Bio-Rad CFX96 Real-Time PCR Detection System
User Guide
University of Puget Sound
Updated June 2022 by Amy Replogle

Table of Contents

1. Disclaimer!

Please note although portions of this guide have been taken from the Bio-Rad Real-Time PCR Applications Guide, this is an abbreviated guide. The manual for this instrument plus the software package is over 150 pages. While it is a good exercise for any student to read the user manual when beginning work on a new instrument, this abbreviated User Guide can serve as a guide for general use of the instrument and experimental design.

In the event that you encounter trouble, need more information, or require assistance, contact the Science Core Facility Technician, Amy Replogle (areplogle@pugetsound.edu; 253-879-2829) or refer to the operator's manual for the instrument and/or software.

The manual and application guide provided by Bio-Rad for this instrument is quite good, and frequent users should familiarize themselves with these booklets both for a review of qPCR concepts and for help with troubleshooting.

This user guide does not cover RNA isolation and cDNA synthesis considerations, both of which are vitally important for successful and reliable qPCR reactions.

2. Responsible Equipment Use

In order to use the CFX96 Real-Time PCR Detection System you must be properly trained! **To set up a training session on how to operate the instrument and use the software**, please contact the Science Core Facility Technician, Amy Replogle. Once you are trained, you must reserve time on the instrument using the Online Instrument Schedule located on the Science Core Facility website (www.pugetsound.edu/sciencecorefacility).

3. Other Training Opportunities

The Science Core Facility Technician offers a 5-day qPCR workshop every summer, usually in June. The goal of this workshop is to cover everything you need to know about setting up, running, and analyzing a qPCR experiment. For more information please contact the Science Core Facility Technician.

4. Safety Considerations

Primary safety concerns with the CFX96 Real-Time PCR Detection System have to do with the high temperatures of the heating block. The thermal cycler generates high heat that can cause serious burns. Always allow the sample block to return to normal temperature before opening the lid and removing samples. Use caution when touching the sample block.

This instrument must be used with care. Pay attention to labels on the instrument; in particular, the lid must always be opened either by pushing the button on the front of the lid, or through the computer software. NEVER force the lid open. This may result in damage to the machine.

5. Real-Time PCR vs. Traditional PCR

In contrast to conventional PCR, in which the final product is detected by end-point analysis on an agarose gel, quantitative real-time PCR (qPCR) allows the detection of the amplified product in "real time", as the product accumulates. This is made possible through the use of fluorescent reporter molecules in the reaction. This method has major advantages over conventional PCR, the most significant of which is the quantitative analysis of starting template.

6. Relative vs. Absolute qPCR

The majority of Puget Sound users currently use relative qPCR to look at gene expression. The differences between the two techniques are detailed below. For more information on how to design an experiment utilizing Absolute qPCR please refer to the Bio-Rad Real-Time PCR Applications Guide.

6.1. Relative Quantification

Relative quantification describes a real-time PCR experiment in which the expression of a gene of interest in one sample (i.e., treated) is compared to expression of the same gene in another sample (i.e., untreated). The results are expressed as fold change (increase or decrease) in expression of the treated sample relative to the untreated sample. A reference gene is used to control for experimental variability in this type of quantification.

6.2. Absolute Quantification

Absolute quantification is the most rigorous of all qPCR experiments and is used when you want to know the exact quantity of nucleic acid per given amount of sample. One example of this is when you want to know the number of viral particles per known amount of sample. Absolute quantification requires the addition of a standard curve in every run to determine the absolute amount of the target nucleic acid of interest. Equivalent amplification efficiencies between the target and the external standard are required for absolute quantification.

7. Amplicon and Primer Design

Before beginning work on the real-time PCR machine, you must first design your primer or primer/probe sets and optimize and validate your reactions. There are a number of different chemistries to choose from. This user guide focuses on DNA-binding dyes such as SYBR Green. For more information on other available chemistries please refer to the Bio-Rad Real-Time PCR Applications Guide.
The most commonly used DNA-binding dye for real-time PCR is SYBR Green I, which binds nonspecifically to double-stranded DNA (dsDNA). SYBR Green I exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1,000-fold when it binds dsDNA. Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present, and will increase as the target is amplified.

The advantages of using dsDNA-binding dyes include simple assay design (only two primers are needed; probe design is not necessary), ability to test multiple genes quickly without designing multiple probes (e.g., for validation of gene expression data from many genes in a microarray experiment), lower initial cost (probes cost more), and the ability to perform a melt-curve analysis to check the specificity of the amplification reaction.

7.1. Primer Design Guidelines

There are two ways to approach designing primers, both are valid, but neither are always 100% successful so always order multiple primer sets!

1. The quick and dirty way is to just use the primers given to you by a software program, without checking secondary structure of amplicon or primer characteristics with IDT Oligo Analyzer.
   --OR--
2. Follow the guideline summary listed below, which will take a little more time, but provide you with a more informed decision.

Primer Design Guidelines Summary* (Steps 1-8 are essential)

*Depending on where you get your guideline information length, temperature, or ΔG cutoffs may differ. The guidelines below are ones that have worked well in practice for the Science Core Facility Technician.

1. Primer and Product size:
   a. Primer should be 18-22bp, we aim for 20bp
   b. Product (amplicon) should be short 70-150bp. For amplicons >250 in length or with high GC or AT content, longer annealing/extension times can be used.
2. Primer Melting Temperature (Tm) should be between 55°- 65°C. Start by telling design programs the optimal Tm is 60°C. If you are having a problem finding a good primer pair, try changing the optimal Tm to 63°C.
3. Primer Annealing temperature is experimentally determined during the optimization process, but is generally 2-3°C below the melting temperature.
   a. The annealing temperature should be similar (no more than 4°C difference) between forward and reverse primers AND between primer sets that are going to be used in the same assay/plate.
   b. If you have been having problems with primer efficiency, try designing primers with an optimal Tm of 63°C (Bustin and Huggett, 2017).
4. Avoid hairpins in primers

    a. The Tm for the strongest hairpin should be at least 50°C below the annealing temperature. If there are hairpins close to the annealing temperature, avoid structures that are ΔG ≥ -3.0 kcal/mol
5. Avoid dimers with primers
    a. A primer pair ΔG ≥ -9.0 kcal/mol will be problematic
6. Avoid secondary structure in the product (amplicon) especially where primers will bind at or above the annealing temperature
7. Placement of primers on gene of interest
    a. Use NCBI BLAST to check that your primers will amplify only your intended gene
    b. Design close to the 3' end of the gene to improve efficiency
    c. Design the primer pair to span an intron/exon junction to avoid amplifying gDNA
8. Optimal GC content of both primers and amplicon is 35-65%
9. Use a GC clamp to encourage complete primer binding
10. Avoid Repeats and Runs
    a. Maximum number of di-nucleotide repeats is 4
    b. Maximum number of runs is 4bp

7.2. Hydrolysis Probe Design Guidelines

1. **Length:** If designing single-quenched probes like TaqMan, ensure that they are 20-30 bases in length. This will help you achieve an idea Tm without increasing the distance between the dye and the quencher such that the quencher will no longer optimally absorb the fluorescence of the dye.
2. **Location:** Ideally, the probe should be in close proximity to the forward or reverse primer, but should not overlap with a primer-binding site on the same strand. Probes can be designed to bind to either strand of the target.
3. **Melting Temperature (Tm):** Preferable, probes should be 6-8°C higher than primers, so that as it cools from 95 to primer annealing, the probe binds first. Usually the annealing temperature for the probe ends up being 66-70°C. If the melting temperature is too low, the percentage of probe bound to target will be low. In this case, the primers may amplify a product, but sensitivity may be compromised as all target sites are not saturated with probe resulting in reduce fluorescence signal that does not truly represent the amount of target present in the sample.
4. **GC Content:** As with primer sequences, aim for a GC content of 35-65% and avoid at the 5' end to prevent quenching of the 5' fluorophore.
5. **Complementarity and Secondary Structure:** Primer and probe designs should be screened for self-dimers, heterodimers against the 2 primers, as well as hairpins. The ΔG value should be weaker (more positive) than -9.0 kcal/mol. Positive numbers indicate that the actual secondary structure shown will not form at all.

8. Assay Optimization and Validation

While optimization and validation of your qPCR reaction does take time, it is time well spent. The resulting assay will have the highest sensitivity and dynamic range, a high efficiency (which correlates with high accuracy), and excellent reproducibility. These factors all lead to confidence in the data. To determine the performance of your SYBR Green qPCR assay you should 1) Identify the optimal annealing temperature for your assay and adjust primer concentrations as needed for specificity to optimize the assay, and 2) Construct a standard curve to evaluate assay performance to validate the assay.

NOTE: Care should be taken when choosing your template DNA for optimization and validation. Ideally the template should be from a sample that contains the PCR target and matches (as closely as possible) those that will be used for the experiment (i.e. the same total RNA or DNA sample).

8.1. Assay Optimization by assessing Annealing Temperature

The annealing temperature is the temperature at which the primers anneal to the target sequence so that extension can occur. In most cases, primers are designed so that they anneal successfully at 60°C. Oligonucleotide sequence, oligonucleotide concentration, and cations present in the buffer all affect the Ta. Annealing optimization needs to be done once for each primer pair, after which you can use the same conditions for future reactions, unless you are using a new Supermix with different cations present, then you would need redo the optimization.

8.1.1. Using a temperature gradient

- In order to determine the optimal annealing temperature for each primer set, reactions should be run on a temperature gradient. Setting up the temperature gradient using the CFX Manager Software is outlined in Section 10.5.
- The range of the gradient may vary according to the T_m of the primer set, but running a gradient from 55°C to 65°C should be sufficient for most well-designed primers.
- A 1:10 dilution of your template cDNA is usually sufficient for determining the optimal annealing temperature.
- Always include a no template control as one of your reactions to control for cross contamination and to easily identify potential problems with primer dimers.
- Be sure that your protocol includes a melt curve (See below), since this an important readout for optimization.
- When performing any qPCR reactions, to minimize variation in experimental results, prepare master mixes for replicate reactions whenever possible. It is recommended to run all samples in triplicate.

