conc. (copies/rxn)		$2.5x10^8$	$2.5x10^7$	$2.5x10^6$	$2.5x10^5$	$2.5x10^4$	$2.5x10^3$	$2.5x10^2$	$2.5x10^1$
Run Date	**Run Number**	**Cq Value**							
SARS-CoV-2 N2 Assay									
29Jun2021	1	13.19	16.39	19.39	22.72	26.18	29.09	32.48	36.28
		12.82	16.00	19.40	22.49	26.31	29.04	32.41	36.38
		12.99	16.07	19.38	22.49	26.40	29.21	32.36	35.22
29Jun2021	2	12.39	15.94	19.38	22.45	27.79	28.86	32.22	36.95
		12.29	15.74	19.09	22.77	27.88	28.88	32.43	35.79
		12.08	15.88	19.28	23.02	27.65	29.12	32.30	36.38
Overall Mean		12.63	16.00	19.32	22.66	27.04	29.03	32.37	36.17
S.D.		0.434	0.221	0.123	0.221	0.816	0.139	0.093	0.592
%CV		3.4	1.4	0.6	1.0	3.0	0.5	0.3	1.6
SARS-CoV-2 sgm RNA Assay									
29Jun2021	1	12.07	14.94	17.97	21.08	24.09	27.40	31.07	34.86
		11.65	14.49	17.82	20.92	24.03	27.44	31.02	34.87
		11.21	14.59	17.79	20.99	24.27	27.75	30.73	33.66
29Jun2021	2	12.81	16.26	19.70	22.94	26.55	29.32	32.94	40.00
		12.77	16.13	19.45	23.14	26.64	29.28	32.93	40.00
		12.55	16.24	19.65	23.31	26.50	29.65	33.15	40.00
Overall Mean		12.18	15.44	18.73	22.06	25.34	28.47	31.97	37.23
S.D.		0.651	0.859	0.958	1.176	1.335	1.049	1.141	3.064
%CV		5.3	5.6	5.1	5.3	5.3	3.7	3.6	8.2

Table 4. Calibration Curve Performance

Run Date	Run Number	% Efficiency	R-Squared	Slope	Intercept
SARS-CoV-2 N2 Assay					
29Jun2021	1	102.1	0.999	-3.273	40.394
29Jun2021	2	97.0	0.992	-3.396	41.075
Overall Mean		99.6	0.996	-3.335	40.735
S.D.		3.606	0.005	0.087	0.482
%CV		3.6	0.5	-2.6	1.2
SARS-CoV-2 sgm RNA Assay					
29Jun2021	1	103.0	0.998	-3.253	38.711
29Jun2021	2	98.2	0.999	-3.366	41.121
Overall Mean		100.6	0.999	-3.3095	39.916
S.D.		3.394	0.001	0.080	1.704
%CV		3.4	0.1	-2.4	4.3

Table 5. Concentration of QC Samples

Positive Control Nominal = 5.00 E +04 Log10 = 4.699					
SARS-CoV-2 N2 Assay			**SARS-CoV-2 sgm RNA Assay**		
Run Number	**1**	**2**	**Run Number**	**1**	**2**
Run Date	**29Jun2021**	**29Jun2021**	**Run Date**	**29Jun2021**	**29Jun2021**
	4.520	4.586		4.641	4.659
	4.555	4.650		4.658	4.699
	4.541	4.637		4.669	4.704
Mean	4.539	4.624	Mean	4.656	4.687
SD	0.0176	0.0338	SD	0.0141	0.0247
CV	0.4	0.73	CV	0.30	0.53
%Bias	-3.4	-1.6	%Bias	-0.9	-0.2

Table 6. Lung Tissue Summary

Treatment Group	Sample ID	N2 RNA (copies/g)	Sgm RNA (copies/g)
Group 1	1001 Lung	1.58E+11	3.66E+09
	1002 Lung	6.15E+10	1.41E+09
	1003 Lung	3.32E+08	7.21E+06
	1004 Lung	1.20E+10	2.88E+08
	1005 Lung	1.27E+10	3.59E+08
	1006 Lung	1.62E+10	5.03E+08
	1007 Lung	9.33E+09	1.95E+08
	1008 Lung	6.92E+09	1.79E+08
	1009 Lung	9.86E+07	1.73E+06
	1010 Lung	2.61E+10	6.14E+08
Group 2	2001 Lung	1.79E+10	4.63E+08
	2002 Lung	1.20E+09	2.50E+07
	2003 Lung	4.37E+09	9.54E+07
	2004 Lung	8.66E+09	1.67E+08
	2005 Lung	3.54E+09	7.56E+07
	2006 Lung	2.39E+09	7.44E+07
	2007 Lung	3.09E+10	5.42E+08
	2008 Lung	1.75E+10	2.84E+08
	2009 Lung	2.40E+10	4.21E+08
	2010 Lung	4.88E+09	1.03E+08
Group 3	3001 Lung	5.35E+08	1.13E+07
	3002 Lung	4.60E+09	7.62E+07
	3003 Lung	1.47E+10	2.94E+08
	3004 Lung	1.10E+09	2.50E+07
	3005 Lung	8.89E+09	1.57E+08
	3006 Lung	5.36E+09	7.74E+07
	3007 Lung	1.29E+10	2.31E+08
	3008 Lung	1.65E+09	2.75E+07
	3009 Lung	4.58E+09	8.50E+07
	3010 Lung	1.69E+10	2.91E+08
Group 4	4001 Lung	3.75E+10	9.53E+08
	4002 Lung	6.51E+09	2.31E+08
	4003 Lung	1.84E+10	4.48E+08
	4004 Lung	5.82E+10	1.46E+09
	4005 Lung	5.33E+07	1.00E+06
	4006 Lung	1.71E+10	4.50E+08
	4007 Lung	4.79E+09	1.85E+08

