Manual

RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix manual

For Laboratory Use.

BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Manual

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1. Introduction

RapiDxFire™ Lyo-Flex 1-Step RT-qPCR 5X Master Mix contains thermostable heat-activated reverse transcriptase, thermostable hot-start Taq DNA polymerase, dNTPs and other components in a glycerol-free, Triton-free buffer specifically formulated for fast, reproducible multiplexing and has been optimised for RT-qPCR. It also contains dUTPs, and therefore UDG can be added to the reaction mix, if desired. A reference dye will need to be included in the master mix if the instrument requires one. The RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix is manufactured in an ISO 13485-certified facility, is a General Purpose Reagent (GPR) and is suitable for further molecular diagnostic test development.

RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix has been optimised for use with hydrolysis probes, including all LGC, Biosearch Technologies' probes (Dual-Labelled BHQ™, BHQplus™, and BHQnova™ which all contain Biosearch Technologies' proprietary BHQ dyes. BHQ dyes have been proven to dramatically reduce low-level background fluorescence due to highly efficient static quenching between reporter and quencher. BHQ dyes are compatible with reporter fluorescent dyes that span the visible spectrum, allowing for broad flexibility in fluorophore selection. RapiDxFire 1-Step Lyo-Flex RT-qPCR 5X Master Mix can also be used with any probe-based RT-qPCR assay. In addition, the Biosearch Technologies RealTimeDesign software and Spectral Overlay Tool are available online to facilitate the design of qPCR assays.

Key features of RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix include:

- 5X formulation offering flexible reaction setups and protocols
- Sensitive detection of both DNA and RNA targets
- Wide dynamic range for multiplexing
- 48-hour reaction benchtop stability (with no RNA template) and eight-hour fully assembled reaction benchtop stability, ideal for automated workflows
- Glycerol-free, Triton-free, high concentration, and bulk formulations for adaptable test development and lyophilisation options
- Manufactured in an ISO 13485-certified facility demonstrating batch to batch reproducibility.

2. Product specifications

2.1. Storage conditions

Storage at temperatures greater than 40 °C is not recommended.

Temperature	Duration of storage
4 °C	Up to 4 weeks
25 °C	Up to 7 days
40 °C	6-24 hours

Table 1. Recommended storage duration times for the RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix at different temperatures.

2.2. Handling recommendations

Avoid repeated freeze-thaw cycles (<10 cycles). To minimise the number of freeze-thaw cycles, we recommend aliquoting the RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix into smaller volumes, using nuclease-free, light-protected tubes/vials.

3. Customer provided reagents

a) Nucleic acid target sequence-specific primers and probes of appropriate T_M (oligonucleotide melting temperature) for example Biosearch Technologies BHQ Probes.
b) Template nucleic acid
c) 10 mM Tris, 0.1 M EDTA, pH 8.0-8.3, or other appropriate oligonucleotide rehydration diluent
d) Molecular-grade, nuclease-free water
e) Passive reference dye (e.g. ROX™), if desired
f) PCR microtitre plates/tubes
g) Optical plate seal
h) qPCR instrument (with filters appropriate for selected dyes)
i) qPCR analysis software

4. General guidelines

a) RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix has been optimised for RT-qPCR applications.
b) For quantification and/or concentration/copy number determination, it is recommended to follow the MIQE guidelines for qPCR.
c) Reaction conditions will vary for different primers/probes and targets. A 60 °C reverse transcription annealing temperature will work for most RNA (cDNA) targets. During primer design, ensure the target-specific oligonucleotides have a T_M appropriate for your chosen reaction temperature for both the heat activated thermostable reverse transcriptase and PCR amplification stage (reverse transcriptase is active at 55-80 °C).
d) The Mg^{2+} concentration may need to be increased prior to lyophilisation for optimal performance of certain assays.
e) Always use good laboratory practice. Wear gloves and use nuclease-free tips and reagents.

5. Recommended reaction volumes and concentrations

5.1. Reaction volumes

The following recommended reaction set-ups have been optimised for good-quality RNA. The table below outlines suggested plate formats for 25 µL, 10 µL and 5 µL final reaction volumes (Table 2).

Reaction volume	Suggested plate formats
10-25 µL	96-well plate
5-10 µL	384-well plate

Table 2. Suggested reaction volumes for different plate formats.