8.1.2. Using a melt curve

- Because SYBR Green I binds to all dsDNA, it is necessary to check the specificity of your qPCR assay by analyzing the reaction product(s).
- At the end of the qPCR run, the thermal cycler starts at a preset temperature (usually above the primer Tm; e.g., 65°C) and measures the amount of fluorescence. The temperature of the sample is then increased incrementally as the instrument continues to measure fluorescence. As the temperature increases, dsDNA denatures becoming single-stranded, and the dye dissociates, resulting in decreasing fluorescence. The change in slope of this curve is then plotted as a function of temperature to obtain the melt curve.
- A specific PCR product will yield a single melt peak.
- Setting up the melt curve using the CFX Manager Software is outlined in Section 10.5.

8.1.3. Analyzing temperature gradient and melt curve

The optimal annealing temperature is determined based on the C_q value and melt curve analysis.

- The C_q value is the cycle at which enough amplified product has accumulated to yield a detectable fluorescent signal above background. You want to pick the warmest

temperature that does not increase the Cq value. In other words, choose the warmest temperature that results in the lowest Cq with a single-peak melt curve.

- This peak should be between the high 70's to low 80's depending on amplicon size and composition.
- Annotating the temperature gradient and melt curve in the software is outlined in Section 10.5.
    - **Helpful Hint:** Use Trace Styles to change the color of the Amplification Curves according to the Temperature.

8.1.4. Troubleshooting the melt curve

If you have more than one peak for your melt curve there are several things you can check to optimize your reaction. However, it's always a good idea to design several primer pairs for the same target to save time on troubleshooting.

a. Do your wells with template have a single peak, but your NTC wells have a broad peak at a lower temperature than the desired product?
    - This means that without any template, or at low concentrations of your template your primers have a tendency to form dimers generated from primers that anneal at their 3'-ends, extend, and then amplify.
    - It is ok to observe a small amount of primer-dimer formation in the NTC wells below the threshold, as long as it doesn't affect primer efficiency.
    - If the primer dimer is above the threshold and if the Cq values in the NTC are within five cycles of any of the unknowns, this may call the validity of the results into question and further optimization via annealing temperature, primer concentration, amplification conditions, or primer redesign may be necessary.

b. Do you have multiple distinct peaks in wells with your template?
    - Multiple melting peaks may indicate one or more of the following
        - Multiple PCR products in this assay.
        - Biphasic melt curve due to GC content.
    - Keep in mind that even a 1^{o}C difference in melt peaks can mean multiple PCR products and will subsequently affect quantification results.
    - To diagnose the problem you can run the PCR product on a gel to verify multiple products.
    - To troubleshoot the problem you can optimize the Mg^{2+} concentration, use a higher annealing temperature, or design new unique primers.

8.2. Assay Validation by Optimizing Efficiency

A standard curve, generated by performing qPCR with a serial dilution of template, is an excellent tool to validate your assay to determine assay efficiency, precision, sensitivity, and working range.

Ideally, the efficiency (E) of a PCR reaction should be 100%, meaning the template doubles after each cycle. In practice, you should strive for an amplification efficiency of 90–105%, however efficiencies of 85-115% are considered usable, with an R squared value >0.980.

Experimental factors such as the length, secondary structure, and GC content of the amplicon can influence efficiency. Other conditions that may influence efficiency are the dynamics of the reaction itself, the use of non-optimal reagent concentrations, and enzyme quality.

8.2.1. Setting up the Standard Curve

1. Prepare a 10-fold serial dilution with five or more points using a sample that contains the PCR target and matches (as closely as possible) those that will be used for the experiment (i.e. the same total RNA or DNA sample). The range of concentrations assayed should span the concentration range expected for the experimental samples.
    - Example dilutions: 1) 1:1 undiluted cDNA template, 2) 1:10 dilution cDNA, 3) 1:100 dilution cDNA, 4) 1:1000 dilution cDNA, and 5) 1:10,000 dilution cDNA)
2. Conduct qPCR with all dilutions and with a no-template control in triplicate reactions, using previously optimized primer annealing temperature and concentrations, if applicable.
3. Be sure that your protocol includes a melt curve to analyze the reaction products.

8.2.2. Analyzing the Standard Curve

1. Set up the plate for the standard curve using the "Editing a Plate Setup for Data Analysis" in step 7E, making sure to Load the **Concentration**.
   Helpful Hint: If you have multiple Targets to analyze on the plate, you'll want to change the **Analysis Mode** from Fluorophore to Target.
2. Review the QC tab according to the instructions in the "Data Analysis for Relative qPCR Runs"
3. With the well contents of the plate properly annotated, the **Standard Curve** graph should be visible in the **Quantification** tab of the **Data Analysis** window.
    a. Assess how close the data are to the fit of the regression line by checking the R-squared value. The R-squared value should be greater than 0.980.
    b. Assess the %Efficiency value displayed in the **Standard Curve** graph. Your assay should have high efficiency 90% - 110%.
4. Ideally, multiple standard curves should be run to verify that this efficiency measurement is reproducible (typical run-to-run variability is in the 5% range).
5. When you perform the actual experiment, after all optimization steps, the PCR efficiencies that were determined for each primer set are used to assist with normalization of the assay results (see section 10.5.13).

8.2.3. Troubleshooting Problems with Efficiency

Efficiencies outside the range of 85–115% may artificially skew results and lead to false conclusions.

- Be aware that not all points of a standard curve will conform to high data quality metrics. Often, the high and low concentration points may not be in range, and elimination of these aberrant concentrations from analysis may results in a high quality assay, across a slightly lower linear quantitative range. If this is the case, you may need to make sure you adjust the template input (lower or higher) so your reactions are in this linear range.
- If PCR efficiency ≤ 85% this indicates suboptimal PCR conditions (low *Taq* activity, incorrect magnesium or primer concentrations) or poor primer design. If you have

taken care in designing your primers it is likely a concentration issue because competition for resources in the tube can produce inefficient reactions.

- If PCR efficiency ≥ 115% indicates excessive primer-dimer, inaccurate pipetting, or inhibition due to poor RNA quality.
- If the Standard Curve is not linear with low levels of sample input and you need that low level of sensitivity you can try to adjust the primer concentrations, or make cDNA with a higher concentration of RNA.
- If the Standard Curve is not linear with high levels of sample input your template concentration is too high resulting in limiting reagents or inhibition from reagents carried over from reverse transcription. If you need high sensitivity you can re-purify the template. Alternatively, those wells with the highest concentration of template can be removed and the standard curve reanalyzed. If the efficiency improves back to under 110%, the assay is fine. Just keep in mind that any concentrations removed from the standard curve may not be used during the actual assay.

8.3. Optimizing Primer Concentrations

For SYBR Green I, relatively low primer concentrations are used to avoid primer-dimer formation. For most SYBR Green I applications, primer concentrations ranging from 50 – 300nM are commonly used, but concentrations can be as high as 600nM.

Note, that not all assays require the testing of this entire range of primer concentrations. Starting out with a new primer set for a gene expression experiment, you can usually start with 250nM of each forward and reverse primer pair. However, if you are having problems with non-specific amplification, excessive primer dimers, or suboptimal efficiency values you can try varying the concentration of both the forward and reverse primers.

To optimize primer concentrations you will need to set up a primer optimization matrix using 50, 150, 300, and 600nM of each forward and reverse primer, then analyze which primer combination gives you the earliest Cq values with a single melt curve.

	1	2	3	4	5	6	7
A	50/50	50/300	150/50	150/300	300/50	600/50	600/300
B	50/50	50/300	150/50	150/300	300/50	600/50	600/300
C	50/50 NTC	50/300 NTC	150/50 NTC	150/300 NTC	300/50 NTC	600/50 NTC	600/300 NTC
D	50/50 NTC	50/300 NTC	150/50 NTC	150/300 NTC	300/50 NTC	600/50 NTC	600/300 NTC
E	50/150	50/600	150/150	150/600	300/150	600/150	600/600
F	50/150	50/600	150/150	150/600	300/150	600/150	600/600
G	50/150 NTC	50/600 NTC	150/150 NTC	150/600 NTC	300/150 NTC	600/150 NTC	600/600 NTC
H	50/150 NTC	50/600 NTC	150/150 NTC	150/600 NTC	300/150 NTC	600/150 NTC	600/600 NTC
	Concentration in nM, Forward primer is listed first						

9. Reaction Set-Up

9.1. Controls for qPCR Experiments

Before setting up the SYBR Master Mix, you need to think about how many samples you will be in the qPCR run. One of those considerations should be what controls are you going to use. The

specific controls that are needed will vary according to the experiment type, but there are certain controls that should be included in every run, such as No Template Controls (NTC). Others should be performed as part of the optimization or validation process, such as the No Reverse Transcriptase (NRT).

9.1.1. No Template Control

NTCs are the most overlooked, but most important control tool you have at your disposal. NTCs provide a mechanism to control for external contamination or other factors that can results in a non-specific increase in the fluorescence signal. Ideally, signal amplification should not be observed in the NTC samples wells. If the NTCs do cross the threshold, their Cq values should be at least five cycles, and preferably more than ten cycles, from the Cq values of your samples. If the Cq values of the NTCs are less than five cycles delayed compared to samples containing template, the Cq values of those samples should not be considered accurate since whatever is causing the fluorescence shift in the NTC wells is likely affecting the fluorescence/amplification in the unknown wells.

9.1.2. No Reverse Transcriptase Control

No RT controls are samples that run exactly as the other reactions, except that the reverse transcriptase enzyme is omitted. Note that if performing a two-step qPCR reaction the no RT should be made from a small aliquot of RNA during cDNA synthesis. No RT controls should show no amplification in the subsequent PCR step since DNA polymerase cannot amplify an RNA template. Amplification occurring in the No RT control wells indicates that there is contaminating gDNA template in the reaction.

9.1.3. Positive Controls

a. Sample known to be positive for the target of interest to show that the Master Mix setup and system setup was correct. In reactions where no positive control is run, if all your unknown samples come up negative, it is impossible to tell if these are true negatives or if some problem in your reagents caused amplification to fail.
b. Synthetic template to determine if samples contain inhibitors.

