genetic PCR solutions™

30 years' experience, packed in microtubes

CoVID-19 N/S
dtec-RT-qPCR Duplex

F100 format

100 tests

Genetic detection of

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

CE

IVD

Sanitary License Number for the Operation of Sanitary Product Installations; 7722-PS

Edition E04 (06/2022)

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DESCRIPTION

The **CoVID-19 N/S dtec-RT-qPCR Duplex** comprises a series of specific reagents, for human clinical diagnosis, designed for **Severe acute respiratory syndrome coronavirus 2** detection by using qPCR. SARS-CoV-2 are enveloped viruses with a positive-sense, single-stranded RNA genome belonging to the Coronaviridae family and the *Betacoronavirus* genus. SARS-CoV-2 causes the CoVID-19 (coronavirus disease 2019), the respiratory illness responsible for the CoVID-19 pandemic, declared on 11 March 2020 by *World Health Organization* (WHO). The virus primarily spreads between people through close contact and via respiratory droplets produced from coughs or sneezes. The assay contains two targets, one for the S gene and another for the N gene, as recommended by the *Centers for Disease Control and Prevention* (CDC). The product is under an intense *Post Market Surveillance Plan* (PMSP), meanly by periodic reviews of sequence data newly described and through test-validation using reference materials.

Both, standard synthetic DNA and RNA have been used to obtain the calibration curve.

PRINCIPLE OF THE METHOD

Polymerase chain reaction (PCR) allows the amplification of a target region from a DNA template by using specific oligonucleotides. In real-time reverse transcription PCR (RT-qPCR), the RNA is first transcribed to complementary DNA by a reverse transcriptase. The accumulating amplified product can be detected at each cycle with fluorescent dyes. This increasing signal allows to achieve sensitive detection and quantification of pathogens.

KIT CONTENT

TargetSpecies dtec-RT-qPCR-mix (AMBER TUBE), contains a mixture of specific forward/reverse primers and probe, to detect **N gene** and **S gene** of **SARS-CoV-2**. 100 reactions

Resuspension buffer (WHITE CAP), 130 µl

DNase/RNase free water (GREEN CAP), 1.5 ml

Internal Control qPCR-mix (AMBER TUBE), contains a mixture of primers, probe and the DNA template to achieve a suitable internal control of PCR. 100 reactions

GPS™-mix-RT (BLUE CAP), it is a 4X mastermix containing a polymerase, retrotranscriptase, dNTPs and buffer. 500 µl, 100 reactions

Positive Control (ORANGE CAP), dehydrated target copies for positive control.

Standard Template (RED CAP), dehydrated target copies only for quantification.

Template buffer (BLACK CAP), exclusive for resuspension of the Positive Control or Standard Template, 150 µl

STORAGE CONDITIONS

All the components of **CoVID-19 N/S dtec-RT-qPCR Duplex** are stable at room temperature for transport. At arrival, if not immediately used, it should be stored at -20 ^{o}C. The product is stable for one year (see expiration date on the label). Once opened and dissolved, to avoid repeated freezing-thawing cycles which may reduce assay sensitivity, we recommend splitting the content in several aliquots and store at -20 ^{o}C.

GPS™-mix-RT (BLUE CAP) is stable at room temperature for transport but should be stored at -20 ^{o}C at arrival.

For **Positive Control** (ORANGE CAP) or **Standard Template** (RED CAP), once dissolved, we recommend storing **in an exclusive box** at -20 ^{o}C.

MATERIALS REQUIRED BUT NOT PROVIDED

- RNA isolation kit, appropriate nucleic acid extraction method needs to be conducted to obtain pure RNA free of PCR inhibitors
- DNase/RNase free water (to prepare standard curve dilution)
- Micropipettes and sterile pipette tips with filters
- qPCR tubes, strips or plates
- Vortex mixer
- Spinner centrifuge
- Cooling block
- Real-time PCR device which must read on the FAM channel (S target), Cy5 channel (N target)and VIC/HEX/JOE channel (Internal Control)

WARNINGS AND PRECAUTIONS

- ⓘ For *in vitro* diagnostics (IVD).
- ⓘ To avoid possible misuse, carefully read the handbook.
- ⓘ Proper training is recommended for correct operation of the kit.
- ⓘ According to good laboratory practices, always wear a suitable lab coat, disposable gloves, and protective goggles.
- ⓘ All the instruments used must been verified and calibrated according to the manufacturer's recommendations.
- ⓘ Samples are potentially infectious materials, take appropriate measures for containment and follow the corresponding biosafety precautions.
- ⓘ Validation of the assay was performed with nasopharyngeal swabs and respiratory samples. Note that any kind of sample containing SARS-CoV-2 can be analysed with this assay after appropriate RNA extraction.

