DETERMINING OPTIMAL RUNNING CONDITIONS FOR qPCR MARKERS

1. INTRODUCTION

1.1. This method describes the initial steps for validating reagents for new qPCR markers. These steps are used to determine the optimal running conditions in the DEP laboratory, including the proper primer/probe and standard curve concentrations, the temperature, timing, and number of amplification cycles.

1.2. This method is essential for determining the efficiency, repeatability, and quantifiable range of a new marker. These results help to determine optimal assay conditions that will ensure higher molecular sensitivity, specificity, and precision.

2. PREPARE REAGENTS

2.1. Primers and probe preparation

2.1.1. Resuspend primers and probe in Tris-EDTA (TE) buffer. The typical stock concentrations are 500 µM for each primer and 100 µM for the probe. The data sheet provided with the primers and probe will list how much TE buffer is needed to reconstitute the primer/probe to a 100 µM concentration. For a 500 µM concentration, divide this number by 5.

2.2. gBlock preparation

2.2.1. Resuspend the gBlock standard. To resuspend, centrifuge the tube briefly then add TE buffer to reach a final concentration of 10 ng/µL. Vortex briefly, then incubate at 50°C for 20 minutes in benchtop incubator. Briefly vortex and centrifuge.

2.3. Measure gBlock standard on Qubit and calculate the concentration for each dilution.

2.3.1. Refer to SOPs PCR-2.0, 3.2, 3.3, 3.4, 3.5, and 3.6 for details on making dilutions, using the Qubit, and performing calculations.

2.3.2. The number of target sequence copies per nanogram of DNA will depend on the size of the gBlock fragment and must be determined for each new gBlock (see equation below).

$$\#\frac{copies}{ng} = (6.022 * 10^{23})/(bp * 650 * 10^{9})$$

Where 6.022×10^{23} is Avogadro's number (mol^{-1}), 650 is the weight of one mole of a bp (g), and 1×10^{9} is the conversion from g to ng. The size of the gBlock in base pairs (bp) is listed on the specification sheet provided by Integrated DNA Technologies (IDT).

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2.4. Dilute DNA gBlock standards to generate a 10-fold curve from 10^9 to 10^0 copies/5 µL.

3. TEST VARIOUS CONDITIONS

3.1. Determine primers/probe concentrations to test. For each assay to test, use published concentrations as a starting point and test out one or two concentrations above and one or two concentrations below this.

3.1.1. For example, the published method uses working concentrations of 300 nM for the primers and 200 nM for the probe. Therefore, you must test in addition to the published concentrations, 150 and 600 nM for the primers, and 100 and 400 nM for the probe (See Table 1).

3.1.2. The concentrations published are generally referred to the final reaction volume of 20 µL. Use the Excel file Primer probe Mix calculator (located in the validation folder: Lab5\Biology\MolecularBiology\Validation) to determine the volume of the stock needed to prepare a primers-probe mix. (See Table 1).

Table 1. *Primers and Probe testing example*

Final concentration		**Primers/Probe mix**				
Forward & Reverse Primers concentration	Probe concentration	Forward Primer volume (500 µM)	Reverse Primer volume (500 µM)	Probe volume (100 µM)	Water colume	Total volume
150 nM	100 nM	1.2 µL	1.2 µL	4 µL	593.6 µL	600 µL
300 nM	200 nM	2.4 µL	2.4 µL	8 µL	587.2 µL	600 µL
600 nM	400 nM	4.8 µL	4.8 µL	16 µL	574.4 µL	600 µL

3.2. Run a standard curve from 10^9 to 10^0 TSC/5 µL for each primers/probe concentration. Include a no template control (water) for each primers/probe concentration. Multiple concentrations of primers/probe can be tested on the same plate.

3.3. Use the default conditions on the QuantStudio3 instrument for initial testing. For TaqMan assays, default run conditions are 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

4. DETERMINE OPTIMAL CONDITIONS

4.1. Determine optimal primers/probe concentration based on initial testing. This concentration should have no amplification in NTCs, robust amplification of standards, and a standard curve with an R^2 value >0.99 and an efficiency of 90–120%. This is typically achieved by using the published concentration of primers/probe.

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4.2. Determine appropriate standard curve range. Choose five consecutive points in the 10-fold dilution series that provide the best R^2 and efficiency values. These points are all in the linear range of amplification and provide accurate quantification of the marker. The highest standard is typically found between 10^5-10^7 TSC/5 µL.

4.3. Determine optimal running conditions. If the default conditions on the StepOne Plus or QuantStudio3 do not yield acceptable results, run additional plates with different conditions. Adjustments can be made to the timing, temperatures, and number of cycles. For all current assays, the default conditions have been sufficient.

4.4. Currently, qPCR tests run on the QuantStudio3 instrument use TaqMan Environmental Master Mix or BactoPure Master mix. Differences in master mixes might account for differences in performance.

5. ALIQUOT REAGENTS

5.1. Enter all reagents into LIMS. Refer to reagent and standard SOPs (PCR-3.x) for guidelines and substitute the appropriate assay name for each reagent.

5.2. Once optimal conditions are determined, aliquot small volumes of primers and probe to freeze at -20ºC as well as standards and spikes to freeze at -80°C.

5.2.1. Designate a tube color to represent the assay.

5.2.2. Determine volumes of primers and probe needed for each reaction mix based on optimal concentration. Aliquot volume needed + 2-3 µL for each reagent. If possible, avoid aliquoting less than 6 µL per tube for storing at -20 °C.

5.2.3. Determine highest standard used for standard curve and aliquot this concentration for spikes (16 µL aliquots) and standards (50 µL aliquots). Lower standards will be prepared fresh for each plate.

6. REFERENCES

6.1. DEP SOP PCR-2.0, *Calibration, Use and Maintenance of Qubit 2.0 Fluorometer*

6.2. DEP SOP PCR-3.2. *Preparation of HF183 Assay Reagents and Standards for qPCR Analysis*

6.3. DEP SOP PCR-3.3 *Preparation of Gull2 Assay Reagents and Standards for qPCR Analysis*

6.4. DEP SOP PCR-3.4 *Preparation of GFD Assay Reagents and Standards for qPCR Analysis*

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6.5. DEP SOP PCR-3.5 *Preparation of BacR Assay Reagents and Standards for qPCR Analysis*

6.6. DEP SOP PCR-3.6, *Preparation of DG3 Assay Reagents and Standards for qPCR Analysis*

Appendix of Changes

08/26/2021 Added equation description.

07/03/2024 Added information about primer probe preparation

08/11/2025 Removed reference to StepOne Plus instrument.

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