B117 RT-PCR Assay

(Research Use Only)

Instructions for Use

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INTENDED USE

The B117 RT-PCR Assay (UK Variant included) is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in nasal swab from individuals suspected of COVID-19 by their healthcare provider (HCP). This test also is for qualitative detection of SRAS-CoV-2 wild type and UK variant virus RNA directly (without RNA extraction) from nasal swab collected in commercially available Viral Transport Media (VTM).

The B117 RT-PCR Assay is only intended for use by qualified laboratory personnel specifically instructed and trained in the techniques of real-time RT-PCR assays and in vitro procedures.

DEVICE DESCRIPTION AND TEST PRINCIPLE

The assay is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test. The SARS-CoV-2 primer and probe sets are designed to detect RNA from SARS-CoV-2 in respiratory specimens from patients as recommended for testing by public health authority guidelines.

The SARS-CoV-2 nucleic acid amplification assay is a real-time (Taqman probe) reverse transcription polymerase chain reaction assay for the amplification and detection of SARS-CoV-2 genomic RNA. Oligonucleotide primers hybridize to specific nucleotide sequences of the UK Variant SARS-CoV-2 (B.1.1.7) N (D3L mutant) gene and RdRp gene of SARS-CoV-2 (both wild type and B.1.1.7 variant). RNA is reverse transcribed and then amplified in the presence of thermostable DNA polymerase (Taq) enzyme and deoxy nucleotide triphosphates (dNTPs). A dual-labeled oligonucleotide probe that is complementary to an internal sequence of the amplification product is also present in the RT-PCR reaction mixture. The 5' exonucleolytic activity of Taq cleaves the fluorescent molecule (FAM) at the 5' end of the dual-labeled probe, thus releasing it from the effects of a fluorescence-quenching molecule (e.g. Black Hole Quencher 1) at the 3' end of the probe.

Fluorescence intensity for both SARS-CoV-2 amplification and internal control amplification is measured in individual wells during each of the 45 amplification cycles. A sample is

considered positive when the signal intensity exceeds a predetermined baseline threshold value. The cycle number at which this occurs is referred to as the cycle threshold C_T. Detection of SARS-CoV-2 RNA in a sample is determined by the CT value.

INSTRUMENTS USED WITH TEST

The B117 RT-PCR (UK Variant included) Assay is to be used with the following instruments: B117 RT-PCR is compatible with real time PCR instruments with FAM, HEX, RED/Cy-5 channels.

Description of Test Steps:

B117 RT-PCR protocol includes the heat inactivation (70^o C for 15 min) of nasal swab sample in VTM, which renders SARS-CoV-2 as non- infectious. Then detection of SARS-CoV-2 wild type/ UK variant RNA is carried out by directly through RT-PCR using B117 RT-PCR kit. The RNA extraction process is optional, and the processed samples can be directly used as template for RT-PCR reaction.

RNA Extraction (if used):

BGX recommends using the QIAampMinElute Virus Spin Kit (Qiagen) Extraction Kit to extract RNA from the samples. Other leading kits, such as the Roche MagNA Pure96 or in-house methods are acceptable for use with this kit, providing that it has been validated prior to use on patient samples.

Materials Required (Provided)

Table 1. **Reagents and Materials *B117 RT-PCR Assay***

Component	Part Number	Quantity Per Vial	Application
B117 Primer & Probe Mix	BPM100	300 µL	Includes targets for N gene (D3L), RdRp and RNaseP
Positive Control (non-infectious)	BPC100	1 x 10^4 copies/µL	Yields a positive result for N gene (D3L), RdRp, and RNaseP
Azura 1-step RT-qPCR master mix, CG	CRT100	1000 µL	-
Azura RTase Mix	CMM100	100 µL	-
Nuclease Free water	NFW100	100 µL	-

Equipment and Consumables Required (But Not Provided)

