Manual

SARS-CoV-2 Variant ValuPanel assay manual

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BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Manual

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Contents

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1. Product description

SARS-CoV-2 Variant ValuPanel™ assays consist of separately delivered probes and primers that are designed for qualitative detection of specific SARS-CoV-2 mutations by reverse transcription-polymerase chain reaction (RT-PCR)-based genotyping. Each SARS-CoV-2 Variant ValuPanel assay amplifies and discriminates between a specific mutation and the wild type SARS-CoV-2 sequence in respiratory tract samples that have previously tested positive for SARS-CoV-2 by diagnostic RT-PCR.

Each SARS-CoV-2 Variant ValuPanel assay contains the following:

- Forward and reverse primers that amplify the SARS-CoV-2 target sequence. One primer also initiates reverse transcription of the target sequence.
- BHQplus™ Probes that specifically detect the mutation site
    - A mutation-specific probe labelled with CAL Fluor™ Orange 560 (CFO560)
    - A reference/wild type-specific probe labelled with FAM

SARS-CoV-2 Variant ValuPanel assays are for research use only, and not intended for SARS-CoV-2 diagnosis. The variant ValuPanel assays are intended for secondary, informational tests only, designed for screening samples that have previously tested positive for SARS-CoV-2 by diagnostic RT-PCR.

Several variants of SARS-CoV-2 have emerged, bringing challenges to diagnostic tests and pandemic eradication efforts. Of particular significance are variants B.1.1.7 (first detected in the United Kingdom), B.1.351 (first detected in South Africa), and P.1 or B.1.1.28 (first identified in travellers from Brazil who arrived in Japan). The ability to quickly and reliably identify SARS-CoV-2 mutations will enhance the ability to gather crucial public health information regarding transmissibility kinetics of new variants, and the efficacy of vaccines and therapeutics.

All assay designs are created with reference to the Wuhan reference sequence NC_045512. Published mutations are then mapped to this sequence. Assay designs are created such that known mutations are excluded from the targeting oligonucleotide sequences. When this is not possible, the oligonucleotides are modified to prevent disruption of the assay. To avoid cross reactivity, all designs undergo an *in silico* screen against a panel of respiratory organisms.

SARS-CoV-2 Variant ValuPanel assays

Cat. no.	ValuPanel assay	SARS-CoV-2 mutation	Variants containing specified SARS-CoV-2 mutation
SCV-E484K-1000	SARS-CoV-2 Variant ValuPanel [E484K]	E484K	B.1.351, B.1.1.28 (P.1)
SCV-del69-70-1000	SARS-CoV-2 Variant ValuPanel [del H69-V70]	ΔH69-V70	B.1.1.7, DK mink Cluster V
SCV-N501Y-1000	SARS-CoV-2 Variant ValuPanel [N501Y]	N501Y	B.1.1.7, B.1.351, B.1.1.28 (P.1)
SCV-P681H-1000	SARS-CoV-2 Variant ValuPanel [P681H]	P681H	B.1.1.7, B.1.1.28
SCV-K417N-1000	SARS-CoV-2 Variant ValuPanel [K417N]	K417N	B.1.351
SCV-K417T-1000	SARS-CoV-2 Variant ValuPanel [K417T]	K417T	B.1.1.28 (P.1)

Table 1. SARS-CoV-2 Variant ValuPanel assay catalogue information.

Panel components

Oligo type	Target	Dye	Quencher	Oligo amount*
Probe	Wild type allele	FAM	BHQ-1 Plus	5 nmol
Probe	Mutant allele	CAL Fluor Orange 560	BHQ-1 Plus	5 nmol
Primer	N/A	N/A	N/A	20 nmol
Primer	N/A	N/A	N/A	20 nmol

Table 2. SARS-CoV-2 Variant ValuPanel assay component information.
* Probes and primers delivered in individual tubes in dried format.

2. Storage conditions

SARS-CoV-2 Variant ValuPanel assays are shipped at ambient temperature. Upon receipt, store at +2 to +8 °C. Once rehydrated, the oligonucleotides should be aliquoted and stored at -30 °C to -15 °C. Multiple freeze-thaw cycles (>10 cycles) should be avoided. Probes should be protected from light.

3. Customer provided materials

Reagents

Reagent	Recommended
Quantitative PCR (qPCR) master mix	RapiDxFire™ qPCR 5X Master Mix GF, Cat. No. 30050-1, 30050-2 (LGC, Biosearch Technologies)
Reverse transcriptase enzyme	EpiScript™ RNase H- Reverse Transcriptase, Cat. No. ERT12925K-ENZ, ERT12925K-1.25ML (LGC, Biosearch Technologies)
PCR passive reference dye	SuperROX™, concentration 15 µM, Cat. No. SR-1000-1, SR-1000-10 (LGC, Biosearch Technologies)
qPCR instrument calibration standard (optional)	CAL Fluor Orange 560 T10 Calibration Standard, 5 nmol, Cat. No. RD-5081-5 (LGC, Biosearch Technologies)

Table 3. Additional reagents that are compatible with the SARS-CoV-2 Variant ValuPanel assays.* Probes and primers delivered in individual tubes in dried format.