	4008 Lung	4.98E+09	1.67E+08
	4009 Lung	5.82E+09	1.87E+08
	4010 Lung	3.74E+09	1.02E+08
Group 5	5001 Lung	4.46E+09	8.27E+07
	5002 Lung	6.09E+10	1.33E+09
	5003 Lung	7.50E+09	1.91E+08
	5004 Lung	8.26E+09	1.94E+08
	5005 Lung	8.13E+08	1.76E+07
	5006 Lung	1.77E+10	4.81E+08
	5007 Lung	5.07E+09	1.10E+08
	5008 Lung	1.30E+09	3.31E+07
	5009 Lung	1.23E+10	2.47E+08
	5010 Lung	2.10E+10	3.50E+08
Group 6	6001 Lung	6.55E+09	1.34E+08
	6002 Lung	1.35E+10	2.04E+08
	6003 Lung	2.42E+09	7.39E+07
	6004 Lung	2.24E+09	4.65E+07
	6005 Lung	2.23E+09	6.33E+07
	6006 Lung	9.62E+09	2.35E+08
	6007 Lung	5.58E+09	7.91E+07
	6008 Lung	1.62E+09	2.42E+07
	6009 Lung	9.12E+09	1.57E+08
	6010 Lung	8.97E+09	2.07E+08
	LLOQ	2.01E+04	

Table 7. Nasal Swab Summary

Treatment Group	Sample ID	N2 RNA (copies/swab)	Sgm RNA (copies/swab)
Group 1	1001 Nasal Swab D3	4.80E+07	7.78E+05
	1002 Nasal Swab D3	3.88E+07	3.56E+05
	1003 Nasal Swab D3	1.54E+08	2.29E+06
	1004 Nasal Swab D3	1.13E+09	2.61E+07
	1005 Nasal Swab D3	3.08E+08	6.16E+06
	1006 Nasal Swab D3	1.26E+08	2.83E+06
	1007 Nasal Swab D3	3.89E+08	1.01E+07
	1008 Nasal Swab D3	2.77E+08	5.15E+06
	1009 Nasal Swab D3	5.91E+08	1.31E+07
	1010 Nasal Swab D3	8.33E+07	1.53E+06
Group 2	2001 Nasal Swab D3	2.63E+08	5.49E+06
	2002 Nasal Swab D3	6.01E+08	1.57E+07
	2003 Nasal Swab D3	1.56E+08	2.26E+06
	2004 Nasal Swab D3	1.50E+08	2.82E+06
	2005 Nasal Swab D3	1.14E+08	1.89E+06
	2006 Nasal Swab D3	8.76E+07	1.49E+06
	2007 Nasal Swab D3	3.07E+08	6.28E+06
	2008 Nasal Swab D3	3.30E+08	6.42E+06
	2009 Nasal Swab D3	5.58E+07	8.81E+05
	2010 Nasal Swab D3	1.58E+07	8.52E+04
Group 3	3001 Nasal Swab D3	1.37E+08	2.92E+06
	3002 Nasal Swab D3	2.23E+08	5.47E+06
	3003 Nasal Swab D3	2.52E+08	4.73E+06
	3004 Nasal Swab D3	1.75E+08	3.54E+06
	3005 Nasal Swab D3	4.71E+08	9.99E+06
	3006 Nasal Swab D3	3.02E+08	6.00E+06
	3007 Nasal Swab D3	4.34E+08	1.02E+07
	3008 Nasal Swab D3	1.60E+08	2.62E+06
	3009 Nasal Swab D3	6.85E+08	1.50E+07
	3010 Nasal Swab D3	3.12E+08	6.98E+06
Group 4	4001 Nasal Swab D3	1.51E+08	1.51E+06
	4002 Nasal Swab D3	2.38E+08	2.80E+06
	4003 Nasal Swab D3	2.02E+08	2.99E+06
	4004 Nasal Swab D3	1.43E+08	2.46E+06
	4005 Nasal Swab D3	2.67E+08	4.81E+06
	4006 Nasal Swab D3	7.54E+07	9.57E+05
	4007 Nasal Swab D3	1.38E+08	2.12E+06