- 1.5 mL polypropylene microcentrifuge tubes (DNase/RNase free) and/or 0.2 mL polypropylene microcentrifuge tubes (DNase/RNase free)
- Racks for 1.5 mL/0.2 mL microcentrifuge tubes
- 70% ethanol (EtOH)
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNA*Zap*™ (ThermoFisher Scientific, catalog #AM9890), or equivalent
- RNase AWAY™ (Fisher Scientific, catalog #21-236-21), or equivalent
- Disposable powder-free gloves and surgical gowns
- Molecular-grade nuclease-free water (RNase/DNase Free) or 10mM Tris pH 7.4-8.2
- -70°C and -20°C freezer(s)
- 4°C refrigerator
- 2 x 96-well cold blocks (-20°C)
- Micropipettors (range between 1-10 µL, 10-200 µL and 100-1000 µL)
- Multichannel Micropipettors (1-10 µL, 5-50 µL)
- Aerosol barrier pipette tips
- MicroAmp™ Fast 8-tube strip 0.1 mL (ThermoFisher Scientific, catalog #4358293) or MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (alternate to 8-tube strips) (ThermoFisher Scientific, catalog #4346906, 4346907, or 4366932)
- MicroAmp™ Optical 8-cap strip (required, do not use film) (ThermoFisher Scientific, catalog #4323032)
- Strip Tubes and Caps 0.1 mL (4 tubes and caps) (QIAGEN, catalog #981103 or 981106)
- QIAcube HT plasticware (QIAGEN, catalog #950067)
- EMAG® Disposables (280135)
- Biohit Pipette Tips (280146)
- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS v1.4.1 Software (ThermoFisher Scientific, catalog #4406985 or #4406984)
- Extraction systems (instruments): QIAGEN EZ1 Advanced XL, QIAGEN QIAcube, QIAGEN QIAcube HT with QIAcube HT Software version 4.17.2 or 4.18, Roche MagNA Pure 96, Roche MagNA Pure Compact, bioMérieux NucliSENS® easyMAG®
- Benchtop microcentrifuge
- Vortex mixer

CONTROLS TO BE USED WITH THE B117 RT-PCR (UKVARIANT INCLUDED) ASSAY

1. A negative (no template) control is needed to eliminate the possibility of sample contamination on the assay run and is used on every assay plate. This control has no extracted nucleic acid added to the rRT-PCR reaction. This control reaction contains RNase-, DNase-free water, the N gene (D3L) and RdRp primers and probes for SARS-CoV-2, as well as the internal control (RNaseP) primers and probes.
2. A positive template control is needed to verify that the assay run is performing as intended and is included in each testing run. The positive control material is cloned plasmid DNA representing the N gene (D3L) and RdRp of SARS-CoV-2 (custom made).
3. Two SARS-CoV-2 positive amplification curve controls (low and high) are included on each amplification plate to ensure that SARS-CoV-2 RNA can be detected by the rRT-PCR test and demonstrate that the anticipated level of sensitivity has been achieved. This control material is *in vitro* transcribed RNA.
4. An internal control is needed to verify that nucleic acid is present in every sample and is used for every sample processed. A primer/probe set detecting human RNaseP is included to ensure an adequate human specimen is present in home-collected specimens.
5. A negative control is needed to monitor for any cross-contamination that occurs during the RT-PCR process.

Table 2: Interpretation of Results for Internal, No Template, Negative and Positive Control Reactions

[a]Sample results can only be interpreted if all control reactions generate valid results.

Control	Valid result[a]	Invalid result
No template control	If amplification signal detected $C_T < 34$	Amplification detected $C_T > 34$
RNase P control	$C_T < 34$	$C_T > 34$[b,c]
Negative control	No amplification signal detected	Amplification detected
Positive control (low)	C_T 28.61 – 29.98	$C_T < 28.61$ or > 29.98
Positive control (high)	C_T 17.34 – 19.54	$C_T < 17.34$ or > 19.54

[b]Specimens with SARS-CoV-2 target "Not detected" (i.e. $C_T > 34$), results are invalid when RNase P control $C_T > 36$.

[c]Specimens with SARS-CoV-2 target "Detected" (i.e. $C_T \leq 34$), RNase P control C_T values are not interpreted.

INTERPRETATION OF RESULTS

The test is run as a multiplex reaction with SARS-CoV-2 N (D3L), SARS-CoV-2 RdRp and RNaseP internal control assays combined in a single tube. All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted, and results cannot be reported.

