Technical note

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

Introduction

RapiDxFire™ Lyo-Flex 1-Step RT-qPCR 5X Master Mix, from LGC Biosearch Techologies™, is a lyophilisation-compatible, 5X concentrated master mix, ideal for both molecular diagnostic (MDx) applications for the detection of both bacterial and viral pathogens, as well as for target-sensitive research projects. With input from MDx assay manufacturers, we have developed a highly sensitive master mix which incorporates a heat-activated thermostable reverse transcriptase, that will allow for the detection of more complex targets (e.g. organisms with a double-stranded RNA genome and targets with inherent RNA-secondary structure). Coupled with a highly efficient *Taq* polymerase for rapid and sensitive target amplification, the RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix may be qualified for a variety of laboratory environments as well as point-of-care (POC) devices using its lyophilised state.

RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix is intended for use as a general purpose reagent (GPR) in molecular diagnostic assays that are based on RT-PCR target detection technologies by clinical laboratory professionals located in the United States. This product must be qualified and validated by clinical laboratory end users for suitability in the detection of any specific target using the procedures in this technical note as guidance. This product is labelled For Laboratory Use (FLU). Outside the United States the FLU statement is equivalent to Research Use Only (RUO).

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GENOMIC ANALYSIS BY LGC

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Once qualified for the assay by the end user, RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix can be combined with gene-specific primers and hydrolysis probes (e.g. probes from the Biosearch Technologies BHQ portfolio) for immediate use in high-throughput laboratory developed tests (LDTs). Provided at 5X concentration without a passive reference dye, this flexible master mix allows more volume for the sample and multiple targets for detection at one time.

In addition, RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix is lyophilisation compatible. Due to the absence of glycerol or other lyophilisation-interfering components, we not only demonstrate robust stability in liquid formulation, but robust stability once lyophilised.

Here we demonstrate the analytical sensitivity and specificity of the RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix for both RNA and DNA genomes, its effectiveness at single-tube multiplexing in an MDx scenario, and the stability of the reaction components under various storage and handling conditions.

Methods

All data were generated using manufacturer-confirmed concentrations of target material (genomic MS2 phage RNA, genomic mouse RNA, AccuPlex™ SARS-CoV-2 synthetic material and rotavirus type A RNA). For the MS2 phage RNA assays, four different regions of the MS2 genome were targeted, each with a unique probe to allow for multiplexing (R6D1, R3D3, R8D1 and R5D1, using probes labelled with FAM, CAL Fluor Orange 560 (or HEX), Quasar 670 (or Cy5) and Quasar 705 (or Quasar 670), respectively), unless otherwise stated. All reactions were carried out in a 20 µL total reaction volume.

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Sensitivity

For the lower limit of detection (LLoD) testing, genomic MS2 phage RNA and genomic mouse DNA were diluted down to 100, 50, 25 and 10 copies per reaction, with 60 replicates at each target concentration. Four MS2 targets were detected (R6D1, R3D3, R8D1 and R5D1). Mouse DNA was detected using an Isg20-specific assay, labelled with a single fluorophore (FAM). Each assay was performed with 60 replicates at each target concentration. The in-house MS2 assays and the mouse Isg20 assay were run on a BIO-RAD CFX, with an annealing temperature of 60 °C.

a)

Assay/fluorophore	Copies/reaction	+/total	Cq average	Cq standard deviation
R6D1/FAM	100	60/60	33.0	0.48
	50	60/60	34.0	0.62
	25	60/60	34.7	0.64
	10	59/60	36.5	0.84
R3D3/CAL Fluor Orange 560	100	60/60	33.4	0.63
	50	60/60	34.4	0.78
	25	57/60	35.3	1.12
	10	38/60	39.7	4.34
R8D1/Quasar 670	100	60/60	34.1	1.06
	50	60/60	35.3	0.89
	25	53/60	36.7	2.78
	10	38/60	40.0	3.60
R5D1/Quasar 705	100	60/60	34.1	0.95
	50	60/60	35.0	0.87
	25	57/60	35.8	1.03
	10	42/60	39.6	3.44

b)

Fluorophore	Copies/reaction	+/total	Cq average	Cq standard deviation
Mouse Isg20	100	60/60	32.6	0.3
	50	60/60	33.5	0.3
	25	58/60	33.4	6.3
	10	59/60	35.9	0.5

Table 1. High levels of sensitivity for detection of both genomic MS2 RNA and genomic mouse DNA, within the same sample type. Genomic targets were diluted to 100, 50, 25 and 10 copies per reaction (20 µL total reaction volume), with 60 replicates at each target concentration. (a) For the MS2 RNA, each MS2-region specific BHQ Probe was assigned a unique fluorophore (R6D1:FAM; R3D3:CAL Fluor Orange 560; R8D1:Quasar 705; R5D1:Quasar 670). (b) For the mouse DNA, an Isg20 detection assay was performed (FAM). The lower-limit of detection (LLoD) is highlighted. LLoD is defined as lowest target level at which 57/60 replicates have Cq<40.

