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 and 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 which will lead to higher molecular sensitivity, specificity, and precision.

2. PREPARE REAGENTS

2.1. Resuspend primers and probe in 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. 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 standard on Qubit and calculate the concentration for each dilution.

2.3.1. Refer to SOP PCR-3.6 and SOP PCR-2.0 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). The size of the gBlock in base pairs (bp) is listed on the specification sheet provided by Integrated DNA Technologies (IDT).

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

2.3.3.1. 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.

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

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3. TEST VARIOUS CONDITIONS

3.1. Determine primer/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 1000 nM for the primers and 100 nM for the probe. Therefore, you might test in addition to the published concentrations, 500 and 1500 nM for the primers, and 50 and 150 nM for the probe.

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

3.3. Use the default conditions on the StepOne Plus or 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. For SYBR Green assays, default run conditions are 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, followed by a melt curve.

4. DETERMINE OPTIMAL CONDITIONS

4.1. Determine optimal primer/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.

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 while assays that are run on the StepOne Plus instrument use the PerfeCTa ToughMix cocktail. Differences in master mixes might account for differences in performance. Test the optimal running conditions on both instruments to determine if the assay can be run on either platform.

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5. ALIQUOT REAGENTS

5.1. Enter all reagents into LIMS. Refer to SOP PCR-3.6 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 -80 °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-3.6 *Preparation of DG3 Assay Reagents and Standards for qPCR Analysis*

Appendix of Changes

08/26/2021 Added equation description.

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