PCR instrument

a. qPCR instrument/plate reader with appropriate filters
b. qPCR and end-point cluster analysis software

Consumables

a. PCR microtitre plates/tubes
b. Optical plate seal
c. Molecular grade, nuclease-free water
d. 10 mM Tris, 0.1 M EDTA, pH 8

Positive control options

Positive template control options for SARS-CoV-2 mutations include:

a. Commercial SARS-CoV-2 or recombinant viral controls
b. Commercial, synthetic RNA controls
c. Commercial DNA controls, such as a synthetic fragment or plasmid
d. Sequence-verified SARS-CoV-2 samples

4. General guidelines

a. For quantification and/or concentration/copy number determination, it is recommended to follow the MIQE guidelines for qPCR.
b. It is recommended to include an appropriate number of both positive (AccuPlex SARS-CoV-2 Verification Panel) and negative (non-template control or NTC) samples on each reaction plate/run to control for assay sensitivity/specificity and contamination events.
c. Further guidance on assay optimisation can be found in Nolan, T., Hands, R. & Bustin, S. Quantification of mRNA using real-time RT-PCR. Nat Protoc 1, 1559–1582 (2006).
d. Use good laboratory practice at all times. Wear gloves and use nuclease-free tips and reagents.
e. For best results, an end-point genotyping protocol is used with cluster plot software.

5. Reaction volumes and number of reactions per kit

Each SARS-CoV-2 Variant ValuPanel assay provides sufficient oligonucleotides to perform at least 1,000 reactions, based on 20 µL reactions containing 200 nM/probe and 900 nM/primer. Our Reaction Estimator is a useful tool to calculate the number of reactions that can be performed, depending on desired reaction volume and oligonucleotide concentration.

Reaction volume (µL)	Number of reactions*	Suggested plate formats
20	1,000	96-well plate
10	2,000	384-well plate
5	4,000	Array Tape™
1.6	12,500	Array Tape

Table 4. Approximate number of reactions from each ValuPanel set.
*Based on reactions containing 200 nM of each probe and 900 nM or each primer.

6. Oligonucleotide preparation

Oligonucleotide re-suspension

Re-suspend the dried probes and primers to make stock solutions. We recommend creating probe stocks of 100 µM, and primer stocks of 300 µM. TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) is recommended but other molecular biology-grade, nuclease-free diluents may also be used. If another concentration is desired, please see our Oligonucleotide Resuspension Calculator for re-suspension assistance.

	Amount of dried oligonucleotide per tube	Re-suspension volume	Final stock concentration
Probe	5 nmol	50 µL	100 µM
Primer	20 nmol	66.7 µL	300 µM

Table 5. Approximate number of reactions from each ValuPanel set.
*Based on reactions containing 200 nM of each probe and 900 nM or each primer.

Preparation of working assay mixes (40x and 80x)

The ValuPanel oligonucleotides are supplied in individual tubes to facilitate optimisation of conditions specific to the reagents and instrument of choice. For most targets, we recommended starting with final oligonucleotide concentrations of 900 nM per primer and 200 nM per probe; however, if required, the final primer concentration can be optimised at 200-900 nM per primer and 100-300 nM per probe.

Please see our Biosearch Technologies website for an Oligo Dilution Calculator, which can assist with any calculations regarding the dilution of the probes and primers for working assay mix generation.
If the final reaction volumes are intended to be ≥5 µL, then 40x assay mix is recommended. For final reaction volumes <5 µL, then 80x assay mix is recommended, to prevent over-dilution of the qPCR master mix with the assay mix.

Component	40x assay mix (for final reaction volumes >5 µL)		80x assay mix (for final reaction volumes <5 µL)	
	Volume	Working concentration	Volume	Working concentration
300 µM primer (each)	12 µL	36 µM	24 µL	72 µM
100 µM probe (each)	8 µL	8 µM	16 µL	16 µM
Diluent	To 100 µL	-	To 100 µL	-
Total volume	100 µL	-	100 µL	-

Table 6. Preparation of 40x and 80x working assay mixes for qPCR to allow for assay set-up with final oligonucleotide concentrations of 900 nM primer and 200 nM probe.

7. Reaction setup

***NOTE**: This product has been shown to accurately genotype SARS-CoV-2 mutations when using the following reaction conditions and reagents. Optimisation may be required for customer-specific reaction conditions and instrument preferences.*

a. Completely thaw reaction components at room temperature. Before use, vortex components and briefly spin the tubes in a microcentrifuge to ensure that the material is collected at the bottom of the tubes.

b. Prepare reaction mixes in sterile, nuclease-free microcentrifuge tubes. For each sample or condition, prepare one reaction mix by multiplying each component volume by the total number of desired reactions (plus extra, typically +10%, to allow for pipetting). Vortex the reaction mix and aliquot one reaction volume into each reaction tube/qPCR reaction plate well.