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Dynamic range

For dynamic range testing, genomic MS2 phage RNA was 10-fold serially diluted from 1 × 10^7 – 10 copies per reaction on which the in-house MS2 R6D1 assay was performed, labelled with a single fluorophore (FAM). Genomic mouse DNA was 10-fold serially diluted from 300,000 – 3 copies per reaction on which the Isg20-specific assay was performed, labelled with a single fluorophore (FAM). Each assay was performed with 6 replicates at each target concentration. The in-house MS2 assays and the mouse Isg20 assay were run on a BIO-RAD CFX, with an annealing temperature of 60 °C.

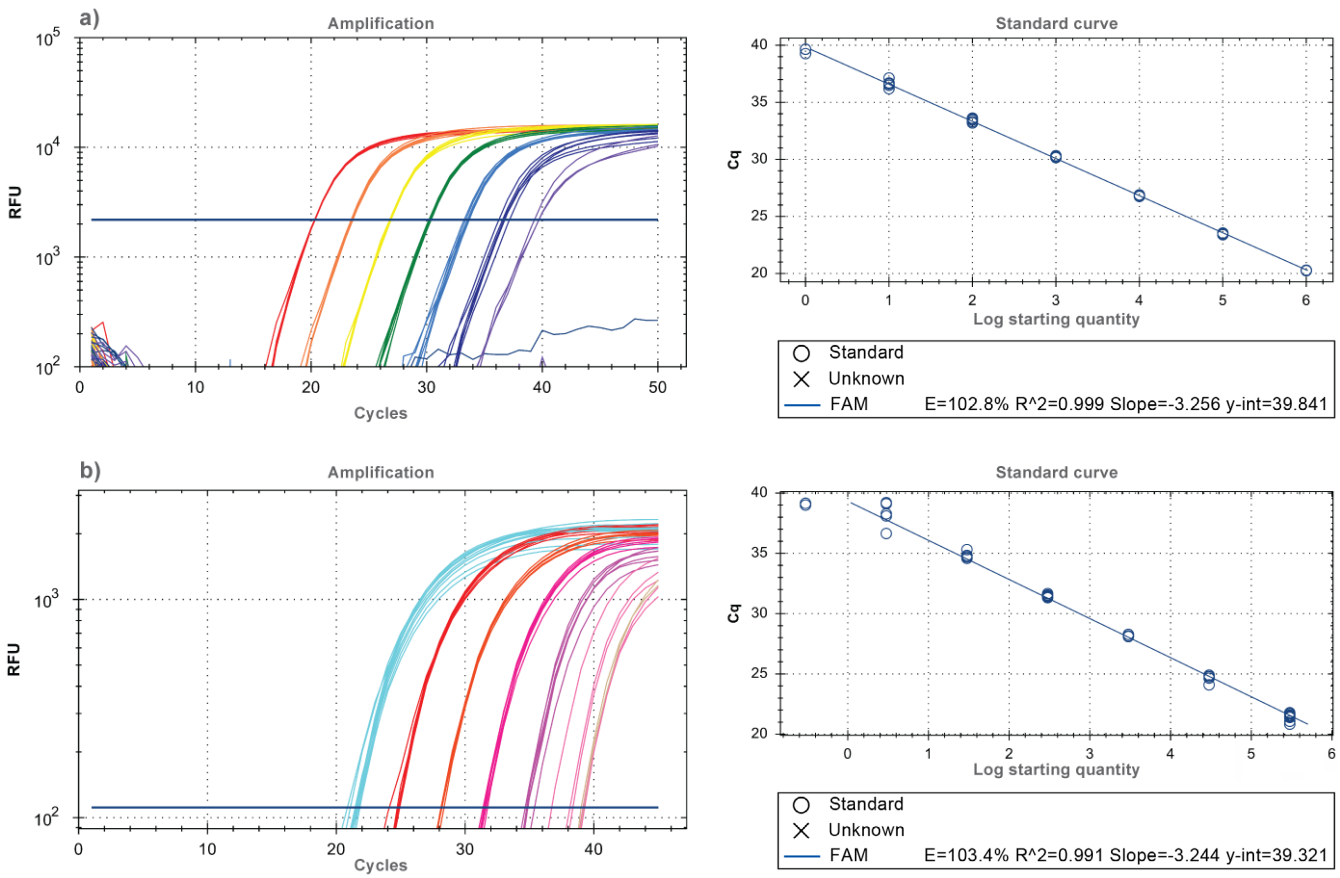

Figure 1. Broad dynamic range for both RNA and DNA targets. (a) SARS-CoV-2 RNA control (Twist) 10-fold serially diluted from 1 × 10^6-1 × 10^0 copies per reaction, on which the UltraDx SARS-CoV-2 N1/N2/RnP assay (Biosearch Technologies) was performed (N1/N2 shown) (20 µL total reaction volume). (b) Genomic mouse DNA 10-fold serially diluted from 300,000 to 3 copies per reaction on which the Isg20 assay was performed (FAM) (20 µL total reaction volume). PCR efficiencies (90-110%) and R^2 values >0.98 for (a) SARS-CoV-2 RNA and (b) genomic mouse DNA assays met PCR criteria.