5.2. Final oligonucleotide concentrations

Final primer and probe concentrations will vary depending on the complexity of the target sequence, and the integrity/concentration of the RNA. Therefore, for all applications, we recommend the following final oligonucleotide concentrations (Table 3):

Oligonucleotide component	Final concentration
Primer	400-900 nM
Probe	200-400 nM

Table 3. Recommended final oligonucleotide concentrations.

5.3. Final RNA concentration

It is recommended to run 5-50 ng/µL RNA per reaction, although lower concentrations may be possible, depending on assay design.

RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix has been shown to work optimally using RNA purification chemistries (e.g. Biosearch Technologies sbeadex™ purification kits).

6. Oligonucleotide preparation and reaction set-up

When working with more complex target sequences, further optimisation may be required. Please see our online Reaction Estimator, to assist with estimating the number of reactions per stock of oligonucleotides and desired reaction conditions.

6.1. Preparation of stock oligonucleotides (100 µM)

Please see our Biosearch Technologies website for an Oligonucleotide Resuspension Calculator, which can assist with any calculations regarding the rehydration and dilution of BHQ Probes.

If ordering BHQ probes from Biosearch Technologies, and these are received as lyophilised stocks, it is recommended to rehydrate all oligonucleotides to 100 µM, from which further working stocks/reaction mixes can be made.

a) To calculate the volume of buffer required to resuspend the lyophilised stock to 100 µM, note the nmol amount (for example, 14.2 nmol). Multiply this number by 10 (14.2 × 10 = 142), and this is the volume, in µL (142 µL), which should be added to the tube to give a final concentration of 100 µM.
b) The recommended buffer for rehydration is 10 mM Tris, 0.1 M EDTA, pH 8.0-8.3. Other appropriate, molecular biology-grade, nuclease-free diluents may also be used for rehydration.

The reaction set-up detailed in Sections 6.2 and 6.3 is intended for guidance only. Conditions will vary for different primers and probes, and their targets. It is recommended to set up any new RT-qPCR protocol following the MIQE guidelines for qPCR. The reaction volumes are scalable, from volumes of 5-25 µL/reaction.

6.2. Preparation of working assays mixes (10X)

Please see our Biosearch Technologies website for an Oligo Dilution Calculator, which can assist with any calculations regarding the dilution of the BHQ probes for working assay mix generation.

It is advisable to prepare working stocks of BHQ assay mixes from the 100 µM rehydrated stocks to ensure standardisation across assays.

The following calculations are based on final concentration of 500 nM primer and 200 nM probe, to generate a 10X working concentration assay mix (Table 4):

Component	1X assay concentration	10X working concentration	10X working concentration volume
100 µM primer (each)	0.5 µM	5 µM	5 µL
100 µM probe (each)	0.2 µM	2 µM	2 µL
Diluent	-	-	To 100 µL
Total volume	**-**	**-**	**100 µL**

Table 4. Example for preparation of 10X working assay mix to allow for assay set-up with final oligonucleotide concentrations of 500 nM primer and 200 nM probe.

6.3. Reaction set-up

The reaction set-ups below are for one target per reaction tube/well, and therefore use a single 10X assay mix per reaction. For multiple targets, add the appropriate volume of 10X assay mix per assay. Both liquid (Table 5) and lyophilised examples below are for a 20 µL total reaction volume.

6.3.1. Reaction set-up using liquid RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix

Keep the reaction mix on ice until use.

Component	1X reaction	Final concentration
RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix	4 µL	1X
Assay Mix (10X)	2 µL	1X primer/1X probe
Template RNA	As required	As required
Total volume	**To 20 µL**	**-**

Table 5. Example of reaction set-up concentrations and volumes for RT-qPCR using liquid RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix.
*Template RNA can be added to bring the reaction to the desired total volume but may vary for different sample matrices i.e. those with inhibitory effects.
**Volume of water to be adjusted to account for any addition of passive reference dye.

NOTE: for multiplexing (>duplex), further optimisation and validation may be required to ensure reproducible assay sensitivity and specificity.

6.3.2. Reaction set-up using lyophilised RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix

Keep the reaction mix on ice until use.

Component	1X reaction	Final concentration
Assay Mix (10X)	2 µL	1X primer/1X probe
Template RNA*	As required	As required
Water**	To 20 µL	-

Table 6. Example of reaction set-up concentrations and volumes for RT-qPCR using lyophilised RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix pellets.
*Template RNA can be added to bring the reaction to the desired total volume but may vary for different sample matrices i.e. those with inhibitory effects.
**Volume of water to be adjusted to account for any addition of passive reference dye.