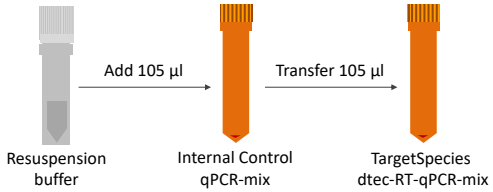
General precautions

- ⚠ To prevent contamination of micropipettes, use sterile tips with filters.
- ⚠ Micropipettes used to dilute the templates should not be used for other PCR reagents.
- ⚠ Extract, store and prepare positive materials (samples, positive controls and PCR products) in a separately laboratory environment.
- ⚠ To decrease the risk of contamination, we recommend that all pipetting be performed in a PCR clean environment. Ideally, this would be a designated PCR lab or PCR cabinet.
- ⚠ To avoid cross-contamination with the positive control, pipette it after closing reaction tubes with negative control and samples.
- ⚠ The workflow in the laboratory should be unidirectional, from clean preamplification area to the amplification area.
- ⚠ Keep components refrigerated in a cooling block.
- ⚠ Protect the primer/probe from prolonged exposure to light.
- ⚠ If any damage is observed in the packaging, contact with technical support for instructions (see CONTACT INFORMATION).

RESUSPENSION PROTOCOLS

Resuspension of TargetSpecies dtec-RT-qPCR-mix with Internal Control

For reconstitution of **Internal Control qPCR-mix** (AMBER TUBE) and **TargetSpecies dtec-RT-qPCR-mix** (AMBER TUBE) and before opening, pulse-spin the tubes in a centrifuge to ensure that lyophilized product goes down and will not be spilt. Add 105 µl of **Resuspension buffer** (WHITE CAP) to the **Internal Control qPCR-mix** (AMBER TUBE), vortex the tube thoroughly (or pipette the solution up and down on the sides of the tube), and harvest by pulse-spin in the centrifuge. Transfer the volume to the **TargetSpecies dtec-RT-qPCR-mix** (AMBER TUBE), vortex the tube thoroughly and harvest by pulse-spin in the centrifuge. Use 1 µl of this suspension in PCR reactions with a final volume of 20 µl.



PREPARATION OF POSITIVE CONTROL

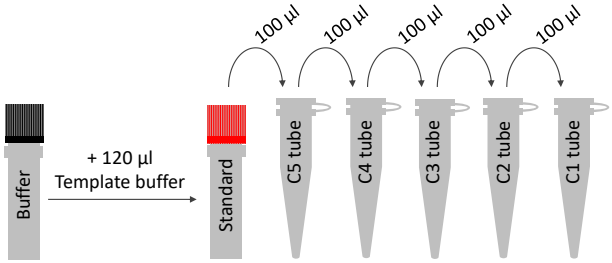
The **Positive Control** (ORANGE CAP) must be prepared only when a detection analysis is required (no quantification). Pulse-spin the **Positive Control** (ORANGE CAP), reconstitute with 120 µl of **Template buffer** (BLACK CAP) and vortex thoroughly.

Pipette 5 µl of template into the well according to your plate set-up. The final volume in each qPCR reaction well is 20 µl

PREPARATION OF STANDARD CURVE DILUTION SERIES

The sealed **Standard Template** (RED CAP) contains a high copy number and there is a significant contamination risk. Discard this tube directly if no quantification is required during the analysis. To avoid contamination, please, follow recommendations of WARNINGS AND PRECAUTIONS section of this handbook.

1) Pipette 900 µl of **DNAse/RNAse free water** (not provided) into five tubes and label as C5 to C1
2) Pulse-spin the **Standard Template** (RED CAP), reconstitute with 120 µl of **Template buffer** (BLACK CAP) and vortex thoroughly
3) Pipette 100 µl of diluted **Standard Template** (RED CAP), into tube C5
4) Vortex thoroughly and pulse-spin
5) Change tip and pipette 100 µl from tube C5 into tube C4
6) Vortex thoroughly and pulse-spin
7) Repeat steps 5 and 6 with the tubes C3 to C1 to complete serial dilution
8) Once the standard curve dilutions are prepared, discard the **Standard Template** (RED CAP) tube



Standard curve dilution series	copies/µl	copies in 5 µl
C5 Tube	2×10^4	10^5
C4 Tube	2×10^3	10^4
C3 Tube	2×10^2	10^3
C2 Tube	2×10	10^2
C1 Tube	2	10

Pipette 5 µl of template into the well according to your plate set-up. The final volume in each qPCR reaction well is 20 µl.