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Benchtop stability

For benchtop stability testing, genomic MS2 phage RNA was 10-fold serially diluted from 1×10^6-1×10^2 copies per reaction, with 6 replicates at each target concentration and time point. The fully assembled reaction mix was kept at room temperature for 0, 2, 4 and 8 hours, before running the assay on RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix, Competitor A Master Mix, and Competitor B Master Mix. Three MS2 targets were detected (R6D1, R3D3 and R8D1). The in-house MS2 assays were run on a BIO-RAD CFX, with an annealing temperature of 60 °C.

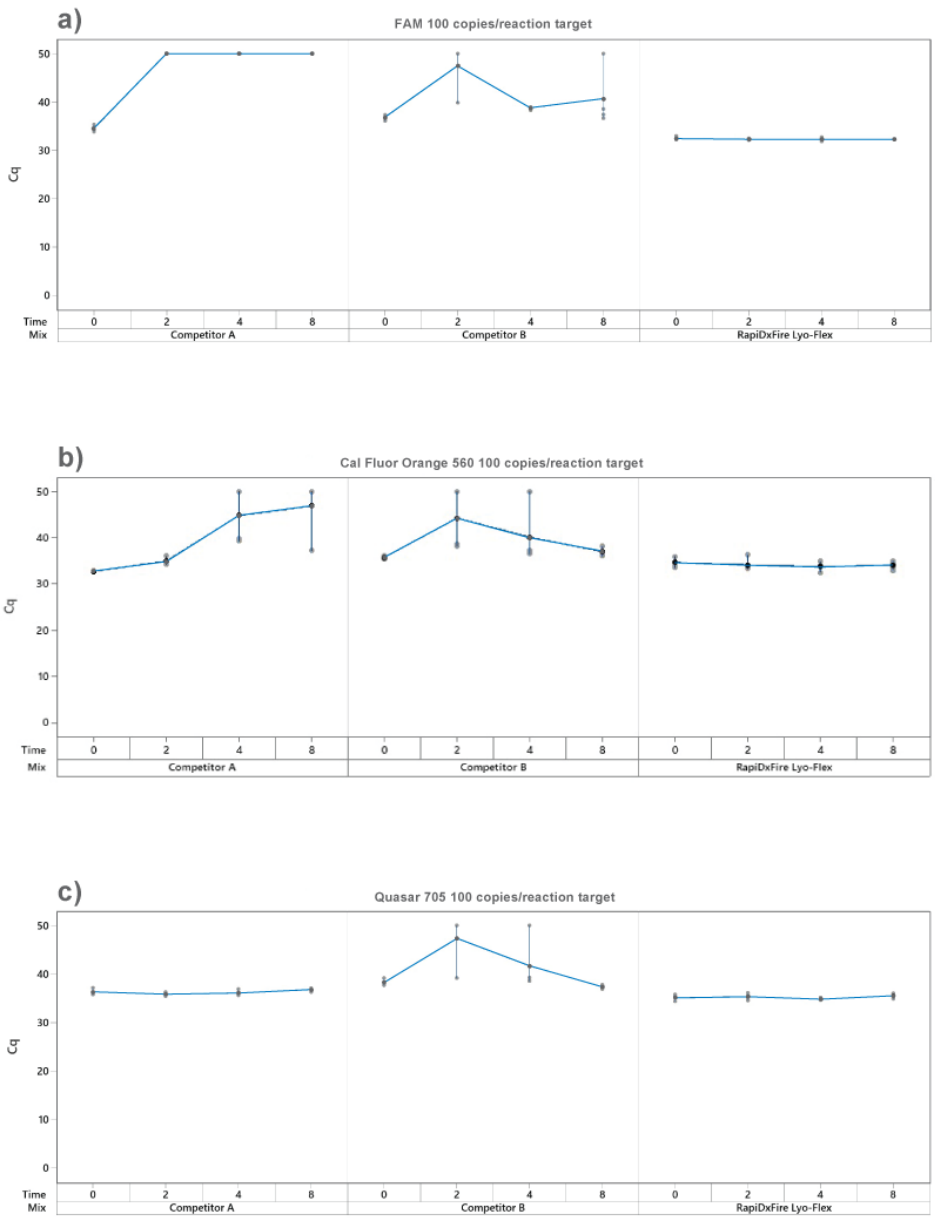

Figure 2. Fully assembled reaction mix at room temperature gives stable and sensitive Cq values for all targets for up to 8 hours . Genomic MS2 RNA (100 copies per reaction, 20 µL total reaction volume) tested in triplex with each MS2-region specific BHQ™ Probe assigned a unique fluorophore ((a) R6D1:FAM; (b) R3D3:CAL Fluor Orange 560 (c) R8D1:Quasar 705). RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix was tested against Competitor A and Competitor B, according to manufacturer's recommendations. The lower the Cq value, the more sensitive the detection. A Cq value >50 is indicative of no amplification.