NOTE: for multiplexing (>duplex), further optimisation and validation may be required to ensure reproducible assay sensitivity and specificity.

7. Protocol

a) Completely thaw reaction components at room temperature. Before use, pipette the RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix. Vortex all other components. 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).
c) Pipette mix the reaction mix and aliquot one reaction volume into each reaction tube/qPCR reaction plate well.
d) Add your samples to the desired tubes/qPCR reaction plate wells and pipette mix.
e) Close the reaction tubes or seal qPCR reaction plate with a qPCR compatible plate seal.
f) 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.
g) 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 the selected probes.
h) Run the protocol until the thermal cycling has reached completion.

8. Thermal cycling protocols

The following thermal cycling protocols are for guidance only, for assays designed under standard conditions, using good-quality RNA. When working with non-standard assay design or with more complex target sequences, further optimisation may be required.

Step	Description	Temperature	Time	Number of cycles
1	RT activation	85 °C	3 minutes	1
2*	Reverse transcription	60 °C	5-15 minutes	1
3	hsTaq activation	95 °C	2 minutes	1
4	Denaturation	95 °C	10 seconds	50
	Annealing/extension	60 °C	30 seconds	

Table 7. Guide for thermal cycling protocol for RT-qPCR.
*Step 2 can be modified to account for the specific T_M of the primers/probes in the specific assay.

9. Troubleshooting

9.1. Poor or no signal

Possible cause	Recommended solutions
Incomplete activation of reverse transcriptase Without activation of the reverse transcriptase, conversion of RNA into cDNA, and then subsequent amplification of the cDNA during the PCR stage will not be possible.	Ensure the thermal cycling temperatures and times are correctly programmed (in particular step 1 in Table 7)
Presence of inhibitors These are typically carried over from the extraction/purification stage. Common inhibitors include phenol, detergents, proteases and organic compounds from the primary biological sample.	Dilute isolated RNA to minimise effect of any inhibitors. Test for the presence of inhibitors using an Internal Quality Control (IQC). Repeat nucleic acid extraction/purification protocol using alternative methods.
Suboptimal reaction components or reaction set-up The reaction set-up may have been performed incorrectly, or the RNA and/or oligonucleotide concentration/sequences may not be optimal.	One or more of the reaction components was not added. Repeat reaction set-up, ensuring all components are added, at the correct volumes. Verify the sequences of the oligonucleotides against target sequence. Ensure instrument setup is correct for each of the fluorophores selected for the reaction. RNA concentration was suboptimal. Quantify RNA to ensure concentration falls within desired ranges.
No RNA present in sample There is no RNA template in the sample.	Include known positive External Quality Controls (EQC) on the run to validate true-negative samples. Enzymatic degradation of RNA has occurred (e.g. via RNAses). Re-purify the RNA or repeat RNA isolation using alternative isolation method.

Table 8. Troubleshooting guidance for poor or no qPCR signal.

9.2. Amplification evident in non-template control (NTC samples)

Possible cause	Recommended solutions
Reaction mix is contaminated with nucleic acid There has been some form of carry-over of nucleic acid into the reaction mix, and/or surface contamination on the equipment. Random contamination is when several NTC wells show varying C_q values. A common (e.g. reagent) contamination is when all NTC wells show a similar C_q value.	Ensure all workstations and equipment are thoroughly cleaned before and after use. Follow equipment manufacturers' recommendations for use of ethanol and UV-light for decontamination procedures. Use nuclease-free consumables (e.g. tubes, plates, pipette tips) and molecular-grade reagents (e.g. water). Use filter-tipped disposable tips to minimise aerosol production during pipetting. Set up all reactions according to PCR best practices, following the recommendations outlined in the MIQE Guidelines (Section 12.3).
Primer-dimer formation Primers may anneal together, forming potential templates for non-target specific amplification.	Verify the sequences of the oligonucleotides against target sequence, checking for secondary-structure primer formation. Reduce primer concentration. Verify presence of primer-dimers via melt-curve analysis.

Table 9. Troubleshooting guidance for amplification evident in non-template control (NTC) samples.