Component	1.6 µL reaction volume	5 µL reaction volume	10 µL reaction volume	20 µL reaction volume	Final concentration
RapiDxFire qPCR 5X Master Mix GF	0.32 µL	1 µL	2 µL	4 µL	1x
EpiScript Reverse Transcriptase, 200U/µL	0.04 µL	0.125 µL	0.25 µL	0.5 µL	5 U/µL
Assay mix (40x or 80x)	0.02 µL (using 80x assay mix)	0.125 µL (using 40x assay mix)	0.25 µL (using 40x assay mix)	0.5 µL (using 40x assay mix)	900 nM primer, 200 nM probe
Template RNA	No more than 1.22 µL	No more than 3.75 µL	No more than 7.5 µL	No more than 15 µL	As required
SuperROX,15 µM (optional)	0.01 µL	0.035 µL	0.07 µL	0.13 µL	100 nM*
Molecular-grade, nuclease-free water	To 1.6 µL	To 5 µL	To 10 µL	To 20 µL	-

Table 7. Example of reaction setup for 1.6 µL, 5 µL, 10 µL and 20 µL reaction volumes.
*Optimisation of SuperROX concentration may be required for certain qPCR instruments.

c. Briefly spin the reaction tubes/plates in a microcentrifuge/plate-centrifuge to ensure that the material is collected at the bottom of the tubes/plates.

d. Place the reaction tubes/plates in a qPCR instrument, pre-set with the desired thermal cycling and data collection settings. Ensure instrument is set to read at the appropriate channels for FAM and CFO560/HEX/VIC™/JOE.

e. Run the protocol until the thermal cycling has reached completion.

8. Thermal cycling protocol

SARS-CoV-2 Variant ValuPanel assays are designed to be compatible with all qPCR instruments that are capable of detecting FAM and CFO560 (VIC/HEX/JOE channel). The mutation-specific probe is labelled with CFO560, while the reference/wild type-specific probe is labelled with FAM. For more information on dye excitation/emission and instrument compatibility, please see our Spectral Overlay Tool and Dye Selection Chart.

For optimal performance, CFO560 dye calibration standards are available to improve signal deconvolution in real-time qPCR thermal cyclers that require spectral calibration. Using the dye calibration as a reference, the analysis software anticipates how much fluorescence to expect from each fluorophore during amplification, and will subtract out signal from inappropriate filter-sets. Calibration with the CFO560 standards therefore reduces the magnitude of crosstalk (see Section 4. Customer provided materials for ordering information).

SNP assay setup	Mutation	Dye	Channel
Allele 1	Wild type	FAM	FAM
Allele 2	Mutation	CAL Fluor Orange 560	VIC/HEX/JOE

Table 8. SNP assay setup and qPCR instrument channel selection.

The results from this assay can be analysed using both real-time PCR and end-point applications. Please ensure that you have the correct data analysis software available before commencing.

Step	Temperature	Time	Number of cycles
1	50 °C	15 minutes	1
2	95 °C	2 minutes	1
3*	95 °C	3 seconds	50
	60 °C	30 seconds	

Table 9. Thermal cycling conditions for the SARS-CoV-2 Variant ValuPanel.

*If performing real-time PCR, ensure that data collection is performed at the end of each cycle. If performing end-point PCR, ensure that data collection is performed once all cycles have completed.

Interpretation of results

Assign calls with the instrument software, preferably using cluster plots/allelic discrimination plots. Based on instrument settings, the X and Y axes will correspond to either the FAM or CFO560 probes. Samples grouping closest to the FAM-assigned axis represent the wild-type cluster. Samples grouping closest to the CFO560-assigned axis represent the variant cluster. NTCs should remain at the cluster closest to the origin, which may also include samples that do not amplify.

Samples that amplify, but are not clearly assignable to a cluster are considered indeterminate and may be due to low RNA concentration or degradation, mutations in primer- or probe-binding regions, PCR inhibitors, or similar causes. When samples have Cq values >30 on N gene-targeted diagnostic assays, the probability of variant assay drop outs may increase. Actual call types/names, colour assignments, etc. will vary by scoring software and respective analysis settings. Only interpret runs with valid quality control representing each cluster.