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Compatibility with difficult targets

To demonstrate RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix compatibility with difficult targets, we used double-stranded RNA (dsRNA) template isolated from rotavirus type A (family *Reoviridae*), in comparison with Competitor C. Rotavirus dsRNA was serially diluted from 50,000 – 5 copies per reaction, with 6 replicates at each target concentration. An assay was designed to detect a single rotavirus. The in-house rotavirus assay was run on a BIO-RAD CFX, with an annealing temperature of 60 °C, with no separate pre-denaturation step. Competitor C master mix was tested according to the manufacturer's instructions.

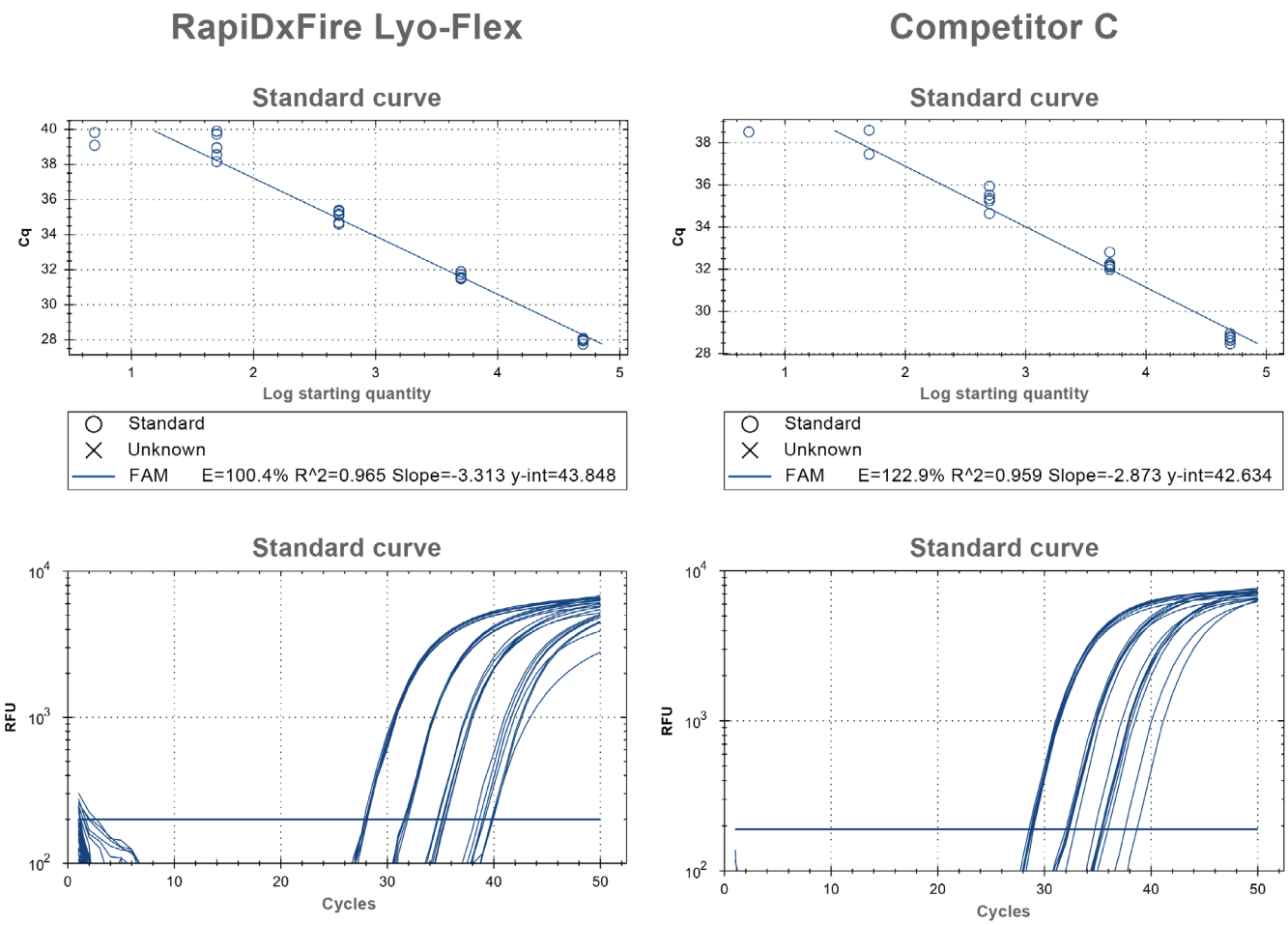

Figure 3. High performance when tested against a complex dsRNA target (rotavirus type A). Rotavirus dsRNA 10-fold serially diluted from 50000 – 5 copies per reaction (20 µL total reaction volume), with the rotavirus-specific BHQ Probe assigned the FAM fluorophore and tested against Competitor C master mix. PCR efficiencies and R^2 values were calculated. All targets met PCR efficiency criteria (90-110%) and demonstrated an R^2 value >0.98, with the only outlier at 0.97. Competitor C master mix was tested according to manufacturer's instructions.