9.1.4. Inter-run Calibration

- Inter-run Calibration is only needed if you cannot follow an All Samples model when planning your Plate Design (see section on Plate Design for whole Experiment).
- Inter-run calibration is automatically attempted in every Gene Study for each target to normalize inter-run variations between targets assayed in separate real-time PCR runs (that is, different .pcrd files generated from different plates).
  NOTE: At least one inter-run calibrator sample must be present in the Gene Study for inter-run calibration to occur. Targets without appropriate inter-run calibrator samples will be processed without correction in the Gene Study (not recommended)
- For the software to recognize a sample as an inter-run calibrator, it must share matching target name, sample name, and if used, biological set name across every plate being compared.
- Inter-run calibrators can be:
  - Pooled RNA or cDNA sample: This will mimic the individual samples because it will be amplified in a similar matrix.
  - Reference material (i.e. stock cDNA, linearized plasmid, synthetic gene block)

- Inter-run calibrators can be run on a single target or using all targets in the assay. For robust inter-run calibration you should repeat multiple samples with all Targets across the multiple plates.

9.2. SYBR Green Supermix

The reaction set-up detailed below is for iTaq Universal SYBR Green Supermix. This only serves as an example and you should always follow the protocols that accompany the Supermix kit you are using. Each time you order replacement SYBR green you should double check that the reaction set-up is still the same.

1. Thaw Supermix and other reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solution at the bottom of tubes, and then store on ice protected from light.

2. Prepare (on ice or at room temperature) enough assay master mix for all reactions according to the recommendations listed in the appropriate supermix protocol or the table below. A Master Mix has all required components except the DNA template (or the component that changes).

   Important Master Mix considerations:
   - The Science Core Facility Technician highly recommends the preparation of a modified Master Mix so that you never have to pipet small volumes repeatedly into the reaction tubes. This will reduce the risk of cross contamination and improve the reproducibility of technical replicates.
   - You always want to prepare more reactions than you'll need due to small volume differences in pipetting and the fact that small amounts of liquid will stick to the tube and pipet tip walls.
     - If preparing a traditional Master Mix, prepare half a reaction or up to 2 reactions more than you need to ensure you have enough assay master mix for all reactions. For example, if I need 10 reactions for my reference gene then I would multiply my mastermix components by 10.5. **NOTE:** If you are using an automatic repeat pippetor you will need to prepare two more reactions than you need.
     - If preparing a modified Master Mix, as recommended by the Science Core Facility there is more planning involved to make sure you have enough Master Mix. Please reach out for assistance.
     - Alternatively you can use the Master Mix Calculator, which can by found under "Tools" in the Bio-Rad CFX Manager Software. However, their default excess reaction volume is 5%, which is WAY more than you need and it always ends up with odd amounts to pipet, so the Science Core Facility recommends you use whole numbers instead of a percentage of the reaction volume.

3. Mix the Master Mix by gently inverting or finger vortexing to ensure homogeneity. Avoid over-vortexing because it can cause bubbles to form, which makes it hard to aliquot the correct amount in the proceeding steps. To help rid the samples of any bubbles that have formed tap the tube firmly on the benchtop, then vortex briefly to collect contents and remove any air bubbles.

4. Dispense equal aliquots into each qPCR tube or into the wells of a qPCR plate. Good pipetting practice must be employed to ensure assay precision and accuracy. Avoid the formation of bubbles as much as possible as they could interfere with fluorescence detection and reduce enzyme efficiency. Some bubbles are okay as they usually pop when the sample is heated. To help rid the samples of any bubbles that have formed tap the tube firmly on the benchtop, then vortex briefly to collect contents and remove any air bubbles.

5. Seal tubes or wells with flat caps or optically transparent film. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture in the vessel bottom.

Component	Volume per 20 µl Reaction	Volume per 10 µl Reaction	Final Concentration
SYBR Green Supermix (2X)	10 µl	5 µl	1X
Forward and Reverse Primers	Variable	Variable	100 – 500 nM each
DNA template*	Variable	Variable	cDNA: 100 fg – 100 ng gDNA: 5 pg – 50 ng
Nuclease Free Water	Variable	Variable	---

* After RT, it is common not to quantify the resulting cDNA by UV absorbance. Instead the cDNA is assigned a concentration unit relative to the original concentration of RNA in the RT reaction. For example, if you used 10 µg of RNA in a 100 µL RT reaction, the designated concentration of the resulting cDNA would be 100ng/µL; which means 1 µL of sample contains the cDNA generated from 100 ng of RNA.

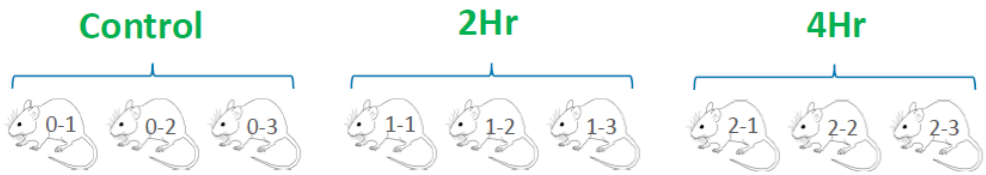
9.3. Plate Design for whole Experiment

Before starting any qPCR run, you need to plan your Plate Design/Reaction Layout so the proper controls are used for accurate gene expression analysis. You need to think about the total number of samples, total number of targets, how many Biological and Technical replicates you will have, and whether or not you'd like to add samples (i.e. time points) or additional genes of interest in the future using the same RNA pools. There are 4 main Plate Design Layouts which are discussed in more detail below.

9.3.1. All Samples and Targets on the same plate

This is where all the samples and all the targets can fit onto one plate for data analysis. No Inter-run calibration is needed because all calculations happen on same plate/run.

For example, if you had an experiment with the following:



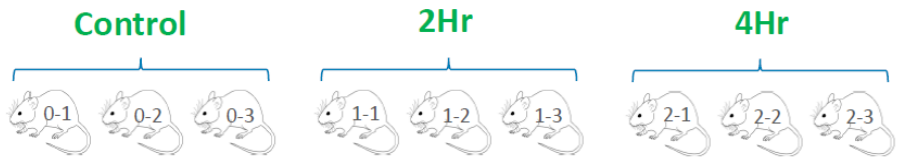
- 3 Biological Groups (Treatments)
- 3 Biological Replicates

- 2 Targets (Gene of Interest and Reference Gene)
- 2 NTC (one for each Target)
- 3 Technical Replicates of all reactions
  = 60 total reactions which will fit on one 96 well plate

9.3.2. *All Samples on the same plate, Targets split across multiple plates*

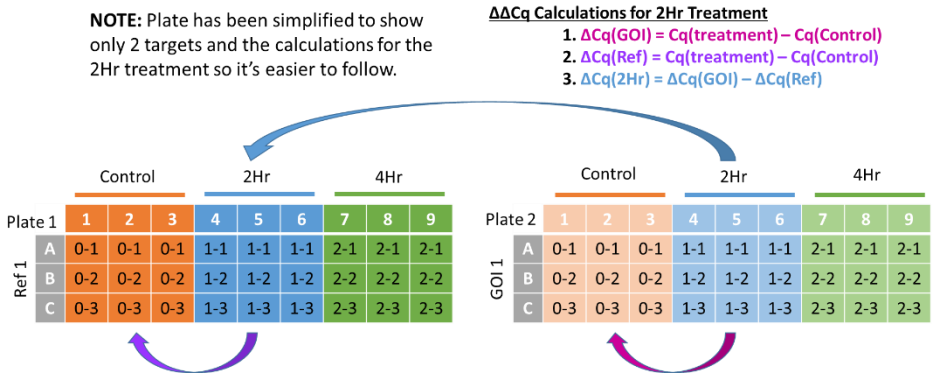
When all reactions cannot fit onto one plate, the **All Samples** model can be used **if all samples have already been collected**. The advantage to this model is that no inter-run calibration is needed, and as additional gene targets are identified a new plate can easily be added to the analysis without having to re-run the Reference Gene if using the same RNA/cDNA.

For example, if we used the same scenario as above, but add 2 additional targets:



- 3 Biological Groups (Treatments)
- 3 Biological Replicates
- **4 Targets (3 Genes of Interest and 1 Reference Gene)**
- 4 NTC (one for each Target)
- 3 Technical Replicates of all reactions
  = 120 total reactions which will require two 96 well plates

If we use the All Samples model, no inter-run calibration is needed because the ΔCq calculation for the GOI and Ref happens on the same plate (see bottom arrows in purple below). The ΔΔCq calculation for each treatment happens across the plates in the same way so each will experience the same variation, so it is normalized out (see top arrows in blue).

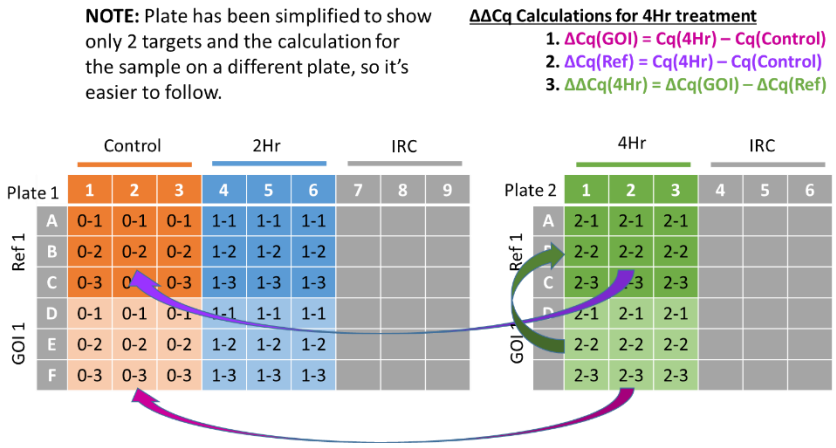


9.3.3. *All Targets on the same plate, Samples split across multiple plates*

When all reactions cannot fit onto one plate, the **All Targets** model can be used **if all target genes have already been determined**. The advantage to this model is that additional samples can be added (like later time points) to the study without re-running

previous samples, as long as an inter-run calibrator is used. The disadvantage to this method, is that Inter-run Calibration is required.