	4008 Nasal Swab D3	2.72E+08	4.25E+06
	4009 Nasal Swab D3	2.26E+08	4.14E+06
	4010 Nasal Swab D3	2.92E+08	3.43E+06
Group 5	5001 Nasal Swab D3	1.28E+08	2.16E+06
	5002 Nasal Swab D3	2.84E+08	4.70E+06
	5003 Nasal Swab D3	8.66E+07	1.29E+06
	5004 Nasal Swab D3	1.31E+08	1.79E+06
	5005 Nasal Swab D3	1.34E+08	2.06E+06
	5006 Nasal Swab D3	2.00E+08	3.11E+06
	5007 Nasal Swab D3	1.27E+08	1.58E+06
	5008 Nasal Swab D3	2.31E+08	3.70E+06
	5009 Nasal Swab D3	6.41E+07	7.86E+05
	5010 Nasal Swab D3	2.97E+08	4.81E+06
Group 6	6001 Nasal Swab D3	2.72E+08	3.54E+06
	6002 Nasal Swab D3	1.57E+08	2.13E+06
	6003 Nasal Swab D3	2.29E+08	3.90E+06
	6004 Nasal Swab D3	3.32E+08	4.26E+06
	6005 Nasal Swab D3	1.02E+08	1.28E+06
	6006 Nasal Swab D3	7.68E+07	1.05E+06
	6007 Nasal Swab D3	1.86E+08	2.66E+06
	6008 Nasal Swab D3	1.57E+08	2.16E+06
	6009 Nasal Swab D3	1.62E+08	2.70E+06
	6010 Nasal Swab D3	1.13E+08	1.51E+06
Group 1	1001 Nasal Swab D5	2.70E+07	4.21E+05
	1002 Nasal Swab D5	7.31E+07	1.49E+06
	1003 Nasal Swab D5	4.00E+07	5.39E+05
	1004 Nasal Swab D5	1.63E+08	2.73E+06
	1005 Nasal Swab D5	3.31E+07	5.47E+05
	1006 Nasal Swab D5	5.09E+07	6.51E+05
	1007 Nasal Swab D5	3.81E+07	6.61E+05
	1008 Nasal Swab D5	1.10E+07	1.32E+05
	1009 Nasal Swab D5	2.02E+08	3.30E+06
	1010 Nasal Swab D5	3.01E+07	3.09E+05
Group 2	2001 Nasal Swab D5	4.04E+07	5.84E+05
	2002 Nasal Swab D5	6.03E+07	7.52E+05
	2003 Nasal Swab D5	7.00E+07	1.30E+06
	2004 Nasal Swab D5	2.56E+07	3.53E+05
	2005 Nasal Swab D5	1.04E+08	2.23E+06
	2006 Nasal Swab D5	1.01E+07	1.43E+05

	2007 Nasal Swab D5	2.07E+07	2.97E+05
	2008 Nasal Swab D5	2.00E+07	2.57E+05
	2009 Nasal Swab D5	2.83E+07	4.23E+05
	2010 Nasal Swab D5	2.34E+08	3.82E+06
Group 3	3001 Nasal Swab D5	1.08E+07	1.40E+05
	3002 Nasal Swab D5	6.45E+07	1.27E+06
	3003 Nasal Swab D5	7.08E+07	1.31E+06
	3004 Nasal Swab D5	6.94E+07	1.13E+06
	3005 Nasal Swab D5	1.35E+08	2.68E+06
	3006 Nasal Swab D5	7.01E+07	9.60E+05
	3007 Nasal Swab D5	5.21E+07	7.93E+05
	3008 Nasal Swab D5	7.31E+07	7.10E+05
	3009 Nasal Swab D5	1.23E+08	1.66E+06
	3010 Nasal Swab D5	2.28E+07	2.69E+05
Group 4	4001 Nasal Swab D5	1.79E+07	2.31E+05
	4002 Nasal Swab D5	9.70E+07	2.14E+06
	4003 Nasal Swab D5	4.08E+07	8.13E+05
	4004 Nasal Swab D5	3.33E+07	4.98E+05
	4005 Nasal Swab D5	6.18E+07	7.99E+05
	4006 Nasal Swab D5	6.65E+07	7.64E+05
	4007 Nasal Swab D5	6.76E+07	8.55E+05
	4008 Nasal Swab D5	7.97E+06	1.18E+05
	4009 Nasal Swab D5	5.39E+07	7.14E+05
	4010 Nasal Swab D5	1.84E+07	2.63E+05
Group 5	5001 Nasal Swab D5	2.18E+07	3.09E+05
	5002 Nasal Swab D5	4.46E+07	6.75E+05
	5003 Nasal Swab D5	2.14E+07	2.47E+05
	5004 Nasal Swab D5	8.21E+07	1.26E+06
	5005 Nasal Swab D5	1.04E+08	2.18E+06
	5006 Nasal Swab D5	7.86E+07	1.29E+06
	5007 Nasal Swab D5	2.38E+07	2.98E+05
	5008 Nasal Swab D5	7.82E+06	1.14E+05
	5009 Nasal Swab D5	1.94E+07	2.56E+05
	5010 Nasal Swab D5	2.41E+07	2.07E+05
Group 6	6001 Nasal Swab D5	2.52E+08	3.80E+06
	6002 Nasal Swab D5	5.09E+07	6.27E+05
	6003 Nasal Swab D5	3.31E+07	5.00E+05
	6004 Nasal Swab D5	1.04E+08	1.99E+06
	6005 Nasal Swab D5	1.41E+08	2.05E+06