B117 RT-PCR (UK Variant included) Assay Controls – Positive, Negative, and Internal:

- Negative (no template control) – the no template control should be negative for all targets detected (C_T Not Detected)
- Positive controls – Each lot of working concentration positive control is analyzed to generate lot specific C_T acceptance ranges. A C_T value within established ranges ensures that the reproducibility and repeatability of the test is consistent between days, equipment and analysts.
- Internal control – The expected C_T value for the RNaseP control is <36. In samples with no SARS-CoV-2 target detected, a C_T value less than or equal to these values for RNaseP RNA demonstrates that effective nucleic acid extraction and rRT- PCR amplification has been achieved.
- Negative control– this control should be negative for the SARS-CoV-2 Assay but positive for the RNaseP internal control
- If any control does not perform as described above, the run is considered invalid and all specimens are repeated from extraction.

Examination and Interpretation of Specimen Results:

Assessment of specimen test results should be performed after the controls have been examined and determined to be valid and acceptable. When all control values are valid as stated above, results of individually tested specimens are reported as shown in Table 3 below;

Table 3: Interpretation of Patient Results from Individually Tested Specimens

Real-time RT-PCR result	Reported result
C_T > 34	SARS-CoV-2 RNA "Not Detected"
$C_T \leq 34$	SARS-CoV-2 RNA "Detected"

ANALYTICAL PROTOCOL

1. In the reagent set-up room, clean the biosafety cabinet, place RT mix, Master Mix and B117 Primer & Probe Mix on ice or cold-block.
2. VTM Tube (with nasal swabs) are subjected to heat treatment in water bath at 70º C for 10 minutes and then quickly placed at 4º C until the moment of amplification.
3. 50 µL aliquots from the VTM tube, reagents and B117 Primer&Probe Mix are centrifuged for 5-10 seconds to collect contents at the bottom of the tube, and then the tubes are placed in a cold rack.
4. Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the negative & positive controls and specimens for pipetting error.
5. Prepare the reaction mix by combining the Master Mix, RT Mix and B117 Primer&Probe Mix as described below:

Component	Volume
Master Mix	10.0 µL
RT Mix	1.0 µL
B117 Primer & Probe Mix	3.0 µL

6. Add 6.0 µL of the processed VTM and controls to the appropriate wells/tubes.
7. Centrifuge for 5-10 seconds and set up reaction tubes or plates in a cooler rack. The samples are now ready for thermal cycling.

Selection of Fluorescence channel

Gene	Dye	Color
UKV Gene	FAM	Green
RDRP Gene	HEX	Yellow
RNase P	Cy5	Red

Thermal Cycling

Step	Cycles	Temperature	Time
cDNA Synthesis	1	50 °C	10 min
Initial Denaturation	1	95 °C	2 min
PCR cycling	45	95 °C	15 Sec
		60 °C	30 Sec

ASSAY PERFORMANCE

Analytical Sensitivity:

Limit of Detection (LoD):

The analytical sensitivity of B117 RT-PCR test was determined in Limit of Detection (LoD) studies. Since no quantified viral isolates of SARS-CoV-2 were available for testing, Custom made (Genescript) plasmid of the SARS-CoV-2 N gene which includes 28280 GAT 28282 to 28280 CTA 28282 mutation were used for spiking into samples negative for SARS-CoV-2. These samples are nasal swab matrices. To determine the preliminary LoD, range finding experiments were performed on three spiked amplification replicates using 2-fold dilutions of IVT in nasal swab matrices. Results are shown in Table 7 below:

Table 7: Summary of Limit of Detection Range Finding Results Using SARS-CoV-2 N Gene plasmid (28280 GAT to CTA mutation)

Nasal swabs		
RNA concentration (copies/mL)	**No. pos./ No. tested**	**C_T mean**
1000	3/3	30.30
100	3/3	32.76
50	3/3	34.55
25	3/3	35.47
13	3/3	35.46
7	0/3	N.A.