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Compatibility with QuickExtract™ DNA Extraction Solution

To demonstrate RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix compatibility with a nucleic acid extraction chemistry, AccuPlex SARS-CoV-2 synthetic material was extracted using QuickExtract DNA Extraction Solution. This was tested against N1, N2 and RNase P targets. N1, N2, and RNase P are US-CDC designs, and the initial CDC EUA was reported with recommended assay conditions; Biosearch Technologies modified the conditions for our EUA/CEIVD assays but not the design of the N1, N2 primer probe combinations. (250, 25 and 2.5 copies per reaction for the N1 and N2 targets and 12.5 copies per reaction for RNase P), with an annealing temperature of 60 °C.

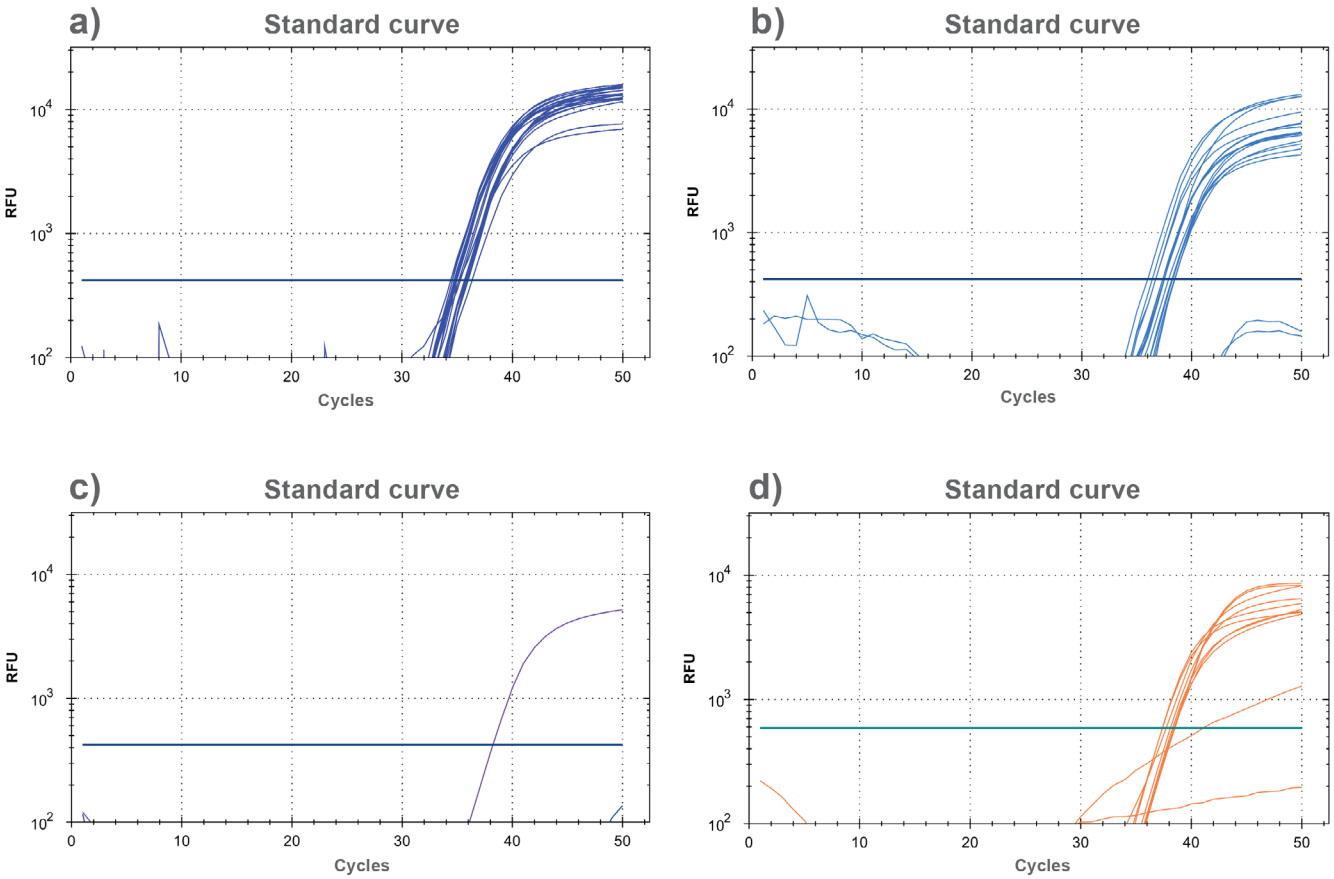

Figure 4. Compatibility with QuickExtract demonstrated by efficient nucleic acid isolation, amplification and detection of the AccuPlex SARS-CoV-2 Verification Panel at (a) 250, (b) 25 and (c) 2.5 copies per reaction by N1- and N2-specific assays (FAM), and (d) 12.5 copies per reaction by RNase P (FAM) (20 µL total reaction volume). With 20 replicates per dilution and a pass criteria of >95% at 100 copies or more, for SARS-CoV-2, 100% detection was achieved for 250 copies per reaction and 75% detection at 25 copies per reaction. All targets met PCR efficiency criteria (90-110%) and demonstrated an R^2 value >0.98.