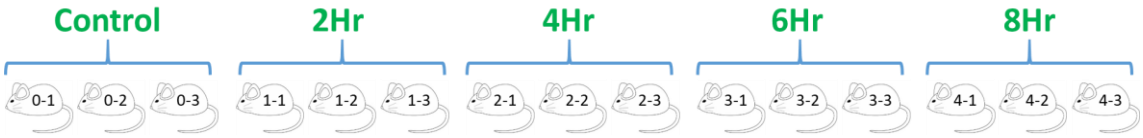
If we use the example above with 120 total reactions and the All Target model, the ΔCq calculation for the GOI and Ref for at least one of the samples is being done across different



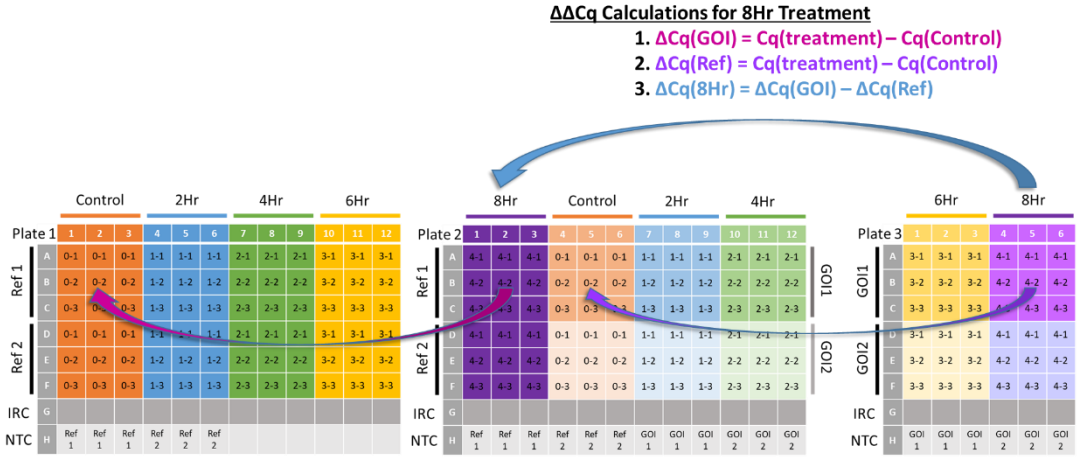
plates, so unlike the All Samples model, there is no normalization for the across the plate ΔCq calculation and thus inter-run calibration is required.

9.3.4. *Samples and Targets on multiple plates*

In very large experiments you may have to split both Samples and Targets across multiple plates. A robust inter-run calibration is required for this situation. Please refer to the Hellemans et. al., paper for more information about when and how to use inter-run calibration: Genome Biology 2007, 8:R19 (doi:10.1186/gb-2007-8-2-r19).



- 5 Biological Groups (Treatments)
- 3 Biological Replicates
- **4 Targets (2 Genes of Interest and 2 Reference Genes)**
- 4 NTC (one for each Target)
- 3 Technical Replicates of all reactions



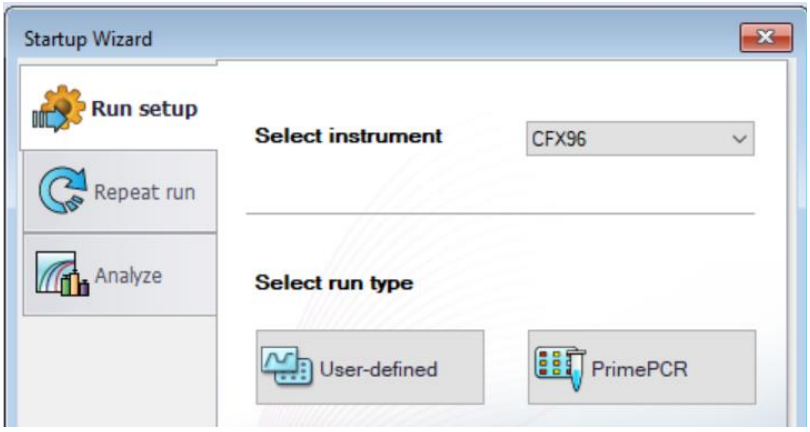
If you wish to compare gene expression across the time points, this plate design would require an inter-run calibration because the ΔCq(Ref) for the different time points will happen across different plates. For example, the ΔCq(Ref) for the 2hr treatments happens on Plate 1, but the ΔCq(Ref) for the 8hr treatment happens across Plates 1 and 2. An inter-run calibrator needs to be run across all three plates to factor in run to run differences.

10. Using CFX Manager Software

The CFX Manager software is run on a PC computer running Windows XP up to Windows 10 to control the CFX96 system. The software is also used to analyze real-time PCR data. If you would like to install the software on your personal computer to analyze your data please check with the Science Core Facility Technician for installation instructions. The software is only available for Windows operating systems.

10.1. Opening the software

1. Open the software by double clicking on the Bio-Rad CFX Manager icon on the desktop.
2. Every time the software is opened, the StartUp Wizard will appear. From this window you can:
   a. Set up a User-defined qPCR Run
   b. Repeat a run using a previous set-up
   c. Analyze your data



10.2. Run Setup

To Set up a User-defined qPCR Run, under **Run Setup** in the Startup Wizard, click **User-defined**. This will open the **Run Setup window** with the **Protocol tab** selected. Here, you will set up the cycling conditions (protocol) for your run. Your protocol will vary depending on assay conditions detailed in kit instructions and determined from assay optimization and validation experiments. If you need to alter the conditions displayed in the Run Setup window, it is recommended to use one of the **Express Load** options so you don't forget to put in a read step or add the melt curve.

10.2.1. Express Load

This is the recommended way to edit qPCR protocols. Select the appropriate cycling conditions by using the drop-down menu. The most commonly used options are:

a. CFX_2StepAmp+Melt: This is a two step cycling process with a denaturation and then annealing and extension occurs at the same temperature. This also automatically adds a melt curve onto a normal 2 Step qPCR run.

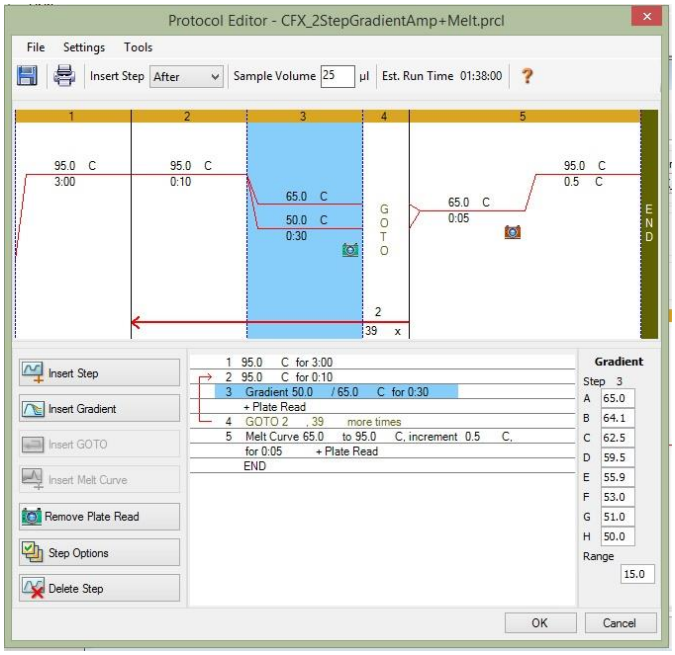
b. CFX_2StepGradientAmp+Melt: This is a two step cycling process that utilizes a temperature gradient for the annealing/extension steps. This also automatically adds a melt curve.

10.2.2. Load existing protocol

If you've used the system before and want to load a saved protocol, choose **Select Existing**… and navigate to the file, then click **Open**. The name of the selected protocol should now appear under the **Selected Protocol** box, and a preview of the selected protocol will load in the **Preview** window. See below for instructions on how to edit the cycling conditions of your selected protocol. Otherwise, click Next at the bottom of the window and proceed to section 10.4.

10.3. Editing a Protocol

To change cycling conditions in your selected protocol, click **Edit Selected…** and the **Protocol Editor** window will open. From here you can modify or add cycling steps to your protocol. It's always important to check the instructions that came with your SYBR Green Supermix to determine the optimal thermal cycling protocol. Below is a screen shot of the **Protocol Editor** with an example of a protocol set up to determine the annealing temperature using Bio-Rad iTaq Universal SYBR Green Supermix and standard qPCR primer design guidelines. Refer back to this image as you read about the different steps you can add or modify in this section.



10.3.1. Sample Volume

At the top of the **Protocol Editor** window you will want to input your sample volume, so the software can better estimate how long it will take for your sample to reach the desired cycling temperatures.

10.3.2. General thermal cycling protocol

The following steps are usually included in a thermal cycling protocol for a two-step amplification of cDNA or plasmid DNA template. Higher temperatures, longer cycle times, or

a separate extension step are needed if using gDNA as a template, if your amplicon is GC rich, or if your amplicon is longer than 150bp.

1. Initial template denaturation and enzyme activation. For iTaq and iQ SYBR Green standard conditions are 95°C for 3 min
2. Denaturation of template at 95°C for 10 sec
3. Annealing and extension + Plate read for 30 sec
4. Repeat Steps 2 and 3, 39 more times for a total of 40 cycles
5. Denaturation for Melt Curve analysis
6. Melt curve

10.3.3. Using the Protocol Editor to edit thermal cycling

The **Protocol Editor** window includes buttons for editing the steps in the protocol and text boxes to edit the temperature or hold time.

a. Editing temperature or hold time

Edit the temperature or hold time by clicking the default temperature or time in the graphic or text view, and entering a new value.

NOTE: Temperature is given in degrees Celsius and time is written as min:sec (i.e., 3:00 means 3 minutes, while 0:30 means 30 seconds).

b. Protocol Editor Buttons

Additional changes to the protocol can be made by using the buttons in the lower left hand corner of the window. First, select a step in the protocol by highlighting it in the graphic or text view, then click one of the Protocol Editor buttons to change the protocol. These buttons allow you to insert the following; a step, gradient, cycle (GOTO), melt curve, or plate read. You can also delete a step, or access additional step options. See descriptions below for more information.

i. Insert Step

Inserts a temperature step before or after the selected step

ii. Insert Gradient

Used to set up a temperature gradient across the plate, where each row is a different temperature.