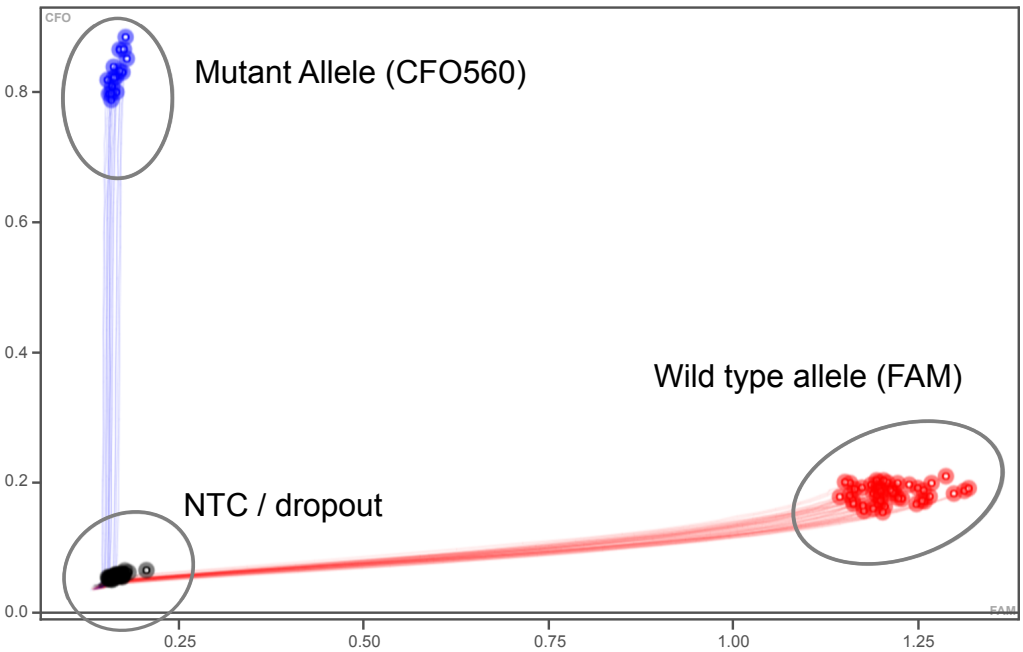

Figure 1. Example of genotyping data plot. Genotyped samples marked red designate the wild type allele (reported with FAM), those marked blue designate the variant allele (reported with CFO560).

9. Analysis of K417T and K417N mutations

Genotyping of mutations at amino acid position 417 of the spike protein can contribute to the identification of multiple variants of concern. For this reason, it is an attractive target for genotyping analysis, but as a Multiple Nucleotide Polymorphism (MNP), it may require additional analysis.

Variant	Amino acid codon	412	413	414	415	416	417	418	419	420	421
K417 wild type	Nucleotide sequence	CCA	GGG	CAA	ACT	GGA	AAG	ATT	GCT	GAT	TAT
	Amino acid	P	G	Q	T	G	K	I	A	D	Y
K417T mutation	Nucleotide sequence						A**C**G				
	Amino acid						T				
K417N mutation	Nucleotide sequence						AA**T**				
	Amino acid						N				

Table 10. K417, T417 and N417 codons. Amino acid position 417 of the spike protein is a Multiple Nucleotide Polymorphism (MNP); a mutation of the 417 codon at position 2 gives rise to the T417 variation, and a mutation of the 417 codon at position 3 gives rise to the N417 variation.

In our performance studies of K417N and K417T assays, we found that the wild type probe can produce a distinct off-target signal in the presence of the alternative variant's template control. For example, the K417T assay can generate a separate N417 cluster when run with N417 template. Likewise, a separate T417 cluster was formed when the K417N assay was run on T417 template material. We recommend that both K417N and K417T assays are used to accurately genotype amino acid 417 of the spike protein. Concordant results from these companion assays can compensate for the challenges of this MNP target.

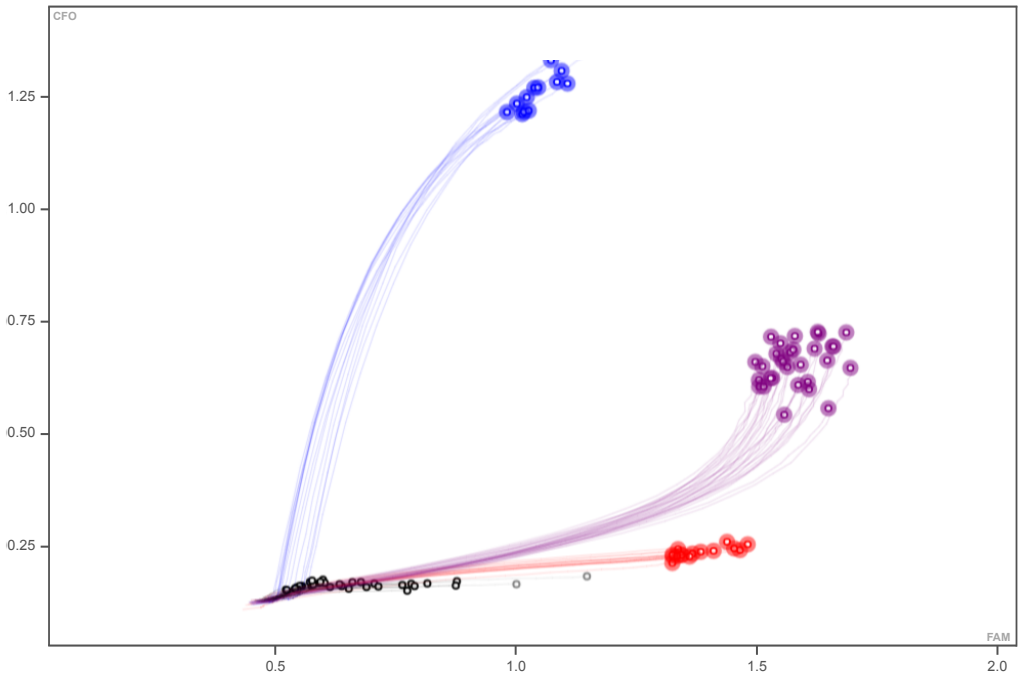

Figure 2. K417N assay genotyping data plot. The blue cluster represents the N417 calls. The purple group represents the K417 cluster. The red cluster represents the K417 probe binding to the off target T417 template.

