Real-Time PCR Research & Diagnostics Core Facility qPCR Tips + Tricks

Separation of Workspace

PCR is one of the most sensitive methods for DNA detection. Because of its sensitivity, it is CRUCIAL to avoid introducing any level of contamination into your workflow. Primers/probes and mastermix should be handled in a clean environment, meaning no DNA/cDNA/RNA/etc. enters the space. Biological samples should be handled in a separate "dirty" room. Once the components that were prepped in the clean room make their way to the dirty room, they can no longer enter the clean area. Plasmids should be handled in a third room as they have the potential to contaminate everything as soon as the tube is opened. Lastly, for each of these 3 areas, you should have designated pipettes that are only used for the aforementioned functions. If different rooms are not an option, have three designated areas within the lab or ask a friend in another lab.

Assay Design and Validation

Once you have designed, ordered, and received your primers and probes, it is critical that you run a 10-fold standard curve validation to determine sensitivity and efficiency using a sample you know and have confirmed to be positive for the assay of interest. We can help with the calculations and a template on how to plot them.

Primers/Probes

Primers should be reconstituted with sterile PCR-grade water in a separate room from where sample extractions and sample handling is performed (clean vs. dirty room). Also, there are several options for probe quenchers/reporters and depending on which one you choose, design parameters may vary.

Pipetting

It takes a lot of practice to get comfortable pipetting in a 384- well format. Here are a few tips that will not only make it easier but will hopefully increase the accuracy of your data:

- Use a multichannel electronic pipette whenever possible, eliminating the variability error of pipetting a single well at a time. (Pro tip: While a 12 channel electronic pipette will not fit in say A1, A2, A3, etc., it WILL fit if you skip every other well. A1, A3, A5, etc.)
- Avoid pipetting small volumes when possible and make any dilutions at a larger scale before pipetting into your well.
- After you've pipetted everything and have sealed your plate, spin it down and look at your wells from below the plate. You want the volumes to be uniform across the board. If there is a lot of variability, it will affect your results. This is also how you can catch if you forgot to add a sample or mastermix.

Cycling Conditions

ALWAYS check the manufacturer's recommendations for your mastermix of choice and start by using those. They can always be adjusted if necessary.

SYBR Protocols/Analysis

When running SYBR assays, keep the following in mind:

- No probe means increased chances of nonspecific binding or primer dimers.
- Therefore, assay design is critical. Include a single positive control and 3 negative water controls for each assay (positive should be positive and all negatives should be negative).

- When analyzing your results, you MUST use the dissociation curve to determine a positive or negative result. The Cq value can still be used for calculations but the Cq alone is not useful for determining a positive/negative status.
- If the dissociation curve isn't visible, do not proceed assuming the Cq values mean a positive result. Adjust your display settings for the X and Y axis until the curves are visible.

Absolute Quantification

This method will require a plasmid (which should be handled in a separate room than your primers/probe AND biological sample handling areas as it will contaminate everything due to its high concentration). See us for ordering help and discounted rates.

Use of Positive + Negative Controls

You should ALWAYS include a known positive AND a water negative control for EVERY assay you run on your plate. If all your samples are negative, there is no way to be sure if they truly are negative or if something went wrong (and if so, where do you start?). Controls allow us to determine what to troubleshoot. Positives should be positive and all negatives should be negative.

Optical Adhesive Placement

ALWAYS make sure to use a plate sealer to press on your seal. Go over the entire plate once, then along the edges, and cut off tabs at perforation.

- NOT sealing the plate properly can result in evaporation of your sample/mastermix.
- NOT removing tabs at perforation will leave sticky residue and cause the robotic arm to malfunction which means your plate will not run and will sit out at room temperature overnight.

Result Analysis

Upon the completion of your run, you will receive an SDS file which will require SDS 2.4 software be installed (PC ONLY) in order to open and proceed. The software has default settings it uses to analyze your data BUT it isn't always correct. ALWAYS check and adjust your baseline AND threshold for every assay on your plate. The baseline represents the amplification cycles and the location at where you see the two flags (generally 3 and 15 by default) indicates that this region contains "junk" or "noise" and should be excluded from analysis. The threshold should be set at about the middle point of the linear phase of the curve.

External Plate Submission Form

Please fill out our form completely. If cycling conditions are different than the default settings listed, please write in the protocol to ensure we make the appropriate adjustments. ALWAYS indicate whether it is a SYBR or probe-based protocol. Lastly, please provide your email AND a phone number where you can actually be reached should we have questions. Otherwise, your plate will not be run overnight.

We hope this list helps set you up to have a successful qPCR experiment! We understand it is challenging and are more than happy to help. Please do not hesitate to schedule a time to chat with one of us. Good luck!