- Upon clicking the “Insert Gradient” button, or when a gradient step is highlighted, the details of the gradient show up on the right side of the screen.
- A gradient runs with the lowest temperature in the front of the block (row H) and the highest temperature in the back of the block (row A).
- The temperature gradient can be controlled by changing the range of temperatures you want to assess (i.e. over a 10 degree range) and by choosing the lowest or highest temperature you want to assay.
    - The range is changed by clicking in the range box at the lower right hand of the window when a gradient is selected.
    - A particular temperature is changed in the gradient by clicking the default temperature in the graphic or text view, and entering a new temperature. Alternatively, click the **Step Options** button to enter a temperature in the gradient range in the Step Options window.

NOTE: The temperatures in the gradient are a range will automatically be adjusted if a new temperature or range is indicated. You cannot manually choose the temperatures you want to assay for each row.

iii. The GOTO step

The GOTO step is used to indicate how many times to cycle through denaturation of template and the annealing/extending step. The default setting is at 39 cycles, which may be sufficient for many cases. However, this number can easily be changed by selecting the number of cycles and typing in a new number. The red arrow indicates where the protocol will cycle back to when the GOTO step is reached.

iv. Insert Melt Curve

The "Insert Melt Curve" button can be used to produce a melt curve for your products after normal qPCR thermal cycling is complete. This is a necessary step for optimization, but should be included in all of your experimental runs because it acts as a quality control for all your assays.

1. Click the GOTO step in the graphic or text display, then click "Insert Melt Curve" to include a melt curve in your protocol.
    - The appropriate temperature series to produce the melt curve, time, and plate read features should be automatically inserted for you after the GOTO step.

v. Adding a Plate Read

For real-time detection, a plate read should be included right before the GOTO step. Default Express Load files should include this plate read, however if you modify, delete or add steps, the plate read may be removed. Thus, be sure that the step before the GOTO step includes a plate read, indicated by a small camera icon. If the icon is not present, click the "Add Plate Read to Step" button when the appropriate step is highlighted in the graphic or text view.

vi. Step Options

There are additional options for each step such as incrementally increasing temperature each cycle (like the melt curve), changing the ramp rate, and extending the time every cycle. To change the step options, follow these instructions:

1. Select a step by clicking on the step in the graphic or text view
2. Click the **Step Options** button to open the Step Options window
3. Add or remove options by entering a number, editing a number or clicking a check box. See below for descriptions of options:
    - **Plate Read:** Check the box to include a plate read
    - **Temperature:** Enter a target temperature between 0.0 and 100°C
    - **Gradient:** Enter a gradient range between 1.0 and 24°C. For example, enter 10 for a gradient that starts at the target temperature (row H) and rises 10°C (row A)
    - **Increment:** Enter an increment temperature between -10 and 10°C/cycle. For example, to raise the temperature 0.5°C each cycle, enter 0.5. To lower the temperature o.5°C each cycle, enter -0.5°C

- **Ramp Rate:** Enter a temperature between 0.1 and 5.0°C/cycle for a 96-well block to instruct the thermal cycler to raise and lower the block temperature at that rate.
- **Time:** Enter the hold time between 1 second (0:01) and 18 hours (18:00:00). For example, type "1" to enter 0:01 seconds and "300" to enter 3 minutes (3:00)
- **Extend:** Enter a time between -60 and 60 seconds/cycle to reduce or extend the hold time in each cycle. For example, enter 0.5 to extend the step by half of a second in each cycle
- **Beep:** Check the box to include a beep at the end of the step

Tip: When you enter a number that is outside the option range, the software changes the number to the closest entry within the range. To find the highest or lowest end of the range, enter any number outside the range for the Step Option to have the software automatically fill in the nearest number within the range.

vii. Delete Step

To delete a step in the **Protocol Editor** window, follow these instructions:

1. Select (highlight) a protocol step in the graphic or text view.
2. Click the **Delete Step** button to delete the selected step
   Warning! You cannot undo this function; you have to manually re-add a step

10.3.4. Saving the Protocol

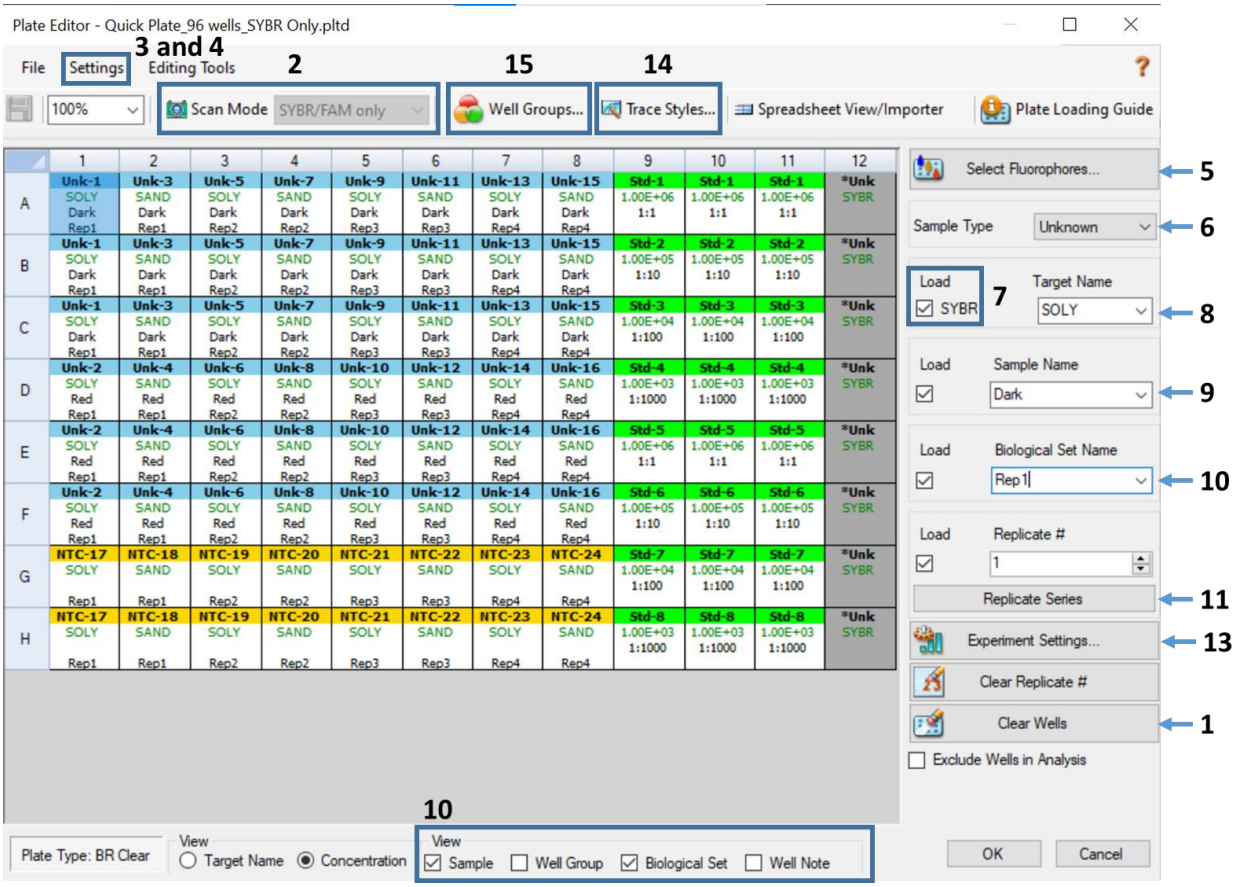
- To save changes to a new protocol, an existing protocol, or Express Load protocol, you will need to click **OK** in the **Protocol Editor** window.
- This will open a new window asking if you want to save changes to the protocol file, click **Yes**.
- A **Save As** window will open asking you to choose a location for your protocol and to give it a name.
  - Navigate to your labs folder in the **qPCR Users** folder which is located on the Desktop
  - Rename your file with your labs file naming convention
  - Click **Save** and this will exit you out of the **Protocol Editor** and take you back to the **Run Setup** window. Click the N**ext >>** button to proceed to the **Plate tab** in the **Run Setup** window and proceed to step D.

10.4. Creating a Plate

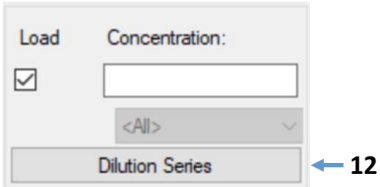
The **Plate Editor** stores information about the content of the sample in each well of the plate. The information instructs the software how to analyze and show the information presented in the **Data Analysis** window. To perform a real-time PCR run, the software requires a minimal amount of information about the contents of your samples (required steps in Step E). For this reason, it is highly recommended that you choose one of the **Express Load** options in the **Plate tab**, instead of creating a new plate on your own. The most commonly used **Express Load** plate is **Quick Plate_96 wells_SYBR Only**. When finished creating and saving your plate, click the **Next >>** button to advance to the Start Run tab.

10.5. Editing a Plate Setup for Data Analysis

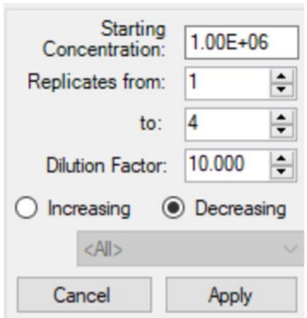
To annotate your plate so that the SCFT can help you analyze your data and for complete data analysis by the software, add more information about the contents in each well. You can add this additional information before, during, or after the run. If you are doing this before the run, click **Edit Selected**. If you are doing this after the run, click **Plate Setup** in the menu bar. Use the screen shot below to help guide you through the next steps. Alternatively, you can import a spreadsheet by clicking the **Spreadsheet View/Importer** in the **Plate Editor Window.** NOTE: If you use the **Express Load** option, steps #2-5 are already done for you.