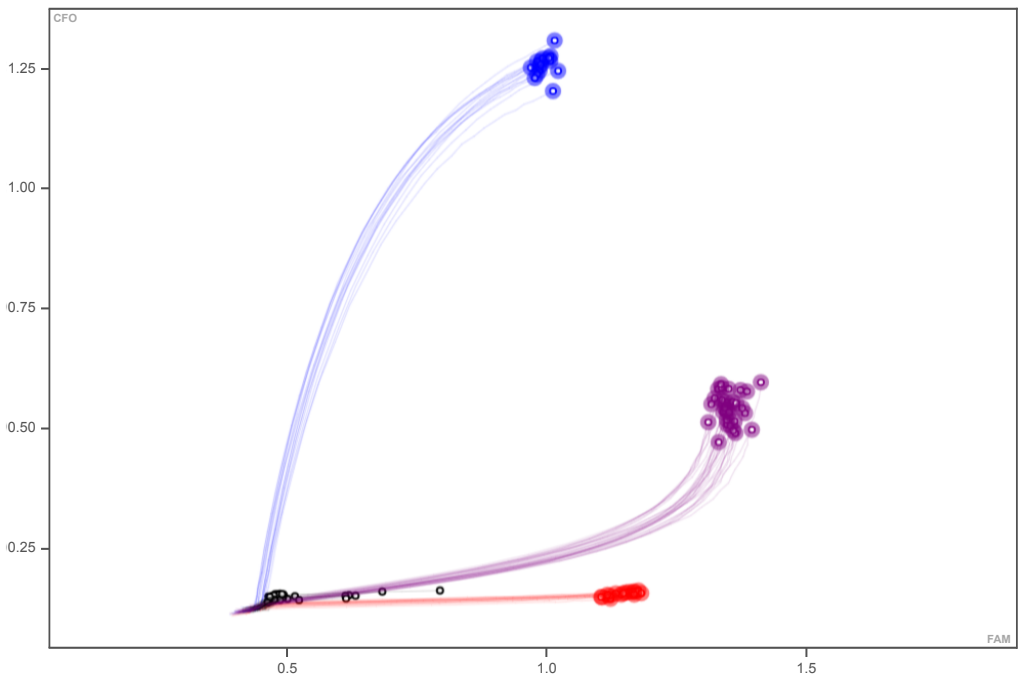

Figure 3. K417T assay genotyping data plot. The blue cluster represents the T417 calls. The purple group represents the K417 cluster. The red cluster represents the K417 probe binding to the off target N417 template.

10. Further support

For any queries about this user guide, please contact: techsupport@lgcgroup.com.

11. Appendix

11.1. MIQE guidelines for qPCR

Condensed and adapted from:
The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Bustin S.A *et al*. Clinical Chemistry 55(4): 611-622 (2009)

Good practice guide for the application of quantitative PCR (qPCR). Nolan T. *et al*. LGC (2013)

11.1.1. Sample purification

Biological sample treatment is crucial to ensure that the extracted (and where applicable, purified) nucleic acid is of sufficient concentration, purity and inhibitor-free. When performing any qPCR applications, co-purified contaminants may influence the final observed result, so care should be taken to ensure that the nucleic acid meets minimum requirements for testing.

11.1.2. Nucleic acid measurement

Once the nucleic acid has been isolated, measurements should be performed to ensure that the minimum quality/quantity requirements are met. Using sub-optimal nucleic acid or an array of samples with different levels of nucleic acid sample integrity within the same assay will result in inconsistencies in the testing chemistry between samples, therefore influencing the final results.

The most common method is to assess the 260/280 and 260/230 spectrophotometric readings, which, by following the Beer-Lambert law, draws a direct correlation between absorbance and concentration. It is known that nucleic acids have a peak absorbance of 260 nm, so measuring the amount of light absorbed at this wavelength can be used to determine the concentration of DNA or RNA in solution. A 260 nm measurement of 1.0 is equivalent to ~40 µg/mL of pure RNA and ~50 µg/mL of pure double stranded DNA.

One commonly used instrument used to measure the 260/280 and 260/230 is the NanoDrop™ (ThermoFisher). However, this instrument measures total absorbance and not just double-stranded nucleic acid. Therefore, should these methods be used to quantify DNA as a result of a PCR reaction, any primers/dNTPs will contribute to the final reading. Therefore, fluorometric measurements, using double-stranded nucleic acid intercalating dyes, (such as SYBR® Green which intercalates between double-stranded DNA), are more commonly used to provide more accurate measurements.