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Consistent lot-to-lot reproducibility

To demonstrate consistency across different lots of RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix, genomic MS2 phage RNA was 10-fold serially diluted from 1 × 10^7 – 1 × 10^2 copies per reaction, with 6 replicates at each target concentration. The fully assembled reaction mix was kept at room temperature for 0 and 4 hours, before running the assays. Four MS2 targets were detected (R6D1, R3D3, R8D1 and R5D1). The in-house MS2 assays were run on a BIO-RAD CFX, with an annealing temperature of 60 °C.

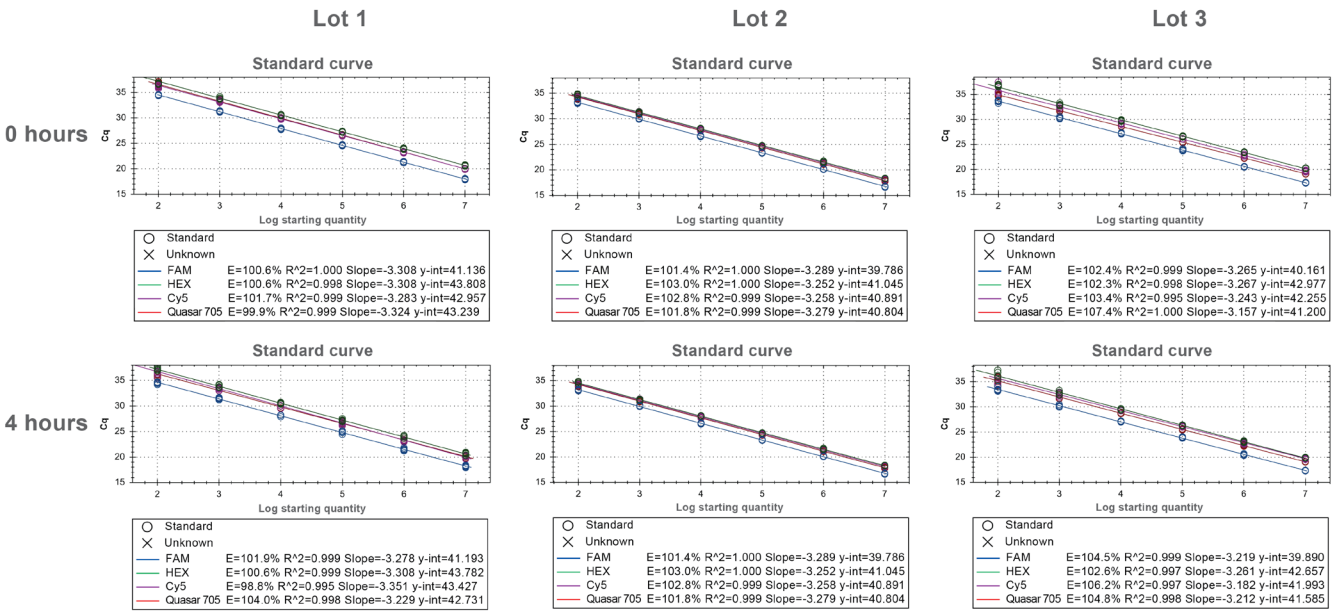

Figure 5. Highly consistent and reproducible results between three different lots of fully assembled reaction mix at 0 and 4 hour time-points. Genomic MS2 RNA 10-fold serially diluted from 1 × 10^7-1 × 10^2 copies per reaction (20 µL total reaction volume, 6 replicates per target), with each MS2-region specific BHQ Probe assigned a unique fluorophore (R6D1:FAM; R3D3:HEX; R8D1:Cy5; R5D1:Quasar 670). PCR efficiencies and R^2 values were calculated for 0 hours and 4 hours for 4 MS2 targets. At both the 0 and 4 hour time points, all 4 targets met PCR efficiency criteria (90-110%) and demonstrated an R^2 value >0.98 (the only outlier being the Cy5 target at 4 hours for lot 28143, which had a PCR efficiency of 112.1%).

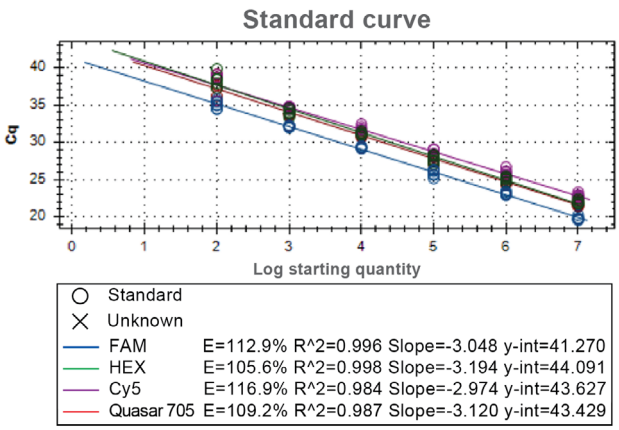

Figure 6. Stability demonstrated for up to fourteen days and 25 °C. Genomic MS2 RNA 10-fold serially diluted from 1 × 10^7-1 × 10^2 copies per reaction (20 µL total reaction volume, 6 replicates per target), with each MS2-region specific BHQ Probe assigned a unique fluorophore (R6D1:FAM; R3D3:HEX; R8D1:Cy5; R5D1:Quasar 670). PCR efficiencies and R^2 values were calculated for 4 MS2 targets. All 4 targets met PCR efficiency criteria (90-110%) and demonstrated an R^2 value >0.98 (the only outlier being the Cy5 target, which had a PCR efficiency of 116.9%).

