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A novel RT-qPCR assay for detection of SARS-CoV-2 variants based on RhAmp technology (IDT technologies)

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ABSTRACT

Brazil was one of the most affected countries during the SARS-CoV-2 pandemic, with more than 10 million cases and more than 300.000 deaths reported. Together with the increasing rate of cases, the emergence of new variants of concern (VOCs) possessing mutations that affect immunogenicity and transmissibility poses a concerning burden in the healthcare system. In Brazil, three such lineages are currently circulating: P.1, a lineage firstly described in Manaus, P.2, firstly described in Rio de Janeiro, both already reported in other cities in the country, and B.1.1.7, a lineage initially identified in England and recently associated to increasing mortality rates.In this scenario, it urges the necessity of a rapid assessment methodology for identification of variants that could contribute to a better epidemiological surveillance and public healthcare administration decisions in dealing with the pandemic. Here, we describe a PCR genotyping approach using RhAmp technology (IDT Technologies) to correctly assess the circulating emergent variants P.1, P.2 and B.1.1.7 in Brazil. Genotyping is a quicker methodology requiring only a real time PCR machine available in most of laboratories preforming COVID-19 molecular diagnosis.

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KEYWORDS

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MATERIALS TEXT

This protocol has been validated using:

- *Quick*-RNA Viral Kit (Zymo Research);
- High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific);
- rhAmp® Reporter Mix with Reference and rhAmp® Genotyping Master Mix (IDT);
- rhAmp® SNP assays (20X) (Single Nucleotide Polymorphism assays/primers) (IDT).

Despite these specification, any comercial RNA extraction and reverse transcription kit should work well. All reactions have been conducted on a 7500 RT-qPCR machine (Applied Biosystems).

Here are the sequences of primers and probes used. The assay was performed using rhAmp® Genotyping Master Mix and rhAmp® Reporter Mix with Reference.

A	B	C
Target	**Sequence**	**Application**
K417T	Forward 5' - /rhAmp-F/TCCAGGGCAAACTGGAAArGATTG/GT3/ (FAM)	rhAmp
K417T	Forward 5' - /rhAmp-Y/CCAGGGCAAACTGGAACrGATTG/GT3/ (HEX/VIC)	rhAmp
K417T	Reverse 5' - GCAGCTATAACGCAGCCTGTAAAATrCATCT/GT2/	rhAmp
E484K	Forward 5' - /rhAmp-F/TGTAAAGGAAAGTAACAATTAAAACCTTCrAACAC/GT3/ (FAM)	rhAmp
E484K	Forward 5' - /rhAmp-Y/TGTAAAGGAAAGTAACAATTAAAACCTTTrAACAC/GT3/ (HEX/VIC)	rhAmp
E484K	Reverse 5' - GCCCTGTATAGATTGTTTAGGAAGTCTAATrCTC AA/GT4/	rhAmp
N501Y	Forward 5' - /rhAmp-F/TCATATGGTTTCCAACCCACTArATGGT/GT2/ (FAM)	rhAmp
N501Y	Forward 5' - /rhAmp-Y/CATATGGTTTCCAACCCACTTrATGGT/GT2/ (HEX/VIC)	rhAmp
N501Y	Reverse 5' - GCGGTGCATGTAGAAGTTCAAAAGAArAGTAC/GT1/	rhAmp

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1 Extract viral RNA using any commercially available kit according to manufacturer's instructions. We used *Quick*-RNA Viral Kit (Zymo Research). Prepare cDNA using the maximum amount of RNA from the previous step allowed by your retrotranscriptase kit. We used High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

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2 Thaw rhAmp® Reporter Mix with Reference and rhAmp® Genotyping Master Mix in ice and rhAmp® SNP assays (20X) (Single Nucleotide Polymorphism assays/primers) at room temperature. Primer sequences are available in *Materials* section. All reagents are stored at -20 °C. rhAmp® SNP assays are shipped as a resuspended solution but can be purchase in 20X or 80X formats. Working concentration is 20X, dilute 80X assays 4-fold in IDTE, pH 7.5 or RNase/DNase-free water. Briefly vortex and then spin down reagents before use.