	6006 Nasal Swab D5	1.25E+07	1.47E+05
	6007 Nasal Swab D5	1.72E+07	2.19E+05
	6008 Nasal Swab D5	2.17E+07	2.72E+05
	6009 Nasal Swab D5	2.01E+08	2.86E+06
	6010 Nasal Swab D5	3.25E+07	4.56E+05
LLOQ		1.25E+03	

5 GENERAL SOPS

The following SOPs on file at Lovelace Biomedical were employed as part of the study sample analysis:

Table 8. SOPs Used During Sample Analysis

SOP TITLE	SOP No.
Operation and Maintenance of the Bio-Rad CFX Real-Time PCR Detection System	TXE-2040
Use and Maintenance of the epMotion	CHP-2136
Use and Maintenance of the KingFisher Flex	CHE-2137

6 COMPUTER APPLICATION PROGRAMS

The computer application program CFX Maestro was used to acquire sample data for this study and for data processing. Microsoft Office Excel was used for statistical calculations. When Excel was used the calculations were 100% QCed.

7 DISCUSSION

The data presented in this biosample analysis report represent data from assay runs that met performance standards and all samples were within the range(s) of the standard curves. Therefore, the data presented in this report are considered accurate and suitable.

8 REFERENCES

- Method MBM-1387: Multiplex Quantitative Real-Time PCR Analysis of SARS-CoV-2 Genomic and Subgenomic Content in Tissues, Feces and Fluids using Trizol Inactivation

9 APPENDICES

Appendix A. Method MBM-1387

LOVELACE BIOMEDICAL	
Title: Multiplex Quantitative Real-Time PCR Analysis of SARS-CoV-2 Genomic and Subgenomic Content in Tissues, Feces and Fluids using Trizol Inactivation	Method No.: MBM-1387-0
Prepared by: Isabelle Katz. Date: 29Apr2021	Page 1 of 13
Reviewed by: Bryan M. Gullick, Ph.D. Date: 29Apr2021	

1 PURPOSE

This method is for performing a reverse transcriptase real-time quantitative polymerase chain reaction (RT-qPCR) assay developed for the quantification of SARS-CoV-2 genomic and subgenomic content in RNA targeting the N gene and E gene of SARS-CoV-2 isolated from animal tissues, feces, and fluids.

Method Summary	
Assay Type	Reverse Transcriptase Real-Time Quantitative PCR
Reference Standards	SARS-CoV-2 N gene RNA SARS-CoV-2 E gene RNA
Species	Hamster, Mouse, NHP, AGM
Sample Treatment	RNA Isolation
Sample Volume (Tissue Homogenate)	400 µL
PCR Run Time	Approximately 1 hour, 20 minutes
Assay Range	5.0E+7 copies/µL RNA to 5.0E+0 copies/µL RNA
Unprocessed Tissue Sample Storage	< -60°C.
Special Conditions Required	N/A

2 EQUIPMENT (as listed or equivalent)

The following equipment, or its equivalent, is utilized during the execution of the method:

Equipment	Vendor/Model, PN, SN, or Cat No.
Class II Biosafety Cabinet (BSC)	Baker Company SG603A-HE or SG603A
Tissue Homogenizer	Tissue Lyser, Qiagen
Nanodrop 2000	Thermo Scientific
Magnetic Extracton System	KingFisher Flex, Thermo Scientific
PCR System	Stratagene MX3005P BioRad CFX384 Touch BioRad CFX 96
Liquid Handling Workstation	epMotion 5075t
Centrifuge	Beckman Coulter
Microcentrifuge	Eppendorf 5430 or 5453
PCR Plate Spinner	VWR 89184-608
Master Mix Workstation	CBS Scientific P-030-20
Nucleic Acid Workstation	CBS Scientific P-030-20
Vortex mixer	VWR Lab Dancer or VWR Analog Vortex Mixer or VWR Mini Vortexer MV1 or Fisher Scientific Mini Roto S56
Plate shaker	Labnet S2020-P4
Minifuge	VWR Galaxy Ministar C1413V-115
Refrigerator	Frigidaire FRU17B2JW13 or FRU17B2JW16
Freezer	Fisher Scientific 301N0001
Ultralow Freezer	Forma Scientific, Sanyo, Panasonic
Single and 12-Channel Pipettes	Various

Title: Multiplex Quantitative Real-Time PCR Analysis of SARS-CoV-2 Genomic and Subgenomic Content in Tissues, Feces and Fluids using Trizol Inactivation	Method No.: MBM-1387-0
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3 REAGENTS AND MATERIALS

The following reagents and materials, or the equivalent, are utilized during the execution of the method:

Reagent	Manufacturer	Cat. No.**
SARS-CoV E-gene RNA	LBRI	N/A
Isolated RNA From Tissues and Fluids	LBRI	N/A
Direct-Zol 96-RNA	Zymo Research	R2055
Direct-Zol 96 Mag Bead	Zymo Reasearch	R2101
Taqman® Fast Virus 1-Step Master Mix	Thermo Fisher	4444432
TaqPath® 1-Step Multiplex Master Mix (no ROX)	ThermoFisher	A28522
N2 Forward#: 5' TTACAAACATTGGCCGCAAA 3'	IDT DNA	N/A
N2 Reverse#: 5' GCGCGACATTCCGAAGAA 3'	IDT DNA	N/A
N2 Probe FAM#: 6FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ-1	IDT DNA	N/A
sgLead Forward#: 5'-CGATCTCTTGTAGATCTGTTCTC-3'	IDT DNA	N/A
E Sarbeco Reverse#: 5'- ATATTGCAGCAGTACGCACACA-3'	IDT DNA	N/A
E Sarbeco HEX Probe#: 6HEX-ACACTAGCCATCCTTACTGCGCTTCG-BHQ-1	IDT DNA	N/A
E Sarbeco Forward#: 5'-ACAGGTACGTTAATAGTTAATAGCGT-3'	IDT DNA	N/A
Nuclease-free Water	BioExpress*	G-3250-125
ELIMINase Decontaminant	Decon Labs Inc.*	1101 or 1102
100% Isopropanol	Sigma Aldrich	190764
100% Ethanol	Sigma Aldrich	SHBK1399
96-well Semi-skirted Reaction Plate or equivalent	USA Scientific*	1402-9700
MicroAmp Optical 8-Cap Strip	Applied Biosystems*	4323032
384-well Hard-Shell PCR Plates, thin wall, skirted, black/white	BioRad *	HSP3865
Microseal 'B' PCR Plate Sealing Film	BioRad *	MSB1001
Aerosol Resistant tips, various sizes	Rainin*	Various
MicrocentrifugeTubes, DNAse/RNAse Free (1.5 mL, 2.0 mL, 5.0 mL)	Eppendorf*	Various
Centrifuge Tubes, Sterile (50 mL)	Fisher Scientific*	06-443-18
Reagent Reservoirs, DNAse/RNAse Free	Phoenix Research*	RRV-2013

** Equivalent manufacturer is acceptable. **Provided Catalog (Cat.) Number may change with changes in quantities, volumes, concentrations, or manufacture/supplier.*
#Individual components may be mixed to create the assay

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4 INSTRUMENT CONDITIONS

4.1 RT-qPCR conditions for amplification of SARS-CoV-2 N-gene and E-gene on Stratagene Instrument:

Parameter	Reverse Transcription	Initial Denaturation	qPCR (40 cycles)	
	Hold	Hold	Denature	Anneal/Extend
Temperature	50°C	95°C	95°C	60°C
Time (mm:ss)	5:00	00:20	00:03	0:30

Total run time for all cycles is approximately 1h 20m

4.2 RT-qPCR conditions for amplification of SARS-CoV-2 N-gene and E-gene on BioRad CFX Instrument:

Parameter	Reverse Transcription	Initial Denaturation	qPCR (40 cycles)	
	Hold	Hold	Denature	Anneal/Extend
Temperature	50°C	95°C	95°C	60°C
Time (mm:ss)	5:00	00:20	00:03	0:30

Total run time for all cycles is approximately 1h 20m

5 Preparations

5.1. RNA Isolation Kit: Obtain a Direct-Zol 96-RNA Kit or Direct-Zol 96 MagBead Kit and prepare the wash buffer reagents according to manufacture instructions. Kits expire 1 year after receipt date. Record receipt date on kit box or packing slip. Store kit components at appropriate temperature based on manufactures recommendations.

5.2. SARS-CoV-2 N gene and E gene RNA: SARS-CoV-2 N gene and E gene RNA is diluted to a concentration of 1E9 copies/µL. Aliquot the diluted stock RNA at an appropriate volume and store at -80°C. Expiration not determined.

5.2.1. Perform serial dilution of SARS-CoV-2 N gene and E gene RNA to generate Standard Calibration Curve. Refer to section 8.2 for dilution calculations.

5.2.2. Prepare a discrete SARS-CoV-2 N gene and E gene RNA amplification control from standard 4. Refer to section 8.2 for dilution calculations.

5.3. SARS-CoV-2 N gene Assay Mix for standard curve, controls, and samples: Prepare N gene assay mix according to the following table

Material (100 µM)	Volume to Add (µL)	Final Concentration
N2-FWD	333	20 µM
N2-REV	333	20 µM
N2 FAM-PRB	83.3	5 µM
TE Buffer	4250.8	NA

5.4. SARS-CoV-2 E gene Assay Mix for standard curve and controls: Prepare E gene assay mix according to the following table

Material (100 µM)	Volume to Add (µL)	Final Concentration
E Sarbeco-FWD	333	20 µM
E Sarbeco-REV	333	20 µM
E Sarbeco HEX-PRB	83.3	5 µM
TE Buffer	4250.8	NA

5.5. SARS-CoV-2 E gene Assay Mix for study samples: Prepare E gene assay mix according to the following table

Material (100µM)	Volume to Add (µL)	Final Concentration
sgLead-FWD	333	20 µM
E Sarbeco-REV	333	20 µM
E Sarbeco HEX-PRB	83.3	5 µM
TE Buffer	4250.8	NA

6 Sample Preparation

6.1. Tissue and Feces Samples

6.1.1. Remove pre-weighed (≤75mg) tissue or feces from frozen storage. Samples should be maintained frozen until addition of TRI reagent (or equivalent). The samples do not need be maintained frozen once in TRI reagent. *NOTE: If tissues have been stored in RNA Later, samples may be allowed to thaw since the RNA Later will stabilize the nucleic acids in the sample. It is also important to blot each sample to remove any residual RNA Later and transfer to a new 2mL safe-lock tube.*

6.1.2. Add 1000 µL of TRI-Reagent to each sample with one 5mm steel bead and disrupt using the TissueLyser set at 25 Hz in two, 5-minute cycles, rotating racks between cycles.