PCR SET-UP PROTOCOL

Reverse transcription and qPCR reaction can be achieved in a single tube by using our **GPS™-mix-RT** (BLUE CAP). One-Step protocol is recommended to prevent contamination, reduce errors, and save significant materials and time.

It is advisable to prepare a reaction pre-mix in a nuclease-free tube on a cooling block, according to the following table. Prepare enough reaction mix for the desired number of reactions, for your samples and the positive/negative controls considered for your experiment.

Reagent	Volume
DNase/RNase free water (GREEN CAP)	9 µl
GPS™-mix-RT (BLUE CAP)	5 µl
TargetSpecies dtec-RT-qPCR-mix (AMBER TUBE)	1 µl
Reaction pre-mix volume	15 µl

Once prepared, pipette 15 µl of this reaction mix into each well or PCR tube according to your experimental platform.

Add 5 µl of extracted sample or, if the case of calibration curves/positive control, the corresponding diluted template to each PCR tube to reach a final PCR volume of 20 µl.

Reagent	Volume
Dispensed reaction pre-mix volume	15 µl
Sample (or DNase/RNase free water)[1]	5 µl
FINAL REACTION VOLUME	**20 µl**

1 In the case of negative control, add 5 µl of DNase/RNase free water (GREEN CAP) instead of sample (see Recommended Reaction Controls section).

AMPLIFICATION REGIME

Once all reactions mixes are ready and gently closed, vortex the tube thoroughly, harvest by pulse-spin in the centrifuge, and place plate or tubes in the block/rotor of the thermocycler programmed to run the cycling regime described on the table. Consider that the fluorogenic signal must be collected by using the FAM channel for the **S gene** target, the Cy5 channels for the **N gene** target and the HEX channel for the **Internal Control**. Probe includes a Dark Quencher which can be set up as any non-fluorescent quencher (NFQ) in the software.

Fast-Cycling	**Step**	Time	Temperature
	Retrotranscription	10 min	50 ºC
	Activation	2 min	95 ºC
40 Cycles	Denaturation	1 sec	95 ºC
	Hybridization / Extension and data collection[1]	10 sec	**60 ºC**

1 Fluorogenic signal should be collected during this step by using the **FAM** channel for the S gene, **Cy5** for the N gene and by using the **HEX** channel for the Internal Control.

GPS™ reagents are compatible with all qPCR devices. The use of a passive dye as ROX is not required.

RECOMMENDED REACTION CONTROLS

These qPCR reaction controls are recommended when considering the guidelines of UNE/EN ISO 17025 standard. When setting-up your qPCR protocol, select the controls considered better suits your quality system. When the Standard Template aliquot is being used for quantification, the C3 dilution must be used for any other control in which Positive Control is indicated.

Negative Control (Ctrl -): Add 5 µl of DNase/RNase free water (GREEN CAP) to 15 µl of reaction mix. Accordingly, this reaction should be negative. A positive result may be considered as a symptom of contamination in some reagents of reaction mix, making the test inconclusive. Reagents must be discarded.

Positive Control (Ctrl +): Prepare the **Positive Control** (ORANGE CAP) as described above. Add 5 µl of the Positive Control to 15 µl of the reaction mix. A positive result indicates that qPCR setup is correct and works. If negative, the test should be carefully repeated after checking the thermal protocol.

Extraction Negative Control (ExtCtrl -): Perform an extraction according to your extraction protocol without addition of sample. Add 5 µl of extraction negative sample to 15 µl of reaction mix. In this case, the test includes the reagents used in the extraction steps. If positive, when the **Negative Control** is negative, a contamination occurs during the extraction process. Extraction reagents must be discarded.

Extraction Positive Control (ExtCtrl +): Perform an extraction according to your extraction protocol adding the **Positive Control** (ORANGE CAP), or RNA extracted from pure cultures into the first extraction buffer. The positive extraction control would include the effectiveness of the extraction method used. A positive result should be expected. If negative, extraction must be carefully repeated, or the extraction method replaced.