11.1.3. Contamination

In regards to qPCR, contamination by the amplified target sequence (amplicon) can give rise to two issues:

a. PCR (including qPCR) can generate billions of targets within a single reaction due to the exponential amplification of the target nucleic acid. These high-copy number amplicons are easily transferred between equipment/workstations, resulting in a high probability of a contamination event occurring.
b. Due to the highly sensitive nature of qPCR (in some instances, assays have the capability of detection down to a single copy of the target), even a single amplicon has the potential to cause a contamination event.

The easiest way to overcome this is to observe good laboratory practice. Many molecular biology laboratories have designated areas (complete with workstations and equipment), solely for the handling of post-PCR products. These areas are separate from where the biological samples are handled and where the pre-PCR reactions are set up.

Other sources of contamination include non-target specific amplicons (for example, those that are generated from alternative PCR reactions). Although these are not derived from the PCR in question, there could be instances of cross-homology or non-specific amplification, which again will result in the presence of false-positives.

The inclusion of both internal and external quality controls will aid with the assessment of any contamination within the assay run.

11.1.4. Inhibition

Inhibition is the action of a product or artefact within the reaction, which can affect the efficiency of the amplification of the target nucleic acid, typically by downregulating the observed result. For example, this causes difficulty in the assigning of genotypes or leads to an incorrect interpretation of relative target quantities.

Common inhibitors include Tris, ethanol, isopropanol, EDTA, guanidine salts (for example, guanidine isothiocyanate, guanidine hydrochloride) and phenol.

One way to assess the presence (if any) of inhibition is to include an internal quality control with each sample to be tested.

11.1.5. Appropriate controls

It is absolutely critical to include controls within each PCR reaction run, as not only will this control for any contamination or inhibition events but their result will confirm that the PCR reaction performed as expected and that the results of the samples tested can be taken as true.

When the external quality controls (EQC) and internal quality controls (IQC), together with the non-template controls (NTC), are assessed individually, and in combination in each reaction run, the validity of the results obtained can be verified, providing confidence and robustness in the results of the test sample.

Therefore, it is possible to pass reaction runs in which various controls have failed, as long as the other controls have shown to be within acceptable detection ranges.

11.1.5.1. Non-template controls (NTC)

These are reactions which contain all of the same PCR components as the other reactions, but with no target DNA (sample buffer or molecular-grade water can be used in place of DNA to ensure all reaction volumes across the run are consistent). In a scenario where there is no contamination, these NTCs will not amplify and therefore generate a negative result. However, in the case of a contamination event, these NTCs will show amplification, suggesting there has been carry-over between each reaction or there has been an external source of contamination introduced (for example, from operators or from the lab environment) during sample processing.

11.1.5.2. External quality controls (EQC)

External quality controls (EQCs) are samples which have a known result and are run alongside the test samples in the reaction, normally with NTCs. Typically, EQCs are included to control for each stage of the experimental process (for instance, an EQC for the extraction, and an EQC for the PCR). In some cases, these EQC can be the same sample carried through each process, or different EQC material can be used for different stages.

EQC result	NTC result	Interpretation
Positive	Positive	Run was a success but evidence of contamination. Only negative test samples can be passed. All positive test samples to be repeated.
Negative	Positive	Run failed, as cannot validate the success of reaction, with evidence of contamination. Test to be repeated.
Positive	Negative	Successful run, so all samples can be passed.
Negative	Negative	Run was not successful, but no evidence of contamination. Only positive test samples can be passed. All negative test samples to be retested.

Table 13: Interpretation of external quality control (EQA) and non-template (NTC) results.

11.1.5.3. Internal quality controls (IQC)

Internal quality controls (IQCs) are additional material artificially introduced (or “spiked”) into the sample being tested, and run in parallel within the same reaction. These controls are typically included to control for inhibition events, to determine a true negative from a false negative.

EQC result	NTC result	Interpretation
Positive	Positive, no inhibition	True positive result.
Negative	Positive, no inhibition	True negative result.
Positive	Positive, with inhibition	True positive result, though some inhibition may be occurring. For accurate quantification, serially dilute DNA sample until IQC is uninhibited to normal levels.
Positive	Negative	True positive result, though inhibition is occurring. For accurate quantification, serially dilute DNA sample until IQC is uninhibited to normal levels.
Negative	Negative	False negative through PCR inhibition. Serially dilute primary sample and extract at different dilutions until IQC is uninhibited to normal levels.

Table 14. Interpretation of sample and internal quality control (IQC) results.

11.1.6. qPCR assay design and optimisation

Varying factors should be taken into consideration when designing a qPCR assay, to ensure that the results obtained are robust and reproducible and that there is confidence in the inferred qualitative and quantitative results.