N.A.-Not applicable

The provisional LoD was 50 copies/mL. To confirm the final LoD, 10 extracted RNA sample from nasal swab specimen/10 direct nasal swab specimen VTM samples matrix at the provisional LoD values identified in the range finding experiment were tested. These results demonstrated detection rates of $\geq$95% at 50 copies/mL for each of the three sample matrices tested. The LoD for each of the three matrices, therefore, is 50 copies/mL.

Direct RT-PCR

The sensitivity of direct RT-PCR of B117 RT-PCR test was determined through the comparison test of 10 positive specimens RNA (extracted with Qiagen Nucleic acid Extraction kit) and 10 heat inactivated direct VTM sample (same specimen). These results demonstrated detection rates of $\geq$95% for each of the ten sample matrices tested.

Table 8: Summary of Finding Results Using SARS-CoV-2 specimen (Direct VTM with

respect to RNA extraction

	#positive replicates	Mean Ct of SARS-CoV-2
RNA extraction (Qiagen Kit) – 50 copies	10/10	22.18
Direct VTM- 50 copies	10/10	23.82

Cross-Reactivity (Analytical Specificity):

The cross-reactivity of the assay has been evaluated previously and, therefore, additional wet lab evaluation was not necessary. The sequences for the N (D3L) gene and RdRp gene primers/probes used in B117 RT-PCR assay are identical to the primer/probe sequences used in the FDA emergency use authorized CDC 2019-Novel Coronavirus (2019-nCoV) Diagnostic Panel. CDC has provided a right of reference to their Cross-Reactivity Study data, which is available at https://www.fda.gov/media/134922/download.

To further assess for potential cross-reactivity of the B117 RT-PCR assay, an *in silico* analysis of the SARS-CoV-2 N (D3L) gene and RdRp sequences was performed using BLASTN 2.10.1 against partial or complete genomes of other common respiratory viral and bacterial pathogens listed in the below table. None of the pathogen sequences displayed greater than 20% homology with any of the SARS-CoV-2 N (D3L) and RdRp primers/probes.

BLAST analysis showed no homology with N (D3L) and RdRp amplicon regions of the B117 RT-PCR test for the organisms listed in the table below.

***In silico* analysis for cross reactivity of targeted pathogen**			
Pathogen	**Gene Accession**	**% identity to SARS-Cov-2 N (D3L mutation) Gene**	**% identity to SARS-Cov-2 RdRp Gene**
Human coronavirus 229E	NC_002645.1	Not reactive	Not reactive
Human coronavirus OC43	NC_006213.1	Not reactive	Not reactive
Human coronavirus HKU1	NC_006577.2	Not reactive	Not reactive
Human coronavirus NL63	NC_005831.2	Not reactive	Not reactive
MERS-coronavirus	NC_019843.3	Not reactive	Not reactive
Adenovirus	MF502426.1	Not reactive	Not reactive
Human Metapneumovirus (hMPV)	NC_039199.1	Not reactive	Not reactive
Parainfluenza virus 1	NC_003461.1	Not reactive	Not reactive
Influenza A	NC_007373.1	Not reactive	Not reactive
Enterovirus C	NC_002058.3	Not reactive	Not reactive

Respiratory syncytial virus	NC_001803.1	Not reactive	Not reactive
Rhinovirus C	NC_009996.1	Not reactive	Not reactive
Chlamydia pneumoniae	HV214386.1	Not reactive	Not reactive
Haemophilus influenzae	NZ_CP009610.1	Not reactive	Not reactive
Legionella pneumophila	NZ_CP013742.1	Not reactive	Not reactive
Mycobacterium tuberculosis	NC_000962.3	Not reactive	Not reactive
Streptococcus pneumoniae	NC_003098.1	Not reactive	Not reactive
Streptococcus pyogenes	NZ_CP010450.1	Not reactive	Not reactive
Bordetella pertussis	NC_018518.1	Not reactive	Not reactive
Mycoplasma pneumoniae	NZ_CP010546.1	Not reactive	Not reactive
Pneumocystis jirovecii (PJP)	NW_017264775.1	Not reactive	Not reactive
Candida albicans	GCA_003454735.1	Not reactive	Not reactive
Pseudomonas aeruginosa	NC_002516.2	Not reactive	Not reactive
Staphylococcus epidermis	MT125873.1	Not reactive	Not reactive
Streptococcus salivarius	NZ_CP009913.1	Not reactive	Not reactive