With Std wells selected, Dilution Series available



Dilution Series Options
Steps 12a through e

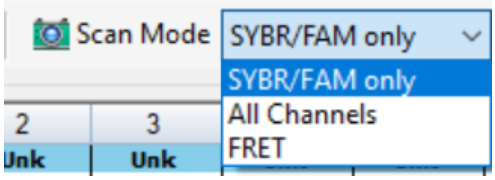


10.5.1. *Clear Wells (optional)*

Select wells with no contents (see step 6a) and click **Clear Wells**. **Warning!** If you clear a well that means the system will not read it, so make sure it is indeed empty. You cannot undo this function; you have to manually re-add all contents of a well.

10.5.2. *Choose a Scan Mode (required for qPCR run)*

Select from the options in the Scan Mode pull-down menu to select the channels that will be used to collect fluorescence data during the run.



Collect data in either All Channels (you can edit it to just the channel you need), SYBR/FAM only, or FRET. **WARNING!** Once you start a run, the Scan Mode cannot be changed. Select the correct scan mode to collect accurate fluorescence data in the correct channel. For example, if you select the FAM/SYBR only scan mode, the instrument only collects data in channel 1, and you will not collect data in the other channels.

a. To prevent data loss, the software default scan made is All Channels.
b. To decrease run time you can select only the scan mode you need to reduce collecting data in unnecessary channels.

Factory Calibrated Fluorophores and Channels:

Channel	Excitation (nm)	Detection (nm)	Calibrated Fluorophores
1	450-490	515-530	FAM™, SYBR Green I™
2	515-535	560-580	VIC®, HEX™, TET™, Cal Gold 540™
3	560-590	610-650	ROX™, TEXAS RED®, Cal Red 610™
4	620-650	675-690	CY5, Quasar 670™
5	672-684	705-730	Quasar 705™
6	450-490	560-580	Accommodates FRET Chemistry

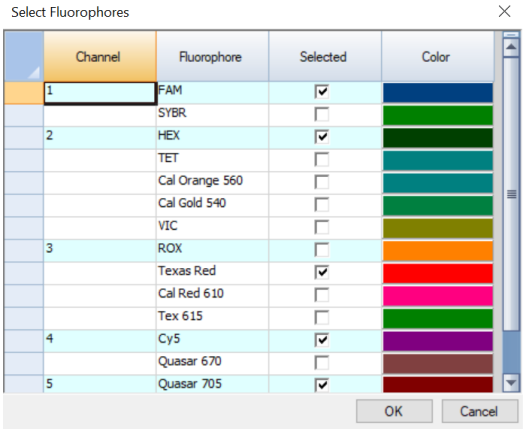
10.5.3. *Choose a Plate Size (required for qPCR run)*

Select Settings > Plate Size in the menu bar. Our instrument can only do 96 well plates. Choose the same setting even if you are using individual 0.2ml tubes or tube strips.

10.5.4. *Choose a Plate Type*

Select Settings > Plate Type in the menu bar. Select either BR White (white wells) or BR Clear (clear wells) for accurate data analysis.

10.5.5. *Select Fluorophores*



If you used Express Load this has already been done for you. but, you can use this step to choose particular channels to help reduce overall time of data acquisition. Click the **Select Fluorophores** button to open the Select Fluorophores window and select the fluorophores from a list of calibrated fluorophores.

10.5.6. *Select a Sample Type for each well (required for qPCR run)*

Select the wells (see step 6a), then select a sample type from the pull-down menu to load it. The sample type choices include Unknown, Standard, NTC (no template control), Positive Control, Negative Control, and NRT (no reverse transcriptase).

c. **How to select wells in the plate:** If you use Express Load this has already been done for you, but you can edit the wells to reduce scan time by indicating which wells don't have any sample in them. Click at least one well to select it, or select more than one well by clicking and dragging across those wells. Alternatively, select a column or row by clicking the column letter or row number, or select the entire plate by clicking the upper right corner of the plate.

IMPORTANT: When you load tube strips into the block, be sure to balance the load to assure that the pressure on the lids of the tubes is even. For example, if you load one strip on the right side of the block, be sure to load another strip (empty or full) on the left side of the block.

10.5.7. *Click the fluorophore Load check box (required for qPCR run)*

If you used Express Load this has already been done for you. but, you can use this step to choose particular fluorophores for particular wells. Click one or more check boxes next to a fluorophore name to load that fluorophore into one or more wells. To add or remove fluorophores in the plate, click the Select Fluorophores button in step 5.

10.5.8. *Load Target Name*

The target name is usually the name of the primer set used or the gene of interest. Select the wells (see step 6a), then select an existing name from the list or type a new name in the pull-down menu. Press the **Enter** key on your keyboard to load the selection. To edit the target names in the list, click the Experiment Settings button.

IMPORTANT: It is critical to write the Target and Sample names consistently. These names appear in the Gene Expression tab of the Data Analysis window, and any change in spelling, spacing, or capitalization will cause them to be analyzed as a separate group.

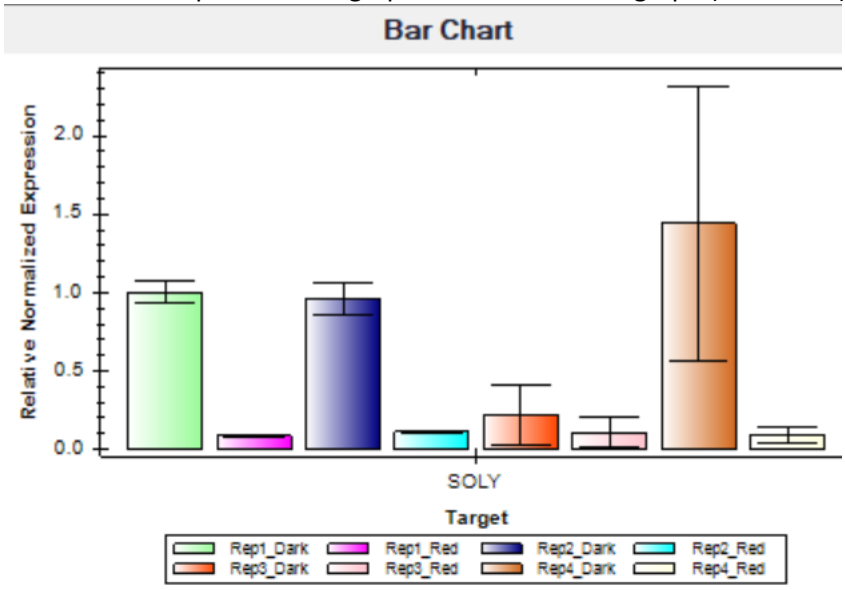
10.5.9. *Load Sample Name*

In order for the CFX Manager Software to organize your data by different samples you need to name the samples present in your experiment. For example, you might be looking at a gene of interest between mutant and wild-type genotype sample types. Alternatively, if you are setting up a standard curve, the sample name could be the dilution of nucleic acid present in those wells. Select the wells (see step 6a), then select an existing name from the

list or type a new name in the pull-down menu. Press the **Enter** key on your keyboard to load the selection. To edit the sample names in the list, click the Experiment Settings button.

10.5.10. Load Biological Set Name (optional)

Biological Set Names are useful when you have replicates of your data on the same plate (or across plates) that you want to view in the Gene Expression tab of the Data Analysis Window as independent bar graphs within the same graph (see below).



a. To enable the Biological Set Name option, check the **Biological Set** box in the **View** portion of the Plate Editor window.
b. Select the wells (see step 6a), then select an existing name from the list or type a new name in the pull-down menu. Press the **Enter** key on your keyboard to load the selection.

10.5.11. Load Replicate #

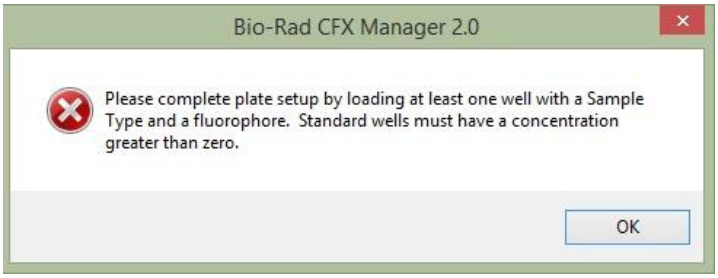
In order for your Cq values, standard deviations, and standard errors to be calculated you need to indicate how many technical replicates of each sample-target combination you prepared. Select the wells (see step 6a) of the same **Sample Type**, then click Replicate Series:

a. **Replicate Size:** Indicate the number of technical replicates present for the same sample
b. **Starting Replicate #:** Usually start with 1, and the software will automatically adjust this number as you add additional replicate series. Sometimes you have to change this number yourself.
c. Click the radial button to indicate if your technical replicates are **Horizontal** (across the row) or **Vertical** down a column.
d. Click **Apply.**
e. If you don't have a standard curve on this plate proceed to **Experiment Settings.**

10.5.12. Load the Concentration

For standard curve generation, select the wells (see step 6a) of the **Standard** within one Target, then click the **Dilution Series** button that has now appeared below the Replicate Series button, and fill in the following information:

a. **Starting Concentration:** If doing relative qPCR the starting concentration does not matter. If doing absolute qPCR you'll want to enter the known concentration of the first replicate.
b. The software should automatically detect which replicates have been highlighted.
c. Enter the **Dilution Factor**. For example, this would be 10 for a 10-fold dilution series.
d. Click he radial button to indicate whether the dilution series is **Increasing** or **Decreasing** across the technical replicates.
e. Click **Apply** to load the dilution series into the selected wells. **NOTE:** A scroll bar appeared when you clicked the dilution series button, you will need to scroll down to click apply. If you accidently click OK in the Plate Editor window instead you will get the error shown below. Click OK to dismiss the error message, then scroll down in the Plate Editor window to click Apply for the Dilution Series.