INTERPRETATION OF RESULTS

If the Standard Template is requested for quantification, the linear regression obtained from the logarithm of the copy number versus Ct gives both constants Y-intercept and slope of the standard curve (equation 1). The number of copies in the sample can be calculated based on the regression (equation 2).

$$Ct = Y\ intercept + Slope\ x\ log(copy\ number) \quad (1)$$

$$Copy\ number = 10^{\frac{(Ct - Y\ intercept)}{Slope}} \quad (2)$$

To obtain the sample quantification directly from the device, the Standard dilution series must be defined in the software of your qPCR device as Standard with the specified copies for each dilution (see PREPARATION OF STANDARD CURVE DILUTION SERIES). Standard curve can be defined as total copy number or copies/µl.

To refer the values obtained with qPCR to the sample material, please consider the elution volume after extraction, the sample volume processed, and any dilution performed.

Ctrl -	Ctrl +	S gene	N gene	I C	ExtCtrl -	Interpretation
+	+ / -	+ / -	+ / -	+ / -	+ / -	PCR reagents contaminated
-	-	+ / -	+ / -	+ / -	+ / -	Experiment fails
-	+	+ / -	+ / -	+ / -	+	Contamination at extraction step
-	+	-	-	+	-	**Negative sample**
-	+	+	+	+ / -	-	**Positive sample**
-	+	+	-	+ / -	-	**Positive sample**
-	+	-	+	+ / -	-	**Positive sample**
-	+	-	-	-	-	PCR inhibition

Key symbols + and - : amplification does or not occur, respectively.

Early scientific reports have indicated that SARS-CoV-2 generates a set of subgenomic RNAs during replication in host cells with different copy numbers depending on the gene. While the S-gene is replicated in a low subgenomic copies, the N-gene may be found in more than 10-fold copies. Consequently, for the same sample, the N-gene assay (Cy5) may show lower Ct values than these obtained in the S-gene (FAM) assay.

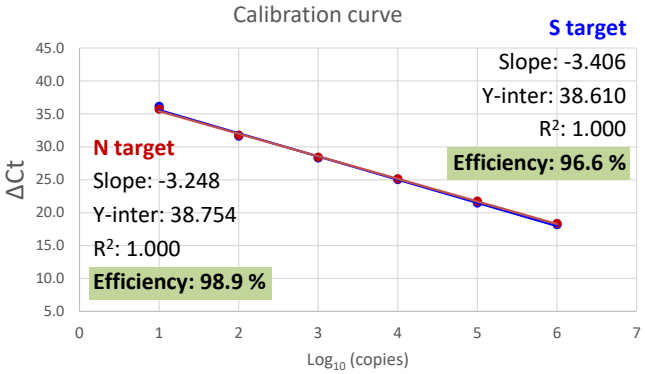
If inhibition is observed, a sample dilution to 1/10 is recommended (if concentration is not close to detection limit) or the purification of the sample must be repeated.

Usually in a qPCR with a 100 % efficiency, 37 cycles are enough to amplify less than 10 copies. That does not mean Ct >37 have not significance, but to accept a higher Ct value (low expression), there is more laboratory bench requirements: contamination prevention, checking inhibition levels and verification along time of the low positive specimens. This evaluation must be done by the customer with the whole protocol, in their laboratory, with their qPCR device, samples and matrices. Only this way a robust criterion can be stablished.

PERFORMANCE EVALUATION AND CLINICAL EVIDENCE

Analytical sensitivity

For the calibration and statistical analysis, the linearity study was carried out with six ranges of decimal dilution from 10^6 copies to 10 copies from the Standard Template (RED CAP). Each parameter was assayed at least 10 times to achieve reproducibility conditions. The required statistical analysis was performed by representing Ct data from dilutions against the copy number logarithm in a graph and the equation parameters were determined for Slope, Y-intersection and Efficiency.



The linear regression analysis gave a correlation coefficient of **R^2 = 1.000** with a slope of -3.248 and Y-intersection of 38.754 for **N gene** target and a correlation coefficient of **R^2 = 1.000** with a slope of -3.406 and Y-intersection of 38.610 for **S gene** target. Amplification efficiency (E) was estimated by using the slope of the standard curves a value of **98.9 %** for N gene assay and **96.6 %** for the S gene assay.

The validation of the linear regression performance was verified with a model based on *Fisher Test* with a confidence interval of 0.05. The calculated experimental values for N and S genes (2.824 and 0.030 respectively) were lower than the theoretical Fisher value (5.318).

Verification of the limit of detection (LOD)

Estimating the PCR limit of detection (LOD) involves knowing the smallest number of target units generating positive result amplification at a 90 % confidence level. A total of 15 measurements were analysed. Both assays, N and S, can detected SARS-CoV-2 at least up to **10 copies** with a **100.0 %** confidence.