9.3. Sigmoidal amplification curves (in logarithmic view)

Possible cause	Recommended solutions
Incorrect baseline correction (subtraction) settings The baseline correction is used to determine the level of fluorescence to subtract from all samples. It is used to reduce any background fluorescence from the results. By setting the baseline correction too low, insufficient background will be subtracted from the samples.	The upper baseline correction setting must be set to accommodate the sample with the earliest C_q value. Auto settings typically do this on a per well basis, meaning each target level will have different baseline settings. Reanalyse the amplification curve using an appropriate baseline correction value. Most qPCR analysis software has a 'Auto Baseline' correction setting, which may be used over 'Manual Baseline' correction to apply the correct baseline correction value.
High levels of fluorescence are detected in the early PCR cycles This can be an artefact of the assay itself, in which a specific assay generates considerably higher fluorescence compared with other assays on the same reaction plate. Suboptimal reaction set-up in which the components are not adequately mixed may also contribute to sigmoidal amplification curves.	The baseline correction can be set manually for the particular assay, to account for the high fluorescence in that specific assay. Ensure all reaction components are thoroughly mixed and centrifuged prior to thermal cycling.

Table 10. Troubleshooting guidance for sigmoidal amplification curves (in logarithmic view).

9.4. Amplification shows suboptimal efficiency

Possible cause	Recommended solutions
PCR efficiency above 110% This is typically due to either suboptimal RNA concentrations or the presence of inhibitors carried over from the nucleic acid extraction/ purification stage. Common inhibitors include phenol, detergents, proteases and organic compounds from the primary biological sample.	Enzymatic degradation of RNA has occurred (e.g. via RNAses). Re-purify the RNA or repeat RNA isolation using alternative isolation method. Dilute isolated RNA to minimise effect of any inhibitors. Test for the presence of inhibitors using an Internal Quality Control (IQC). Repeat extraction/purification protocol using alternative methods.
PCR efficiency below 90% The reaction set-up may have been performed incorrectly, or the RNA and/or oligonucleotide concentration/sequences may not be optimal. Oligonucleotide concentration may be limiting the rate of the reaction, particularly in multiplex reactions.	Verify the sequences of the oligonucleotides against target sequence. Verify integrity of reagents used (e.g. RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix has not undergone >10 free-thaw cycles). Ensure baseline is set appropriately. RNA concentration was suboptimal. Quantify RNA to ensure concentration falls within desired ranges. Ensure each target is validated as a singleplex reaction before combining in a multiplex, to determine the limiting oligonucleotide set.

Table 11. Troubleshooting guidance for suboptimal PCR efficiency.

10. Ordering information

Description	Volume	Product code
RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix	1 mL	30060-1
	10 mL	30060-2
	100 mL	30060-3

Table 12. Ordering information for RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix.

11. Further support

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

12. Appendix

12.1. Fluorophores and BHQ dye selection chart

Excitation and emissions spectra for commonly used fluorophores with their corresponding recommended BHQ dye.

	Fluorophore	Alternate dyes	DYE-5' - T_{10} Excitation	DYE-5' - T_{10} Emission	Recommended quencher	BHQ™ dye quenching range
○	**Biosearch Blue™**		356	454	BHQ-1	**BHQ-0** *430-520 nm*
	FAM		495	520	BHQ-1	
	TET		521	536	BHQ-1	
○	**CAL Fluor™ Gold 540**	*VIC®/TET/JOE*	522	544	BHQ-1	
○	**CIV-550™**	*VIC*	530	550	BHQ-1	
	VIC	*CIV-550/CAL Fluor Orange 560*	530	550		
	JOE		529	555	BHQ-1	
	HEX		535	556	BHQ-1	**BHQ-1**
○	**CAL Fluor Orange 560**	*VIC/HEX/JOE/CIV-550*	538	559	BHQ-1	*480-580 nm*
○	**Quasar™ 570**	*Cy 3*	548	566	BHQ-2	
	Cy™3		549	566		
	TAMRA		557	583	BHQ-2	
○	**CAL Fluor Red 590**	*TAMRA*	569	591	BHQ-2	**BHQ-2***
	ROX™		586	610	BHQ-2	
○	**CAL Fluor Red 610**	*Texas Red/ROX/Alexa Fluor® 594*	590	610	BHQ-2	
	Cy 3.5		594	612		
	Texas Red®		597	616		
	LC Red® 640		619	637		
○	**CAL Fluor Red 635**	*LC® Red 640*	618	637	BHQ-2	
	Cy 5		646	669		
○	**Quasar 670**	*Cy 5*	647	670	BHQ-2*, BHQ-3	
○	**Quasar 705**	*Cy 5.5*	690	705	BHQ-2*, BHQ-3	**BHQ-3** *620-730 nm*
	Cy 5.5		694	711		

○ Indicates Biosearch Technologies' proprietary dyes

Dyes in **BOLDFACE** are available modifications for labeled oligos.