10.5.13. Edit Experiment Settings

Change the **Experiment Settings** to add information for data analysis. For example, select reference genes, control samples, and indicate primer efficiencies. You can also change the color of the targets or samples graphed in the Gene Expression chart. **NOTE:** Depending on what type of analysis you are doing, i.e. Temperature Gradient, Primer Efficiency, Gene Expression, or Absolute Quantification, you may not need to use all the settings.

a. To open the **Experiment Settings** window, follow one of these options:
   i. While setting up a run, click the **Experiment Settings** button in the Plate Editor
   ii. While analyzing data in the Data Analysis window you can either select **View/Edit Plate f**rom the **Plate Setup** button, then click the **Experiment Settings** button. Or, in the Gene Expression tab, click the **Experiment Settings** button.

b. In the **Experiment Settings** window there are several global data analysis options:
   i. Click the **Show Analysis Settings** box in the Experiment Settings window to view more parameters for each target or sample.
   ii. Under **Biological Set Analysis Options** choose the best way to display your data, if you setup Biological Sets in the Plate Editor window.
   iii. Choose which sample types to exclude from the Gene Expression analysis. The most common sample to exclude is the NTC.

c. In the **Target** Tab:
   i. Select the target(s) that are being used as reference genes in your data analysis. Click the box in the **Reference** column next to the Name for that target.
   ii. For easier visual analysis, click a color in the **Color** column to change the color of the targets graphed in Gene Expression tab of the Data Analysis Window.

iii. Enter a number for the efficiency of each target in the **Efficiency(%)** column. Select **Auto Efficiency** if the data include a standard curve, and the software calculates the efficiency for you.

d. In the **Samples** Tab:
   i. Select the sample as a control sample, click the box in the **Control** column next to the Name for that sample.
   NOTE: Only one control can be selected per well group. Multiple well groups on a plate can each have a control sample, but when All Wells is selected from the data analysis drop-down menu, only one control box can be checked in the Experiment Settings window. If more than one control is selected, the bar chart data will be plotted with "None" as the control sample. To change this setting for the bar chart, select a control sample from the drop-down menu in the settings pane.
   ii. For easier visual analysis, click a color in the **Color** column to change the color of the samples graphed in the Gene Expression tab of the Data Analysis Window.

e. Click **OK** when finished to return to the Plate Editor or Data Analysis windows.

10.5.14. Define Trace Styles (optional)

Use the Trace Style window to adjust the appearance of traces in the amplification and melt curve charts on the Quantification and Melt Curve tabs. There are two ways to open this window:

a. In the Plate Editor window, click the **Trace Styles…** button
b. In the Data Analysis window menu bar, select **Settings > Tracee Styles**

Use the tools in the Trace Styles window to adjust the appearance of traces, and preview the changes in the well selector at the bottom of the window. There a several options to change your trace styles:

a. Select a specific set of wells by using the well selector at the bottom of the window. Alternatively, select wells that contain one sample type in the pull-down menu in the Wells column, including Unknown, Standard, NTC Positive Control, Negative Control, NRT, or your custom name sample types. Click a box in the **Color** column to select a color for the trace then click **OK**, the new color will appear in the Well Selector.
b. If desired, you can add a symbol in addition to the color (or instead of changing the color). To do this, select a symbol from the pull-down menu in the **Symbol** column.
c. Click one of the **Color Quick Set** buttons to apply colors as specified:
   i. **Random by Well:** Random colors for each well
   ii. **Random by Replicate:** Random colors for each replicate
   iii. **Use Fluor Colors:** A color is given for each fluorophore
   iv. **Use Target Colors:** A color is given for each target
   v. **Use Sample Colors:** A color is given for each sample

10.5.15. Create Well Groups (optional)

Set up well groups when you want to analyze sets of wells within the same plate in the Data Analysis window. For example, create well groups when you want to find the best primer set by comparing the data in different standard curves.

Creating well groups also allows you to use them for independent data analysis. You can analyze each well group with a different baseline threshold method or standard curve.

a. To open the click the **Manage Well Groups** button (red/yellow/green circles icon) in the toolbar of the Data Analysis window, or open the Plate Editor and click the **Well Groups** button in the toolbar.

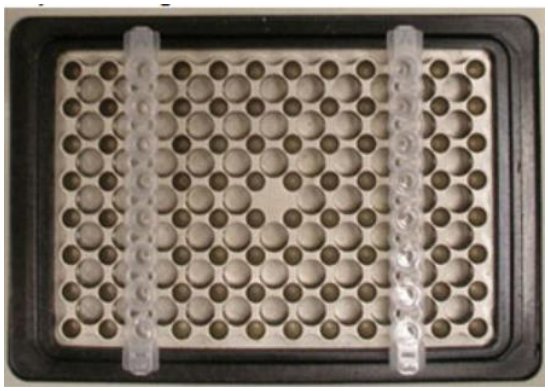
b. To create Well Groups in the Well Groups Manager follow these instructions:
   i. Click **Add** to create a new group. The drop-down menu shows the group name as **Group 1** for the first group.
   ii. Select the wells that will compose the well group in the plate view. Selected wells change color (turn blue).
   iii. Change the name of the croup by selecting the group name in the pull-down menu and typing a new name.
   iv. Create more well groups by repeating the first two steps. You can review the groups, but selecting the group name in the drop-down list.
   v. Delete well groups by selecting the group name in the drop-down list, and clicking the **Delete** button.
   vi. Click **OK** to finish and close the window.

10.5.16. Save Plate Setup

When you are finished with the Plate Editor window, click **OK** at the bottom of the window. The software will ask if you want to Apply changes, click **Yes**.

10.6. Start Run

1. After saving your Plate Setup, click the **Next >>** button to advance to the Start Run tab.
2. If you haven't done so already, open the lid of the instrument by clicking the **Open Lid** button or pressing the physical button on the instrument. **IMPORTANT:** Never for the lid open or closed with your hands. Always press the appropriate button and let the lid open or close on its own.
3. Place the PCR reactions in 0.2ml microplates or tube stripes for your run into the instrument block.
   NOTE: When you load tube strips into the block, be sure to balance the load across the block to assure that the pressure on the lids is even. For example, if you load one tube strip on the left side of the block, be sure to load another tube strip (empty or full) on the right side of the block.



4. Close the lid of the instrument by clicking the **Close Lid** button or pressing the physical button on the instrument. **WARNING:** Opening the lid during a run will pause the protocol and might alter the results of the run.
5. Click the **Start Run** button to start the run.
6. Review the progress of the run and view the accumulating data in this window. To make changes to the plate layout during the run, click the Plate Setup button on the Real-time Status tab and select **View/Edit Plate**.
7. When the run finishes, the software automatically saves the data file, and opens the Data Analysis window.

10.7. Data Analysis for Relative qPCR Runs

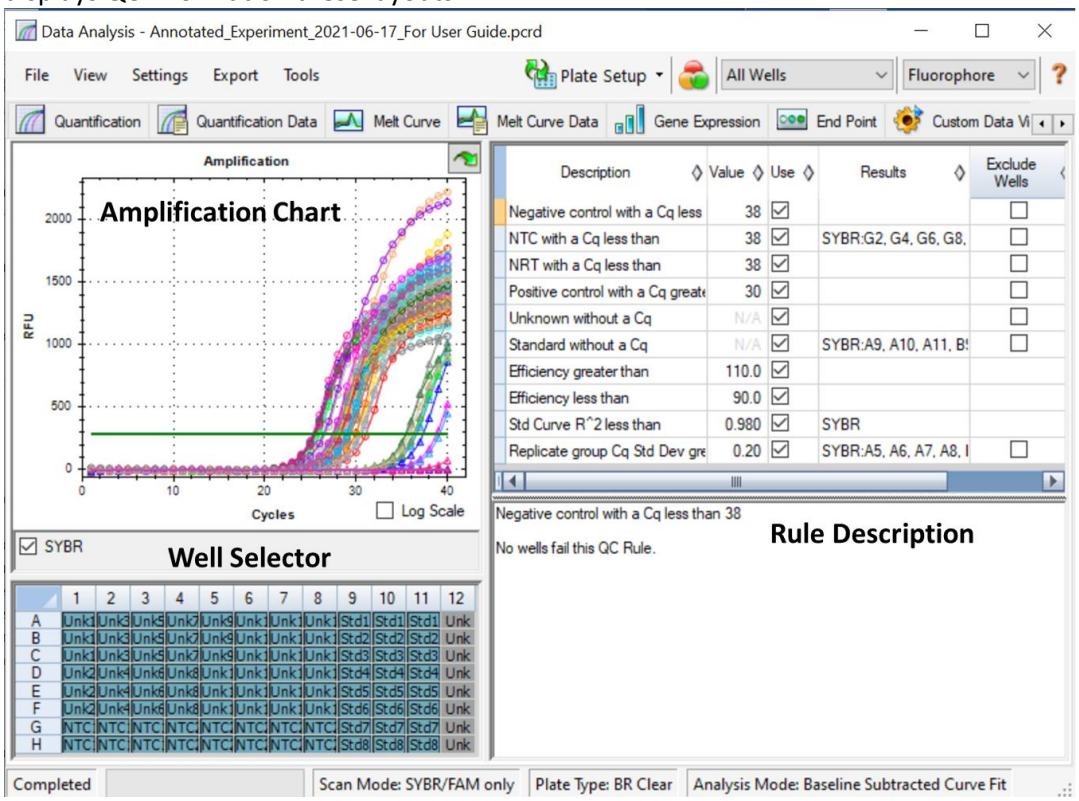
The Data Analysis window includes a menu bar, toolbar, and up to eight tabs. These tabs show analyzed real-time data from one run (a protocol and plate file run on one instrument). The software only displays a tab if the data are available for that type of analysis. This user guide will focus mainly on the steps needed for analyzing optimizing, validating, and expression data for relative qPCR runs. The first two steps are things you need to do regardless of how you are analyzing your data:

10.7.1. Edit contents of the plate

If you haven't done so already, edit the contents of your plate. Click **Plate Setup** in the menu bar and select **View/Edit Plate**, then follow section 10.5 of this user guide.