6.1.3. Incubate samples at room temperature for 15 minutes to allow for viral inactivation.

6.2. Swab and BALF Supernatant Samples (or other liquid samples)

6.2.1. Remove sample from frozen storage. Add 1000 µL of TRI reagent to 300µL of sample. Volumes may be scaled as long as ratios are maintained. *The samples do not need be maintained frozen once in TRI reagent.*

6.2.2. Mix samples well by vortexing or shaking in a rack on plate shaker.

6.2.3. Incubate samples at room temperature for 15 minutes to allow for viral inactivation.

7 RNA ISOLATION PROCEDURES

Before beginning, ensure that Direct-Zol RNA Prewash, RNA Wash Buffer and DNAse I have been prepared per the manufacturer's directions. Perform the purification procedure at room temperature (18 to 25ºC), unless otherwise stated.

7.1. Direct-Zol 96 Procedure

7.1.1. Thoroughly clean all counter tops and non-sterile supplies with ELIMINase, or equivalent reagent, prior to use.

7.1.2. Centrifuge homogenates at 10,000-16,000 x g for 5 minutes.

7.1.3. Transfer 400µL of tissue homogenate or liquid sample to a clean collection plate. Store remaining sample at -80ºC.

7.1.4. Add an equal volume of ethanol to sample lysed in TRI Reagent (or equivalent) and mix well by pipetting up and down or using a plate shaker.

7.1.5. Transfer each mixture to a discrete well of Zymo-Spin I-96 plate mounted on a new collection plate and centrifuge at ≥2500 x g for 5 minutes at room temperature. Note: well capacity is 800µL. Spin and reload plate to process larger volumes.

7.1.6. Mount the Zymo-Spin I-96 Plate onto a new collection plate and discard the flow through.

7.1.7. Add 400µL RNA Wash Buffer to each well of the plate and centrifuge at ≥2500 x g for 5 minutes at room temperature.

7.1.8. Prepare DNAse I according to the following table. Prepare at least 10% excess.

Component	Volume per sample
DNAse I	5µL
DNA Digestion Buffer	35 µL
Total Volume	40µL

7.1.9. Add 40µL of prepared DNAse I and DNA Digestion Buffer Mix directly to the column matrix of each well.

7.1.10. Incubate at room temperature for 15 minutes.

7.1.11. Add 400µL Direct-zol RNA PreWash to each well and centrifuge at ≥2500 x g for 5 minutes at room temperature.

7.1.12. Discard flow through and wash a second time with 400µL Direct-zol RNA PreWash in each well.

7.1.13. Centrifuge at ≥2500 x g for 5 minutes at room temperature and discard flow through.

7.1.14. Add 800µL RNA Wash Buffer to each well and centrifuge at ≥2500 x g for 5 minutes at room temperature.

7.1.15. Discard flow through.

7.1.16. To ensure complete removal of wash buffer centrifuge the empty plate at ≥2500 x g for 5 minutes at room temperature.

7.1.17. Mount Zymo-Spin I-96 plate onto an Elution Plate.

7.1.18. Add 100µL of DNAse/RNAse free water to each well and centrifuge at ≥2500 x g for 5 minutes at room temperature to elute RNA.

7.1.19. Store the purified nucleic acid on ice for same day use, at -20ºC for up to 1 month, or at -80ºC for long-term storage.

7.2. Direct-Zol 96 Mag Bead Procedure

Before beginning, ensure that MagBead DNA/RNA Wash 1, MagBead DNA/RNA Wash 2 and DNAse I have been prepared per the manufacturer's directions. Perform the purification procedure at room temperature (18 to 25ºC), unless otherwise stated.

7.2.1. Thoroughly clean all counter tops and non-sterile supplies with ELIMINase, or equivalent reagent, prior to use.

7.2.2. Centrifuge homogenates at 10,000-16,000 x g for 5 minutes for tissue and feces and 1 min for liquid samples.

7.2.3 Transfer 400µL of tissue homogenate, feces homogenate, or liquid sample to a clean King Fisher Deep Well plate. Store remaining sample at -80ºC.

7.2.4 Add equal volume of ethanol (95-100%) to each sample.

7.2.5 Add 20µL MagBinding Beads to each sample. Seal plate and set aside until ready to load into the KingFisher Flex.

7.2.6 Add 500µL of DNA/RNA Wash 1 (previously diluted according to manufacturer's instructions) to each sample well in a KingFisher Deep Well Plate. Label appropriately and set aside until ready to load into the KingFisher Flex.

7.2.7 Add 500µL of DNA/RNA Wash 2 (previously diluted according to manufacturer's instructions) to each sample well in a KingFisher Deep Well Plate. Label appropriately and set aside until ready to load into the KingFisher Flex.