Table 13. Excitation and emission spectra for commonly used fluorophores. Fluorophores (with their corresponding recommended BHQ dye) highlighted in bold are available from Biosearch Technologies.
Many dyes are also available in the form of nucleic acid chemistry reagents.
*BHQ-2 dye is recommended for **Quasar 670** and **Quasar 705** fluorophores due to static quenching.

12.2. Instrumentation calibration standards

Calibration standards are available from Biosearch Technologies to allow for improved signal deconvolution in qPCR instruments and fluorescent plate readers and enables the instrument to store relevant fluorescent profiles of each dye to control for crosstalk between filter channels. Calibration standards are available for FAM, CAL Fluor and Quasar dyes, and are all available in 5 nmol scales. Please see our Dye Calibration Standards webpage for further details.

12.3. 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)

12.3.1. 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.

12.3.2. 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.

12.3.3. Contamination

Regarding 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 (i.e. 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.

12.3.4. 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. This causes difficulty in, for example, the assigning of genotypes or lead to an incorrect interpretation of relative target quantities.

Common inhibitors include Tris, ethanol, isopropanol, EDTA, guanidine salts (e.g. 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.

12.3.5. Appropriate controls

It is 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, if the other controls have shown to be within acceptable detection ranges.

12.3.5.1. Non-template controls

These are reactions which contain all the same PCR components as the other reactions, but with no target nucleic acid (in some instances, molecular-grade water can be used in place of nucleic acid 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.

12.3.5.2. External quality controls (EQC)

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 (i.e. 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.
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 (EQC) and non-template control (NTC) results.

12.3.5.3. Internal quality controls (IQC)

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.

Sample result	IQC 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 nucleic acid sample until IQC is uninhibited to normal levels.
Positive	Negative	True positive result, though inhibition is occurring. For accurate quantification, serially dilute nucleic acid 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.

12.3.6. 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.

12.3.6.1. 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 (either 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 (e.g. different patients) or different sample types (e.g. 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 (e.g. multiple qPCR reactions from the same nucleic acid 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 (e.g. no temperature variations across a thermal cycling heat block).

12.3.6.2. Assay optimisation

Assay optimisation is crucial to ensure that the qPCR is performing at its optimal efficiency, and there are several 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 ideal 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 T_M optimisation is not possible. Three-step cycles (denaturing, with separate annealing and extension stages) are preferable for more complex target sequences and allows for T_M optimisation.

The concentration of magnesium chloride ($MgCl_2$) 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 and
- 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).

12.3.7. 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.
- Ruggedness – 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.
- 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.

12.3.7.1. 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 C_q values are determined from the amplification plot. These C_q 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 is 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 (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 C_q determined, and the concentration/copy number extrapolated from the standard curve.

12.3.7.2. Comparative quantification

Comparative quantification is used to measure the relative change in expression levels between samples under different experimental conditions or over 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 C_q (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 be incorrectly assumed and secondly, comparing C_qs from different assays is problematic, as C_q is an arbitrary value rather than a defined unit. Therefore, the following equation is applied to take into account these inaccuracies:

$$\text{Ratio} = \frac{(E_{target})^{\Delta CP\ (control - sample)}_{target}}{(E_{target})^{\Delta CP\ (control - sample)}_{ref}}$$

12.3.8. 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.

12.3.8.1. 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 section Nucleic acid measurement).

12.3.8.2. Assay normalisation

Assay normalisation is most easily achieved by the inclusion of external and internal quality controls (see section 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.

12.3.8.3. Analysis normalisation

Should there have been a dispensing error 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 what is known as 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 be measured and then used to normalise the fluorescence values of the fluorophores associated with the target-specific amplification. One commonly used passive reference dye is ROX.

12.3.9. Data analysis

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

12.3.9.1. 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.

12.3.9.2. 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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