1) ***Endogenous Interference Substances Studies***

Nasal swab samples that were collected from healthy donors and confirmed negative for SARS-CoV-2 were used in the interference substances studies. Four nasal swab samples, each in 3 mL of VTM, were pooled to obtain a total of 16 mL each of negative swab sample. For each interfering substance listed in the Table below, 1 mL of negative swab sample was aliquoted into microcentrifuge tubes. 1 mL of sample in each tube was spiked with 50% of the relevant interfering substance. From each SARS-CoV-2 negative swab samples containing 50% interfering substance, 200 µL dilutions were prepared with final interfering substance concentrations of 10%, 1%, and 0.1%, as shown in Table. All dilutions were spiked with quantified SARS-CoV-2 virus to a final concentration of 1000 copies/µL and then real-time RT-PCR was performed with B117 RT-PCR kit.

According to the results of the interfering substance inhibition tests, Chlorhexidine at 10% (v/v) and all other substances at 50% concentration may interfere with the B117 RT-PCR.

Interfering Substances	Concentration		N (D3L)		RdRp		RNase P		Results
			Avg. Ct (40)	SD	Avg. Ct (40)	SD	Avg. Ct (40)	SD	
No interfering substances	0		34.72	0.09	37.15	0.02	34.85	0.05	Positive(+)
OTRIVIN, Adult Nasal Spray	v/v%	50	Not detected						Negative(-)
		10	34.64	1.08	38.73	0.95	36.4	0.52	Positive(+)
		1	33.04	0.22	36.95	0.42	33.4	0.78	Positive(+)
		0.1	33.12	0.16	36.99	0.38	33.02	0.15	Positive(+)
Mucin (Sigma)	w/v%	50	Not detected						Negative(-)
		10	35.36	0.88	37.43	0.82	36.54	0.54	Positive(+)
		1	34.09	0.32	36.45	0.47	34.24	0.68	Positive(+)
		0.1	33.14	0.17	35.97	0.33	33.11	0.16	Positive(+)
Strepsils (throat lozenges)	w/v%	50	Not detected						Negative(-)
		10	35.81	0.15	39.5	0.68	33.77	0.25	Positive(+)
		1	35.38	0.88	39.2	0.59	33.31	0.38	Positive(+)
		0.1	34.12	0.23	38.54	0.52	33.12	0.19	Positive(+)
Amoxicillin	w/v%	50	Not detected						Negative(-)
		10	35.64	0.35	38.97	0.72	33.37	0.45	Positive(+)
		1	33.21	0.22	37.97	0.68	33	0.29	Positive(+)
		0.1	33.15	0.18	37.54	0.43	32.62	0.36	Positive(+)
Pencillin G	w/v%	50	Not detected						Negative(-)
		10	35.51	0.29	38.51	0.52	33.1	0.35	Positive(+)
		1	34.5	0.45	37.82	0.78	32.46	0.42	Positive(+)
		0.1	34.12	0.19	36.42	0.38	32.31	0.41	Positive(+)
Lactic Acid Bacillus	w/v%	50	Not detected						Negative(-)
		10	35.86	0.69	38.42	0.12	34	0.39	Positive(+)
		1	35.7	0.25	38.36	0.35	31.99	0.49	Positive(+)
		0.1	35.21	0.25	38.15	0.19	31.58	0.25	Positive(+)
Cefadroxil	w/v%	50	Not detected						Negative(-)
		10	35	0.59	39.76	0.42	34.12	0.52	Positive(+)
		1	34	0.47	38.17	0.85	32.75	0.19	Positive(+)
		0.1	34.09	0.13	38.02	0.05	31.42	0.15	Positive(+)
Azithromycin	w/v%	50	Not detected						Negative(-)
		10	35.3	0.28	38.73	0.65	33.72	0.25	Positive(+)
		1	34.93	0.25	36.77	0.21	33.27	0.39	Positive(+)
		0.1	34.12	0.21	36.18	0.19	33.08	0.26	Positive(+)