10.7.2. Review QC tab

a. Select the **QC** tab to quickly assess the quality of the experimental data. The software displays QC information these layouts:



i. **Amplification Chart:** Shows the relative fluorescence (RFU) for each well at every cycle. Each trace in the chart represents data from a single fluorophore in one well.
ii. **QC rules:** Shows the currently applied QC rules and the settings that define each rule. You can turn on or turn off rules by clicking the check box next to the rule in the **Use** rule column. You can modify the cutoff for the rule, by double clicking in the **Value** box for the rule you wish to modify. Available rules:
    - **Negative Control with a Cq less than** – Indicates if you had some kind of contamination resulting in early amplification in the negative control.
    - **NTC with Cq less than** – Also indicates if you had some kind of contamination resulting in early amplification in the negative control.
    - **NRT with a Cq less than** – Indicates if you have some gDNA contamination
    - **Positive control with a Cq greater than** – Indicates if there is a problem with the reaction because you didn't get expected amplification in your positive control.
    - **Unknown without a Cq** – Indicates when amplification has not occurred
    - **Standard without a Cq** – Indicates when amplification has not occurred, this is a problem if it occurs in the standard curve. Sometimes this is a common occurrence in the lowest concentration tested, which indicates that is the limit of detection.
    - **Efficiency greater than** – Indicates when your primer efficiency is outside the acceptable range.
    - **Efficiency less than** – Indicates when your primer efficiency is outside the acceptable range.
    - **Std Curve R^2 less than** – Indicates when your data does not fit the regression line
    - **Replicate group Cq Std Dev greater than** – Indicates when there is a problem with your technical replicates.
iii. **Well Selector:** Selects the wells with the fluorescence data you want to show.
iv. **Rule Description:** Shows the selected QC rule and highlights wells that fail the rule.

b. Wells failing a QC criteria are listed in the **Results** column of the QC rules table and in the Rule Description pane. These wells can be excluded, or included, in analysis by checking or un-checking the appropriate **Exclude Wells** checkbox.
    i. You really want to scrutinize the rules "**NTC with a Cq less than"**, and "**Replicate group Cq Std Dev greater than"** because these can indicate problems with optimization, validation, and contamination. These types of problems have a huge effect on accurately determining primer efficiency or expression values.
    ii. For the "Replicate group Cq Std Dev greater than" if you have three technical replicates, it's possible just one of those replicates is causing all three wells to fail QC. You may want to visually inspect your technical replicate Cq values to see if just one is 0.5 cycles away from the others. If one is 0.5 cycles away from the others, right click on the well in the Well Selector then select **Well No., > Exclude from Analysis**. This does not get rid of the fluorescence data, just excludes the Cq value from the analysis.

c. As part of the QC process, you also always want to check the **Melt Curve** tab for wells that are failing a QC rule to help diagnose the problem. Even if wells aren't failing a QC

rule, you should always check the **Melt Curve** tab to see if there is any unexpected amplification.

10.7.3. Gene Expression Tab

Now that the data has been properly annotated (see section 10.5) and cleaned up using the QC rules (see section 10.7.2), you can look at the gene expression data by clicking on the **Gene Expression** tab.

a. Bar Chart

The bar chart displays gene expression data as vertical bars that are proportional in size to the normalized fold expression of each target for the sample indicated.

To adjust the data you view in the Bar Graph Chart tab, follow these methods:

- Select a well group to view and analyze a subset of the wells in the plate. Select each well group by name in the Well Group menu in the toolbar
- Select the Analysis Mode (Fluorophore or Target) in the menu bar by selecting **Settings > Analysis Mode**
- Change the order of the bars in the chart, by right clicking and selecting the **Sort** option
- Select a Mode to analyze the data using **Normalized expression (ΔΔCq)** or **Relative quantity (ΔCq)**.
- Select a **Graph Data** option to graph the data **Relative to control**, or **Relative to zero**. When you select a control sample in the Experiment Settings, the software automatically defaults to calculate the data relative to that control.
- Select a Control sample to use for gene expression calculations
- Click the **Show Chart Settings** box for more options:
  - Select an **X-axis** option to show data from the Target or the Sample on that axis.
  - Select a **Y-axis** option to graph the data as Linear. Log 2, or Log 10 scale
  - Select a **Scaling** option to choose unscaled to leave the data unscaled. Select **Highest** to scale the expression data to the highest value or select **Lowest** to scale the expression data to the lowest value.
  - Select an **Error Type** to choose Standard Error of the Mean (default, SEMs), or Standard Deviations (Std Devs)
  - Select a **Chart Error Bar Multiplier** to adjust the error bars for SEMs or Std Devs.
- Click the **Experiment Settings** button to open the Experiment Settings window and change information about the targets or samples listed in the run.
- Click the **Target Stability Value** button to determine the stability of reference targets used in the run. Target stability values can be calculated whenever more than 1 reference gene is used. The software calculated two quality parameters for the reference genes:
  - **Coefficient of Variation (CV)** of normalized reference gene relative quantities. A lower CV value denotes higher stability.
  - **M-value**: A measure of the reference gene expression stability. Acceptable values for stably expressed reference genes according to Hellemans et al. (2007):

Samples	CV	M
Homogeneous	<0.25	<0.5
Heterogeneous	<0.5	<1

b. Clustergram

The clustergram shows the data in a hierarchy based on the degree of similarity of expression for different targets and samples as follows:

- Up regulation (higher expression) by a red square
- Down regulation (lower expression) by a green/blue square
- No regulation by a black square
- The lighter the shade of color, the greater the relative expression difference.

On the outer edges of the data plot is a dendrogram, which indicates the clustering hierarchy. Targets or samples that have similar expression patterns will have adjacent branches while those dissimilar patterns will be more distant.

c. Scatter Plot

The scatter plot shows the normalized expression of targets for a control versus an experimental sample as follows:

- Up regulation: red circles
- Down regulation: green/blue circles
- No change: black circles

d. Volcano Plot

The volcano plot shows the change in expression (regulation) of a target for an experimental sample compared to a control and indicates the degree of significance based on p-value. Data with low p-values will appear towards the top of the plot. Data typically of most interest will appear in the upper left and right regions of the plot, which correspond to data of high statistical significance and large changes in expression.

The plot image shows the following changes in regulation based on the threshold setting:

- Up regulation: red circles
- Down regulation: green/blue circles
- No change: black circles

e. Heat Map

The heat map shows the regulation of a target for an experimental compared to a control sample based on relative normalized expression and its position on a plate. **NOTE:** For better visual impact (i.e. greater variation in color shading), Log2 of Relative Normalized Expression is used.

A legend below the heat map shows the range of normalized expression which corresponds to the following:

- Up regulation: red
- Down regulation: green/blue
- No change: black

f. Results

This table describes the information shown in the Results tab:

Information	Description
Target	Target Name (amplified gene)
Sample	Sample Name
Mean Cq	Mean of the quantification cycle
Mean Efficiency Corrected Cq	Mean of the quantification cycle after adjusting for the reaction efficiency
Normalized Expression	Target expression normalized to a reference target (ΔΔCq)
Relative Normalized Expression	Normalized expression relative to a control sample
Regulation	Change in expression relative to a control sample
Compared to Regulation Threshold	Up, down, or no regulation based on a threshold setting
P-Value	The probability that a difference in expression is significant
Exceeds P-Value Threshold	Indication of whether or not the p-value for a target exceeds a threshold

10.8. Organize and Export Spreadsheet Data

To organize data in the spreadsheet, click any column header to sort the data by the values in that column. Sort the data by up to three columns.

To export the results to an Excel spreadsheet or text file you have two main options:

1. In the main menu bar, there are four options from the **Export** drop-down menu. Two of which you might commonly use:
    a. Select **Export > Export All Data Sheets to Excel** to export all the spreadsheet views from every tab of the CFX Manager software into individual excel formatted files.
    b. Select **Export > Custom Export** to open a window in which the fields to be exported and the file format can be customized.
2. Right click in the spreadsheet you want to export to select an option to export the results to an Excel spreadsheet or text file.

10.9. Quick Start Guide for Experienced Users

To create and perform a run in CFX Manager software, follow these instructions:

1. Open the Run Setup window

Open this window by clicking the **User-defined** button on the Run Setup tab of the Startup Wizard or select **File > New > User-defined Run** in the menu bar.

2. Click the Protocol tab

Open this tab to select an existing protocol, to run or edit a protocol, or to create a new protocol in the Protocol Editor window.

3. Click the Plate tab

Open this tab to select an existing plate to run or edit, or you can create a new plate in the Plate Editor window.

TIP: To get started quickly, select a "QuickPlate" from the Express Load list, then load the well contents during or after the run. The QuickPlates instructs the instrument to collect data using All Channels or SYBR scan mode, depending on the plate selected.

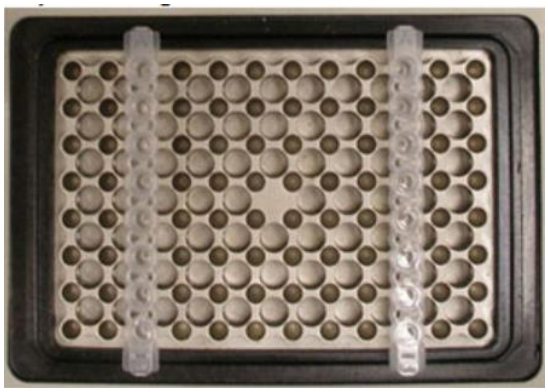
4. *Click the Start run tab*

Open this tab to enter notes (optional), check the run settings, select one or more instrument blocks, and begin the run.

5. *Load PCR reactions in the block*

Place the PCR reactions in 0.2ml microplates or tube stripes for your run into the instrument block.

NOTE: When you load tube strips into the block, be sure to balance the load across the block to assure that the pressure on the lids is even. For example, if you load one tube strip on the left side of the block, be sure to load another tube strip (empty or full) on the right side of the block.



6. *Click the Start Run button*

Begin running the protocol on the selected instruments by clicking the **Start Run** button on the Start Run tab. When the run starts, the software opens the Run Details window.

7. *View the Run Details window*

Review the progress of the run and view the accumulating data in this window. To make changes to the plate layout during the run, click the Plate Setup button on the Real-time Status tab and select **View/Edit Plate**. When the run finishes, the software automatically opens the Data Analysis window.

8. *Analyze your data*

Open the tabs in the Data Analysis window to review and adjust the data. Export the data in a data Report, or in images, spreadsheets, and text files.