11.1.6.1. Replicates and randomisation

For quantitative applications, it is generally accepted that a minimum of six replicates is required to obtain reasonable confidence in a result. However, the decision on the number of replicates (be they biological replicates or technical replicates) chosen is dependent on the aims of the experiment. Biological replication is when multiple biological samples are tested. These could be different sources of the sample (for example, different patients) or different sample types (for example, different cell types from the same patient). Technical replication is when the nucleic acid is isolated from a single source, but there are several replicates at each stage of the testing process (for example, multiple qPCR reactions from the same DNA eluate).

Randomisation of the arrangement of samples may also be incorporated into the assay design, to ensure there is no bias within the experimental setup (for example, no temperature variations across a thermal cycling heat block).

11.1.6.2. Assay optimisation

Assay optimisation is crucial to ensure that the qPCR is performing at its optimal efficiency, and there are a number of factors which can be adjusted to improve the sensitivity, specificity and precision. It is therefore paramount to perform in-house optimisation and validation of each qPCR assay prior to routine use to ensure that each assay is working as optimally as possible.

There may be instances where the primer and/or probe concentrations have to be adjusted from the standard protocol. The idea is to use the oligonucleotides at concentrations where there is the highest technical reproducibility at the lowest limit of detection, with any NTCs remaining a true-negative.

Cycling conditions also play an important role. Typical qPCR thermal cycling protocols will run for a total of 25 to 45 cycles and can consist of either a two-step or three-step cycle. Two-step cycles (denaturing and a single annealing/extension stage) are more flexible in accommodating assays with varying properties; however, this limits the scope for oligonucleotide design, as Tm optimisation is not possible. Three-step cycles (denaturing, with separate annealing and extension stages) are preferable for more complex target sequences and allows for Tm optimisation.

The concentration of magnesium chloride ($MgCl_2$) has its presence in a qPCR reaction has a three-fold effect:

- Influences the hybridisation of the oligonucleotides to the target
- Affects the processivity of the DNA polymerase enzyme
- Impacts the rate of hydrolysis of the exonuclease moiety

Hence, too little $MgCl_2$ may result in a sub-performing assay; however, too much $MgCl_2$ may result in non-specificity. Conventional PCR reactions require approximately 1-2 mM standard $MgCl_2$ concentration, whereas hydrolysis probe-based qPCR applications may require as much as 3-5 mM $MgCl_2$ to achieve sufficient probe cleavage (and therefore generation of a fluorescent signal).

11.1.7. Assay evaluation

Once the assay is optimised, and the most specific and sensitive conditions identified, it is important to assess the assay efficiency and technical dynamic range.

When assessing the performance of an assay, there are two commonly used quantification methods applicable to qPCR. These are standard curve quantification and comparative quantification.

***NOTE**: The terms absolute quantification and relative quantification have been applied to qPCR, both of which can be carried out with or without the inclusion of a standard curve, and have been used interchangeably in molecular biology. In the interest of adhering to MIQE guidelines and to avoid confusion the aforementioned terms have been avoided.*

Whilst performing assay validation, it is also important to assess the various performance parameters that could affect the overall efficiency, and therefore robustness and reproducibility of the qPCR assay:

- Precision – The closeness of agreement between independent measurements.
- Bias – The difference between the expected test measurement and an accepted reference value.
- Robustness – Guard-railing against potential experimental and/or operator errors, which could accumulate over time.
- Specificity – The extent to which the methods can detect the target without interference from other, similar components.
- Sensitivity – The reproducibility to identify the lowest, defined limits of detection (LoD).
- Working range and linearity – Interval between the upper and lower concentrations of the target, deemed suitable for the assay, and the assay's ability to generate a result directly proportional to the concentration of the target.
- Measurement uncertainty – The estimated range of values within which the true value of the measurement resides, indicating the reliability of the assay.

11.1.7.1. Standard curve quantification

Standard curves used in qPCR applications allow for the quantification of a target within a sample. They are typically serial-dilutions of a known positive, generated *in vitro* and used in each PCR reaction. The results from each of the serial dilutions are then used to generate what is known as a standard curve, from which the concentrations (or copy number) in each test sample can be extrapolated. The samples used to generate the standard curves tend to be reference genes, such as endogenous reference targets, plasmids containing the target of interest, or cell-culture grown controls.

DNA of a known concentration or a known copy number is serially diluted, typically in 10-fold dilutions, and the Cq values are determined from the amplification plot. These Cq values are then plotted against the logarithm of the concentration/copy number to generate a standard curve (linear relationship). The assay efficiency is calculated from the slope (m), derived from the line of best-fit, described by the equation:

$$y = mx + c$$

And where the efficiency as calculated as: $E = 10^{(-1/m)} - 1$
The efficiency of an assay should be a value close to 1, with 1 indicating a 100% efficient reaction.

The correlation coefficient (R^2) provides an estimate of the "goodness" of the line of best fit of the data point in the linear trendline, and if each sample was tested in replicates (a minimum of triplicate reactions are recommended), the values for each replicate should be highly reproducible, with $0.98 > R^2 \leq 1$. The intercept (c) of the standard curve on the y-axis should provide a theoretical sensitivity of the assay, correlating to the number of cycles required to detect a single unit of measurement.