7.2.8 Prepare four ethanol plates. Add 500 µL100% Ethanol to each sample well in a KingFisher Deep Well Plate. Label appropriately (Ethanol 1-4) and set aside until ready to load into the KingFisher Flex.

7.2.9 Prepare DNase Treatment Plate. Add 50 µL of DNase I Reaction Mix (previously prepared according to manufacturer's instructions) to each sample well in a KingFisher Deep Well Plate. Label appropriately and set aside until ready to load into the KingFisher Flex.

7.2.10 Prepare elution plate. Add 100 µL DNase/RNase free water to each sample well in a KingFisher Elution Plate. Label appropriately, seal plate and set aside until ready to load into the KingFisher Flex.

7.2.11 Select program "R2101_Direct-zol RNA_KingFisherFlex_DNase" on King Fisher Flex.

7.2.12 Load the plates into the KingFisher Flex according to the prompts.

7.2.13 Press Start to begin the run.

7.2.14 When prompted, remove the DNase Treatment plate from the King Fisher Flex and add 500 µL RNA Prep Buffer to each sample well and return plate to King Fisher Flex. Press start to resume run.

7.1.20. When prompted, remove the sample plate from the King Fisher Flex. Remove seal from elution plate and place in the King Fisher Plate. Press Start to resume run.

7.1.21. When run is complete remove elution plate and seal immediately. Store the purified nucleic acid on ice for same day use, at -20ºC for up to 1 month, or at -80ºC for long-term storage.

7.2.15 Follow prompts to remove all plates from KingFisher Flex and discard.

8 RNA QUANTIFICATION PROCEDURES

NOTE: If downstream quality of extracted sample is in question, sample may be analyzed for concentration and quality using spectroscopy instruments (NanoDrop, SpectraMax i3x, Bio-Tek FLx800 or equivalent), or re-extracted. Below are the instructions for the NanoDrop.

8.1. Remove the extracts from storage and maintain cold on the benchtop.

8.2. Clean NanoDrop with ~2 µL of nuclease-free water. Remove with Kim Wipe. Set up NanoDrop 2000 for concentrations presented in ng/µL.

8.3. Blank the instrument with appropriate diluent. Remove blank before reading samples with a Kim Wipe.

8.4. Read ~2µL of the first sample. Label the sample as noted in the "NanoDrop Read Key" above. Save the workbook in the electronic study folder with at the least the following information: [study number – (tech initials and date)]

8.5. Remove the sample from the pedestal with a Kim Wipe after each reading. Continue reading samples (see "NanoDrop Read Key" for number of replicates per sample type) with removing of the previous sample after each reading.

8.6. Highlight all samples and review the report. Ensure the 260/280, 260/230, instrument information, software, and firmware information are included. Set graphs to "overlay".

8.7. Export the data file and save to the electronic study file and print the report.

8.8. Clean NanoDrop with ~2µL of nuclease-free water. Remove with Kim Wipe.

8.9. Return sample(s) to appropriate storage.

9 REAL-TIME QUANTITATIVE PCR PROCEDURES

9.1. Master Mix Preparation:

9.1.1. Clean the Master Mix PCR Workstation and any non-sterile supplies with ELIMINase, or equivalent reagent, prior to use.

9.1.2. Transfer ~3mL of DNAse/RNAse free water into DNAse/RNAse free 5 mL tube for use during preparation.

9.1.3. Remove N gene and two E gene assays from storage and allow to thaw. Vortex vigorously and centrifuge until liquid is collected at the bottom of the tube.

9.1.4. Remove Taqman® Fast Virus 1-Step Master Mix, TaqPath 1-Step Multiplex Master Mix or equivalent from storage and allow to thaw. Swirl or invert the bottle before use.

9.1.5. Use the "Master Mix Preparation" excel worksheet to calculate the amount of each master mix to prepare based on the number of samples to be analyzed.

9.1.6. Prepare required master mix in an appropriately sized PCR clean tube according to the Tables below.

Master Mix with N gene and Sarbeco E gene Assays for Standard Curve			
Reagent	**Stock Conc.**	**Vol. for Single Reaction[a]**	**Final Concentration per Reaction**
TaqMan Fast Virus 1-Step Master Mix TaqPath 1 Step Multiplex Master Mix or equivalent	4X	5 µL	1X
N gene Assay Mix:	premixed	1.5 µL	1X
Sarbeco E gene Assay Mix:	premixed	1.5 µL	1X
DNase/RNase Free Water	N/A	8.5 µL	N/A
Master Mix Total Volume per Well		15 µL	
RNA, Standard, or Control Sample Amount per Well		5 µL	
Reaction Total Volume per Well		20 µL	

[a]*Note: Preparation of additional material of master mix (e.g. 10%) is recommended to account for loss during manipulations.*

Master Mix with N gene and sgRNA Leader E gene Assay for Samples			
Reagent	**Stock Conc.**	**Vol. for Single Reaction[a]**	**Final Concentration per Reaction**
TaqMan Fast Virus 1-Step Master Mix TaqPath 1 Step Multiplex Master Mix or equivalent	4X	5 µL	1X
N gene Assay Mix:	premixed	1.5 µL	1X
sgRNA Leader Assay Mix:	premixed	1.5 µL	1X
DNase/RNase Free Water	N/A	8.5	N/A
Master Mix Total Volume per Well		15 µL	
RNA, Standard, or Control Sample Amount per Well		5 µL	
Reaction Total Volume per Well		20 µL	

[a]*Note: Preparation of additional material of master mix (e.g. 10%) is recommended to account for loss during manipulations.*

9.1.7. Vortex master mix solution to mix well. Transfer prepared master mix solution to PCR workstation or epMotion liquid handler workspace.