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Stability during transport and storage

To demonstrate stability of the RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix for up to fourteen days when stored at 25 °C (to replicate transport and storage conditions), genomic MS2 phage RNA was amplified from 10-fold serially diluted target ($1 \times 10^7 – 1 \times 10^2$ copies per reaction). Four MS2 targets were detected (R6D1, R3D3, R8D1 and R5D1). The in-house MS2 assays were run on a BIO-RAD CFX, with an annealing temperature of 60 °C.

Lyophilisation compatibility and stability

To demonstrate RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix compatibility with lyophilisation, lyobeads were manufactured by Vendor A (supplemented with 4 mM $MgCl_2$). Genomic MS2 RNA was phage RNA was 10-fold serially diluted from 1×10^7-1×10^2 copies per reaction, with 6 replicates at each target concentration. Four MS2 targets were detected (R6D1, R3D3, R8D1 and R5D1). The in-house MS2 assays were run on a BIO-RAD CFX, with an annealing temperature of 60 °C.

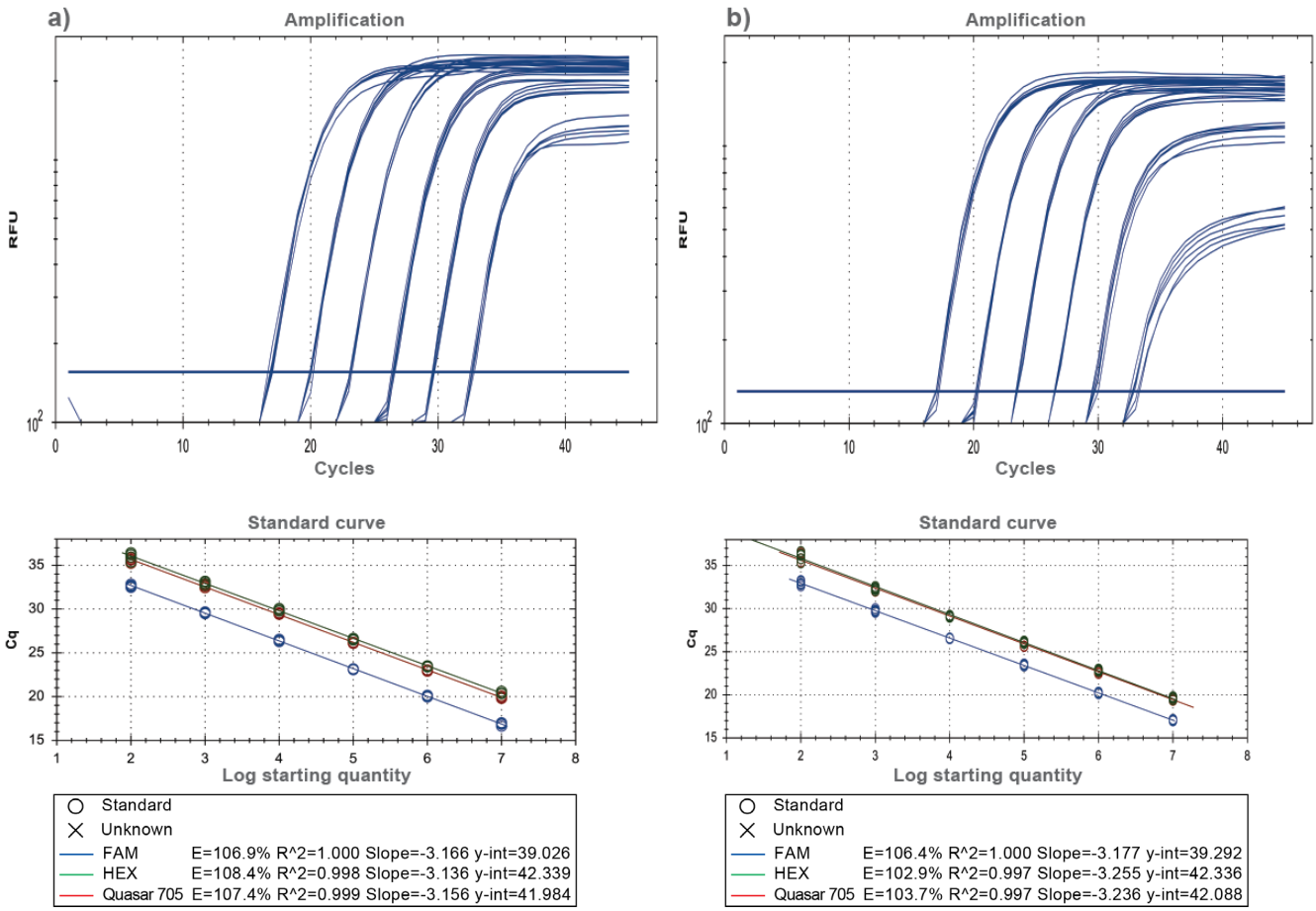

Figure 7. Consistent performance of RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix post-lyophilisation by Vendor A, supplemented with 4 mM $MgCl_2$. Lyophilised spheres were tested against genomic MS2 RNA 10-fold serially diluted from 1×10^7-1×10^2 copies per reaction (20 µL total reaction volume), with each MS2-region specific BHQ Probe assigned a unique fluorophore (R6D1:FAM; R3D3:CAL Fluor Orange 560; R8D1:Quasar 705). (a) Pre-lyophilisation and (b) post lyophilisation amplification curves and standard curves for all targets tested with the R6D1:FAM assay, with PCR efficiencies (90-110%) and R^2 values >0.98, meeting PCR criteria.

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Results and discussion

The LLoD, as defined by the Minimum Information for Publication for Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin, *et al.* 2009), is “the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure”. In figure 1 we demonstrate the RapiDxFire Lyo-Flex 1-Step RT-qPCR Master Mix has an LLoD of between 10 and 50 copies per reaction for the MS2 RNA targets (figure 1a) and an LLoD of 10 copies per reaction for the mouse DNA Isg20 target (figure 1b).

Reproducibility of replicates of a known target copy number across a range of Cq values is used to evaluate the dynamic range across a range of 10-fold serial dilutions. In figure 2 we demonstrate that the dynamic range of the RapiDxFire Lyo-Flex 1-Step RT-qPCR Master Mix across both the MS2 RNA and mouse DNA targets is comparable, indicating high reproducibility and sensitivity from 1×10^7-1×10 copies per reaction for RNA (figure 2a), and from 300,000 – 3 copies per reaction for DNA (figure 2b). This is also reflected in the PCR efficiency and R^2 data (table 1).

When working on large-scale instrumentation platforms or with high-sample numbers, it is important to ensure that any pre-assembled reaction mixes are stable for an extended period, which will allow for flexibility in laboratory processes. In figure 3 we demonstrate that a fully-assembled RapiDxFire Lyo-Flex 1-Step RT-qPCR reaction mix is stable at room temperature (20-25 °C) for up to 8 hours. All three MS2 assays met performance criteria for PCR efficiency and R^2 calculations after 4 hours at room temperature, and three out of four assays met performance criteria for PCR efficiency and R^2 calculations after 8 hours at room temperature.