Amplicon accumulation is proportional to 2^n, where n is the number of amplification cycles. Therefore:

2^n = fold dilution

2-fold dilution n~1

10-fold dilution n~3.323

Therefore, when a 10-fold serial dilution is performed, the amplification plots for each dilution should be ~3.3 cycles apart.

A sample of unknown concentration/copy number is then run on the same reaction as the serial dilutions, the Cq determined, and the concentration/copy number extrapolated from the standard curve.

11.1.7.2. Comparative quantification

Comparative quantification is used to measure the relative change in expression levels between samples under different experimental conditions or over a period of time. The concentration of the gene of interest is compared against a validated reference gene(s) to normalise against operator-introduced variation.

The comparative quantification method is also known as the delta delta Cq (termed as $2^{-\Delta\Delta Cq}$) and uses a standard curve (the validated reference gene) to verify the reaction efficiencies. It is therefore important that the amplification efficiencies of both the gene of interest and the reference genes are virtually identical and close to 100%.

However, this method has its drawbacks. Firstly, the PCR efficiencies could differ from 100% and secondly, comparing Cqs from different assays is problematic, as Cq is an arbitrary value rather than a defined unit. Therefore, the following equation is applied to take into account these inaccuracies:

$$\text{ratio} = \frac{(E^{\text{target}})^{\Delta Cq}\ \text{target}\ (^{\text{control}\ -\ \text{sample}})}{(E^{\text{target}})^{\Delta Cq}\ \text{ref}\ (^{\text{control}\ -\ \text{sample}})}$$

Normalisation

Normalisation is the process by which technical variation is accounted for (or removed) from the analysis, to allow for a true result and the determination of genuine biological variation.

Any normalisation applied should account for any technical variability from each step in a multifactorial qPCR protocol, from initial biological sample handling through to the analysis. However, it should be noted that an individual normalisation step may not account for any technical variability at an earlier or later stage, so multiple normalisation stages are recommended.

11.1.7.3. Biological sample normalisation

Most biological samples are inherently heterogeneous, differing in cell count, nucleic acid concentration and composition, with a greater variation noticeable when comparing healthy and diseased samples. While this is unavoidable due to the nature of the starting material, normalisation of the extracted nucleic acid will greatly assist in ensuring equivalent qualities/quantities of nucleic acid are tested across a panel of samples. This can be achieved by routine measurement using absorbance-based or fluorescence-based measurement methods (see 11.1.2. Nucleic acid measurement).

11.1.7.4. Assay normalisation

Assay normalisation is most easily achieved by the inclusion of external and internal quality controls (see above 11.1.5. Appropriate controls). By including controls of which their concentration/copy number are known, assessments can be made as to whether there are factors associated with each sample which is affecting the assay's PCR efficiency.

11.1.7.5. Analysis normalisation

Should there have been a "miss-dispense" with the amount reaction mix added to the tube or well, or variation in the optics shuttle light-path between wells when reading the fluorescence, this may affect the total amount of signal read, therefore affecting the results.

One way to account for these potential discrepancies is to include a passive reference dye in the reaction mix. This reference dye does not interfere with the chemistry of the PCR reaction or have any influence on the fluorescence generated from a genuine amplification event. The purpose of this reference dye is to normalise variation in instrument detection of the fluorescence values of the fluorophores associated with the target-specific amplification. One commonly used passive reference dye is ROX.

11.1.8. Data analysis

There are many factors which can be taken into account and adjusted during the run analysis to ensure the results obtained are as accurate as possible.

11.1.8.1. Baseline correction

qPCR measurements are based on amplification curves that are sensitive to background fluorescence. An increased baseline fluorescence may hinder the quantitative comparison of different samples, so therefore it is important to correct for this variation.

There are many factors which could contribute to this background fluorescence, including, but not limited to:

- Choice of plasticware in which the qPCR reactions were performed
- Unquenched probe
- Signal carryover into the neighbouring sample wells

One common way to account for this background fluorescence is to use the fluorescence observed in the early stages of the qPCR run (for example, within the first 3-10 cycles), identify the linear component and normalise the rest of sample signals against these readings. By using more cycles for the baseline fluorescence, the potential accuracy for the linear component increases. However, as the cycles progress so will the fluorescence (due to target amplification), therefore making these readings unsuitable for baseline correction.

11.1.8.2. Setting a threshold

The setting of the threshold is based on the principle that information related to the target quantity is available during the log-linear phase of the amplification curve. By reading the cycle for each log-linear curve, quantities for each sample can be determined. It is important for samples to be compared on the same reaction run - the threshold is set at the same point for all samples tested. It is important to ensure that the threshold is set:

- Above the fluorescence baseline, so no amplification curves cross the threshold prematurely due to background fluorescence.
- As low as possible, to ensure that the threshold crosses the log-linear phase of each sample, and not the plateau phase.

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