3 Dilute rhAmp® Reporter Mix with Reference 1:20 in rhAmp® Genotyping Master Mix to make the Combined Master Mix. As an example, pippet 50 µl of rhAmp® Reporter Mix with Reference in 1 ml of rhAmp® Genotyping Master Mix. We recommend scale the Combined Master Mix for the number of reactions that will be performed, considering 1 negative control (no cDNA) and 2 positive controls (1 for each allele of each target SNPs).

4 Prepare the genotyping reaction mix as follows:

A	B	C
Reagent	**1 reaction (µl)**	**100 reactions (µl)**
Combined Master Mix	5.3	530
SNP assay 20X (for K417T or N501Y assays)	0.5	50
Sample cDNA	4.2	-
Total volume	10	1000

5 Some modifications were performed specifically for the E484K SNP assay. Prepare the Genotyping Reaction Mix for E484K assay as follows:

A	B	C
Reagent	**1 reaction (µl)**	**100 reactions (µl)**
Combined Master Mix	5.3	530
SNP assay 20X (for E484K assay)	0.75	75
Sample cDNA	3.95	-
Total volume	10	1000

6 Add 5.8 µl of Genotyping Reaction Mix to each well and then add 4.2 µl of cDNA for K417T or N501Y assays, or 3.95 µl for E484K assay. After applying the cDNA, pipette the total volume up and down 10 times to mix. Each reaction is performed in final 10 µl volume. Seal the plate and briefly centrifuge to collect the liquid at the bottom of the plate.

7 Set your Real-Time PCR System for the “genotyping” module and checkbox the software to perform pre-amplification, amplification and post-amplification reads. Although only the post-amplification read is compulsory to perform in a Real-Time PCR System, we strongly recommend that the amplification also be performed in such systems (with data collection at each cycle) to validate the exponential amplification of the sample through multicomponent curves (exponential absolute fluorescence curves).

8 Set the fluorophore for the wild-type (WT) or mutant (Mut) alleles for each SNP assays in the software as follows:

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A	B	C
Mutation	**FAM (WT) allele**	**HEX/VIC (Mut) allele**
K417T	A	C
N501Y	A	T
E484K	G	A

9 We recommend running only 1 SNP assay per plate for better visualization of the clustering results. The cycling can be performed with 55 cycles; however, 80 cycles are recommended for better visualization of results. Set the cycling parameters for K417T or N501Y as follows:

A	B	C	D
Step	**Repetition**	**Temperature**	**Time**
Enzyme activation	1	95 °C	10 min
Denaturation	55-80	95 °C	10 sec
Annealing	55-80	60 °C	30 sec
*Extension	55-80	68 °C	20 sec

*Set to collect amplification step data here.

10 For E484K SNP assay, annealing temperature and time were modified to improve the results. Set E484K SNP cycling parameters as follows:

A	B	C	D
Step	**Repetition**	**Temperature**	**Time**
Enzyme activation	1	95 °C	10 min
Denaturation	55-80	95 °C	10 sec
Annealing	55-80	57 °C	1 min
*Extension	55-80	68 °C	20 sec

*Set to collect amplification step data here.

11 After the run is completed, the results should be displayed in a cartesian dot plot graph showing the samples clustering patterns in which the wild-type allele should be in one axis and the mutant allele in the other one. For interpretation of the genotypes, use the following chart:

A	B	C	D
Genotype	**K417T**	**N501Y**	**E484K**
P.1	Mut (C)	Mut (T)	Mut (A)
P.2	WT (A)	WT (A)	Mut (A)
B.1.1.7	WT (A)	Mut (T)	WT (G)
B.1.351	WT (A)	Mut (T)	Mut (A)
Others	WT (A)	WT (A)	WT (G)
Others	Mut (C)	WT (A)	Mut (A)
Others	Mut (C)	WT (A)	WT (G)

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