Chlorhexidine (Mouth Wash)	v/v%	50	Not detected						Negative(-)
		10	42	0.8	42	0.19	43.68	0.19	Negative(-)
		1	38.08	1.02	40.54	0.55	38.82	0.59	Positive(+)
		0.1	37.89	0.13	39.21	0.35	38.26	0.29	Positive(+)
Colgate toothpaste	w/v%	50	Not detected						Negative(-)
		10	38.81	0.78	38.25	0.17	38.55	0.27	Positive(+)
		1	36.05	0.12	35.49	0.25	36.12	0.15	Positive(+)
		0.1	35.98	0.35	35.29	0.22	36.05	0.08	Positive(+)

ASSAY STUDY

Assay evaluation of the BT117 RT-PCR assay was performed by spiking confirmed (whole genome sequencing) positive sample given by Center for DNA Fingerprinting & Diagnostics (CDFD, Hyderabad) into known negative samples at concentrations ranging from 2x LoD through the range of the assay. The negative samples consisted nasal wash swabs. For nasal wash, 100% agreement was achieved for 30 samples spiked at 5 concentrations, ranging from 2x LoD to 2,000 x LoD. All 5 negative specimens were negative for SARS-CoV-2 and each had an internal control CT value <35. For nasopharyngeal swabs, a signal was detected for all samples at each spiking concentration. An agreement of 100% was achieved for all 30 spiked nasal swabs samples at different concentrations. For nasal swabs all 5 negative specimens were negative for SARS-CoV-2 and each had an internal control CT value <35.

Table 11: Assay performance of the B117 RT-PCR Assay in nasal wash

Fold of LoD	RNA concentration (copies/mL)	No. pos./ No. tested	Agreement	SARS-CoV- 2 Mean C_T		
				N (D3L) gene	RdRp gene	RNaseP gene
Negative	N.A.	5/5	100%	N.D	N.D.	32.54
1x	50	5/5	100%	34.99	34.99	34.99
2x	100	5/5	100%	32.95	32.95	32.95
10x	500	5/5	100%	29.25	29.25	29.25
20x	100	5/5	100%	26.04	26.04	26.04
200x	10,000	5/5	100%	22.56	22.56	22.56
2000X	1,00,000	5/5	100%	19.34	19.34	19.34

[1]Not applicable [2]Not detected

Adding population screening of individuals without symptoms or other reasons to suspect COVID-19 to an authorized test

Data was provided to support testing of a screening population consisting of individuals with mild symptoms or other reasons to suspect COVID-19. Testing using the B117 RT-PCR Assay was performed directly heat –inactivated nasal swab sample VTM, and RT-PCR amplification by the Biorad CFX 96 Real-Time PCR system, Qiagen RotorGene Q.

Samples were collected from individuals exhibiting or not exhibiting any signs characteristic of SARS-CoV-2 infection (i.e. individuals being screened) or other reasons to suspect COVID-19. The samples tested were nasal swab specimens collected during community screening events with initial testing by BioGenex Laboratories. A total of 35 samples were tested with the B117 RT-PCR Assay. Of these 15 consecutively collected positive specimens and 20 consecutively collected negative specimens were also tested with the comparator assay. All samples were stored at -20˚C (or colder) after collection, and all samples were fully de-identified prior to re-testing with the comparator assay. A summary of results is shown in the table below.

Table 12: Results of performance evaluation individuals with mild symptoms or other reasons to suspect COVID-19- Biorad CFX 96plex RT-PCR

		RUO Authorized Assay		
		Positive	**Negative**	**Total**
BT117 RT-PCR	Positive	15	0	15
	Negative	0	20	20
	Total	15	20	35

Table 13: Results of performance evaluation individuals with mild symptoms or other reasons to suspect COVID-19- Qiagen RotorGeneQ

		RUO Authorized Assay		
		Positive	**Negative**	**Total**
BT117 RT-PCR	Positive	15	0	15
	Negative	0	20	20
	Total	15	20	35

Limitations:

The performance has not been established in all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the performance evaluation. Performance at the time of testing may vary depending on the variants circulating, including newly emerging strains of SARS- CoV-2 and their prevalence, which change over time.

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