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Description automatically generated **Figure S1.**Establishing optimal probe, primer and Taq polymerase concentrations with B47 and the CoV-E assay. **A.** Amplification plots and Cq values recorded with a range of probe concentrations using protocol P1. **B.** Amplification plots and Cq values recorded with a range of primer concentrations using protocol P1. **C.** Amplification plots and replicate Cq values recorded with 0.06 and 0.12U of MyTaq per 2.5µL reaction across the denaturation gradient protocol P2. **D.** Amplification plots and replicate Cq values recorded with 0.06 and 0.12U of MyTaq per 2.5µL reaction across the polymerisation gradient protocol P3.

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**Figure S2.**Amplification of SARS-CoV-2 cDNA with assay CoV-E, B47 and MyTaq run on BioRad CFX Connect or BMS Mic qPCR instruments. **A.** ∆Cq values (±95% CI) recorded at the indicated cycling temperatures versus protocol P1 for the combined runs on the BioRad instrument. Cq values are listed in the supplementary data file. **B.** Amplification plots and Cq values for standard (P8) and fast protocols (P9) on the BMS Mic. **C.** ∆Cq values (±95% CI) recorded for P9 compared to P8.

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**Figure S3.**  Amplification of SARS-CoV-2 PCR amplicons with DNA primers, Pentabase primers (PB), Probe G and B47. **A.** Amplification plots and Cqs recorded on a denaturation gradient using protocol P10. **B.** Amplification plots and Cqs recorded on a polymerisation gradient using protocol P3. **C.** ∆Cq values (±95% CI) recorded at the indicated cycling temperatures versus protocol P1 for the DNA (blue) and Pentabase (brown) primers.

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**Figure S4.** Linearity and efficiency of 4E-2 (G) qPCR assay. **A.** Amplification plots and Cq values recorded under standard PCR conditions with SensiFast master mix. **B.** Amplification plots and Cq values recorded under reduced ∆T conditions with buffer 47 and MyTaq polymerase. **C.** Standard curves for SensiFast (blue) and B47 (pink) with 95% CI.

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**Figure S7.**  2-step RT-qPCR reactions with SARS-CoV-2 gRNA reverse transcribed with native RT buffers or B47 and using the CoV-E assay. **A.** Amplification plots and Cq values recorded for mRNA reverse transcribed with EpiScript (ES) in its native buffer (n=7) or B47 (n=7) and amplified with SensiFast on a Hybrid PrimePro 48 instrument. **B.** ∆Cq values (±95% CI) recorded for the combined mRNA samples reverse transcribed with buffer 47 versus the Cqs recorded from mRNA samples reverse transcribed with native buffers. **C.** Amplification plots recorded for mRNA reverse transcribed with UltraScript-2 (US-2) in its native buffer (n=4) or B47 (n=4) and amplified with B47 on a BioRad Opus instrument. **D.** ∆Cq values (±95% CI) recorded for the combined mRNA samples reverse transcribed with buffer 47 versus the Cqs recorded from mRNA samples reverse transcribed with native buffers.

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**Figure S8.**  1-step RT-qPCR reactions with human breast cancer mRNA using NEB Luna or EpiScript/B47 with MyTaq or GoTaq or ExTaq DNA polymerases 1-step master mixes. **A.** Amplification plots and Cq values for TSG-6 (FAM) and HGF-1 (HEX) on the PrimePro 48. **B.** Amplification plots and Cq values for TSG-6 (FAM) and HGF-1 (HEX) on the BioRad CFX Connect. **C.** Plot of Cq values (±SD) for TSG on the PrimePro 48. **D.** Plot of Cq values (±SD) for TSG on the BioRad CFX Connect. **E.** Plot of Cq values (±SD) for HGF-1 on the PrimePro 48. **F.** Plot of Cq values (±SD) for HGF-1 on the BioRad CFX Connect.

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**Figure S9.**  Comparison of 1-step RT-qPCR reactions carried out targeting human breast cancer RNA using duplex TSG-6/HGF-1 assays with and without a dedicated RT step. **A.** 1-step RT-qPCR protocol (R2) run on a BioRad CFX Connect. **B.** Amplification plots and Cq values for PCRBio Clara, NEB Luna and B47/ES/MyTaq 1-step master mixes using the standard protocol**. C.** 1-step RT-qPCR protocol R3 without a dedicated RT step. **D.** Amplification plots and Cq values for PCRBio Clara, NEB Luna and B47/ES/MyTaq 1-step master mixes using the modified protocol. **E.** ∆Cq values (±95% CI) of the reactions carried out without a dedicated RT step (RT-, protocol R3) versus those carried out with a dedicated RT step (RT+, protocol R2).