Verification of the limit of quantification (LOQ)

Estimating the PCR limit of quantification (LOQ) involves knowing the smallest number of target units generating a repeatability result of quantification. The validation is based on *Student's T-test*. At least up to 10 copies of SARS-CoV-2 can be quantified according to the values calculated from 15 Standard Template dilutions. Obtained t_{value} of **0.821** and **0.035** (N and S targets) are less than the $t_{student}$ value of 2.145; the **LOQ** at **10 copies** is validated.

Precision of measurement

Reliability is the ability of a method to produce results free of random errors. This parameter is estimated through the assessment of repeatability and reproducibility of the kit. **Repeatability**. A total of 1 dilution with 10 replicates of the six ranges of the standard curve were ran with the same device, the same day, the same technic in the same run. **Reproducibility**. Values obtained from standard curves were made by two different technicians, on different dates with different devices. The coefficient of variation (CV) for Repeatability and Reproducibility are detailed for each level of the dilution series.

	Coefficient of variation % (CV)			
	N gene target		S gene target	
Copies	**Repeatability**	**Reproducibility**	**Repeatability**	**Reproducibility**
1000000	0.35 %	0.49 %	0.28 %	0.30 %
100000	0.37 %	0.30 %	0.36 %	0.33 %
10000	0.16 %	0.16 %	0.26 %	0.25 %
1000	0.26 %	0.23 %	0.29 %	0.28 %
100	0.42 %	0.36 %	0.42 %	0.58 %
10	0.97 %	1.03 %	1.57 %	1.66 %

Analytical Specificity

Specificity is the ability of the method to recognize the target specifically and distinguish it from similar targets. A first primer check, for each assay, was performed *in silico* after the designing work by similarity search in hundreds of genomes using the appropriate software of the *National Center for Biotechnology Information* (NCBI) and *Global Initiative on Sharing All Influenza Data* (GISAID) databases. Additionally, once primers were synthetized, the specificity was experimentally tested with six synthetic RNA genomes of the SARS-CoV-2 (Wuhan and variants from UK, South Africa and Brazil) for inclusivity. All the sequences showed amplification.

Clinical performance

A total of 145 clinical samples were analysed with the assay to calculate the Diagnostic sensitivity (proportion of positives correctly identified) and Diagnostic specificity (proportion of negatives correctly identified).

CoVID-19 N/S dtec-RT-qPCR Duplex		**Reference technique**			
Total samples	145	Positive	127	Negative	18
Positive	125	True Positives	125	False Positives	0
Negative	20	False Negatives	2	True Negatives	18

Diagnostic sensitivity was evaluated with 127 positive samples for SARS-CoV-2, with a value of 98.4 %. Diagnostic specificity was evaluated with 18 negative samples for SARS-CoV-2, with a value of 100.0 %. The overall Diagnostic efficiency was 98.6 %.

CERTIFICATION OF ANALYSIS

All batches are calibrated with a standard curve from 10^6 to 10 copies with our Standard Template. Diverse parameters are evaluated: Ct, slope, R^2 and efficiency. All this information is available in the **Quality Certification** provided to the customer by GPS™.

REGULATORY REQUIREMENTS

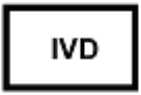
The product complies with the provisions of Real Decreto 1662/2000 (European Directive 98/79/CE) which regulates the manufacture of medical devices for *in vitro* diagnostics.

CONTACT INFORMATION

For any question and technical support, contact support@geneticpcr.com. For quotes, orders, or new target designs, please contact orders@geneticpcr.com.

HARMONISED SYMBOLS AND ABBREVIATIONS

DNA	Deoxyribonucleic acid	qPCR	Real-Time polymerase chain reaction
RNA	Ribonucleic acid	RT	Reverse transcription
RT-qPCR	Reverse transcription polymerase chain reaction		

	In vitro diagnostics		Manufacturer
	CE mark		Date of manufacture
	Batch code		Expiring date
	Caution		Range of temperatures
	Consult instructions for use		Protect from exposure to light

Genetic PCR Solutions™
by GENETIC ANALYSIS STRATEGIES S.L
Av. Dr. Gómez-Pardo Ródenas, 1
03300-Orihuela (Alicante)
☎ Phone: +34-965429901
💻 Web: www.geneticpcr.com
✉ e-mail: info@geneticpcr.com