In figure 4 we demonstrate, when compared to Competitor C, RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix achieved equally robust PCR efficiency and R^2 calculations when testing for dsRNA rotavirus targets, even down to 5 copies per reaction.

We demonstrate compatibility of RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix with our QuickExtract DNA Extraction Solution using the AccuPlex SARS-CoV-2 Verification Panel (figure 5). Efficient amplification and detection at 250 and 25 copies per reaction for N1 and N2 SARS-CoV-2 targets and robust PCR efficiency and R^2 calculations were achieved

Lot-to-lot stability is essential to ensure reproducibility. In figure 6 we demonstrate that serially diluted MS2 RNA targets were successfully amplified and detected at both 0- and 4-hour time points across 3 different lots, and apart from a single Cy5 outlier at 4 hours, all targets met performance criteria for PCR efficiency and R^2 calculations.

Here we have demonstrated that RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix has the capability of delivering high performance results under different experimental conditions. figure 7 demonstrates robustness of this master mix is shown by high levels of sensitivity and specificity after prolonged storage of 14 days at 25 °C, when compared against Competitor C, when tested for 4 MS2 RNA targets.

Lyophilisation of RT-qPCR master mixes is an important property for customer considering non-standard shipping, transport and/or point-of-care testing. In figure 8 we demonstrate does RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix show robust performance post-lyophilisation as demonstrated in all cases by all assays meeting performance criteria for PCR efficiency and R^2 calculations.

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Summary

As demonstrated here, RapiDxFire Lyo-Flex 1-Step RT-qPCR 5X Master Mix is a robust, sensitive and specific 1-step, 1-tube master mix, applicable for the detection of genomic targets from a range of organisms, comprising of either RNA or DNA genomes. We have shown that we can accurately define a LLoD to 50 copies per reaction and lower, along with reproducible and consistent amplification efficiency down to either at least 10 copies per reaction for RNA or 3 copies per reaction for DNA. In addition, stability at room temperature of up to 8 hours allows for flexibility in laboratory workflows. Coupled with the option for lyophilisation in a concentrated 5X working stock, robustness for complex targets, and compatibility with extraction chemistries, such as our QuickExtract DNA Extraction Solution, RapiDxFire Lyo-Flex 1-Step RT-qPCR Master Mix is an ideal choice for highly sensitive MDx, POC and research applications.

References

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

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.

This product is classified as a general purpose in vitro diagnostic device reagent in the United States as defined in: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?FR=864.4010.

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