9.1.8. Aliquot the nuclease-free water diluent for the standard curve and the controls according to the following preparation table. Alternatively, this step may be performed by epMotion liquid handler.

9.2. Standard Calibration Curve and Quality Control Preparations:

9.2.1. Clean the PCR Workstation and non-sterile supplies with ELIMINase, or equivalent reagent, prior to use.

9.2.2. Remove the SARS-CoV-2 N gene and E gene RNA (1.0×10^9 copies/µL) from frozen storage and allow to thaw.

9.2.3. Aliquot the nuclease-free water diluent for the standard curve and the controls according to the "Standard Calibration Curve and Controls" table.

9.2.4. Serially dilute the SARS-CoV-2 N gene and E gene RNA (1.0×10^9 copies/µL) to create the standard calibration curve samples according to the "Standard Calibration Curve and Controls" table. Alternatively, this step may be performed by epMotion liquid handler.

9.2.5. Prepare assay controls according to the "Standard Calibration Curve and Controls" table.

9.2.6. All calibrators and controls must be prepared on the day of use.

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Standard Calibration Curve and Controls					
Standard Name	**RNA Concentration (copies/µL)**	**Diluent Volume (µL)**	**Source Volume (µL)**	**Source**	**Source Conc. (copies/µL)**
Standard 1	5×10^7	180	10 (per RNA standard)	SARS-CoV-2 N gene/E gene RNA (1.0×10^9copies/µL)	1.0×10^9
Standard 2	5×10^6	90	10	Standard 1	5×10^7
Standard 3	5×10^5	90	10	Standard 2	5×10^6
Standard 4	5×10^4	90	10	Standard 3	5×10^5
Standard 5	5×10^3	90	10	Standard 4	5×10^4
Standard 6	5×10^2	90	10	Standard 5	5×10^3
Standard 7	5×10^1	90	10	Standard 6	5×10^2
Standard 8	5×10^0	90	10	Standard 7	5×10^1
Positive AmplificationControl-SARS-CoV-2 RNA	1×10^4	80	20	Standard 4	5×10^4
No Template control	N/A	100	0	TE Buffer	N/A

9.3. PCR Plate Preparation

9.3.1. PCR plate preparation may be performed manually in a clean PCR workstation or using the epMotion liquid handler.

9.3.2. Add 15.0 µL of master mix into appropriate sample wells of a PCR plate; refer to plate map.

9.3.3. Add 5 µL of the RNA extracts to the designated wells for study samples; refer to plate map

9.3.4. Add 5 µL of the controls to the designated wells; refer to plate map.

9.3.5. Add 5 µL of TE Buffer to the designated "NTC" wells; refer to plate map.

9.3.6. Seal the PCR plate as appropriate to the plate type.

9.3.7. Spin the plate briefly using the plate spinner until bubbles at the bottom of the wells are absent. Ensure there are no smears on the caps or seal before placing the plate on the Stratagene MX3005P or BioRad CFX384 Touch instrument.

9.3.8. Analyze the extracts on the Stratagene MX3005P or BioRad CFX384 Touch instrument using the defined instrument settings.

10 SYSTEM SUITABILITY

Each assay plate will be analyzed independently. The quantification cycle (Cq) set for one plate may not necessarily apply to another without review of the amplification results. System suitability and therefore plate acceptance will be assessed by a series of samples on each plate: a standard calibration curve, a positive control (PC), and a no template control (NTC) containing TE Buffer. For an assay to be accepted the following system suitability criteria must be met:

10.1. Standard calibration curve must have a slope between -3.6 and -3.1, and a best fit line with an R^2 value $\geq$0.98. At least six concentrations including the highest concentration and the LLOQ must be used in analyzing the standard curve.

10.2. Positive control wells must amplify with a $\log_{10}$ transformed calculated value ± 15% of the target value.

10.3. No Template Control must not amplify or amplify with an average Cq value greater than the average Cq value of the LLOQ wells.

11 DATA ANALYSIS

Summary results from the PCR run will be exported to a suitable Excel data file for reporting.

A semi-logarithmic calibration curve is obtained by plotting the Cq values against the logarithm of RNA concentration. The mathematical equation and the parameters characterizing the semi-log curve fitting are as follows:

$$y = A + B\,[\log(x)]$$

Where:

y = response variable (Cq value)

x = concentration of analyte

A = y - intercept

B = slope

12 FORMS

The following form templates will be used during method validation and study sample analysis. These forms are subject to change to accommodate updates in the method.

- RT-qPCR Preparation Workbook

13 REVISION HISTORY

Current Version	Description of Change(s)	Supersedes
MBM-1387-0	Initial	None