

Guide to using the Bio Rad CFX96 Real-Time PCR Machine



Kyle Dobbs and Peter Hansen

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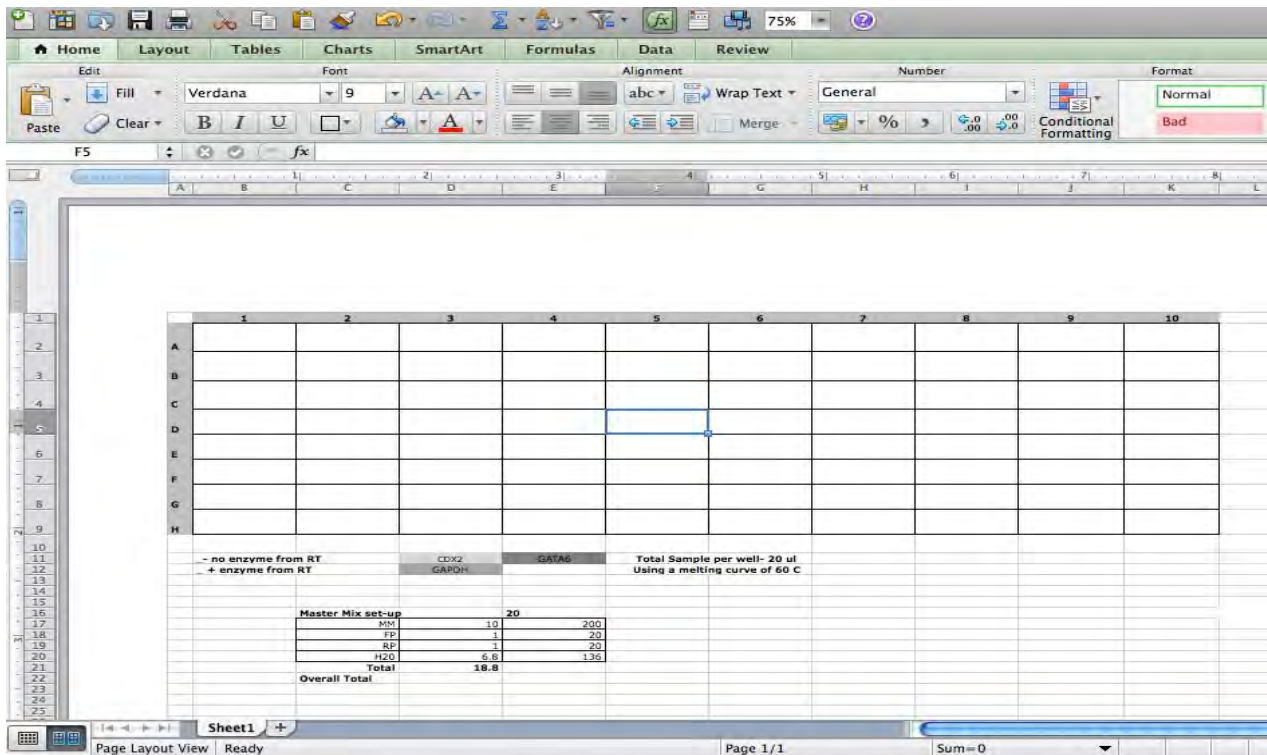
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Overview

- This pictorial guide to using the Bio-Rad real-time PCR machine is meant to supplement the Polymerase Chain Reaction guide that is also on Dr. Peter Hansen's website (http://www.animal.ufl.edu/hansen/Protocols/PCR_protocol%20Fear%20et%20al.pdf). Screen shots and pictures contained in this guide are more informational purposes only. For more information, please see the instruction manual located on the desktop computer for this machine.
- In general, we suggest that you setup you plate on the computer using excel first followed by setting up the plate on the Bio-Rad computer software before actually pipetting anything.
- **ALWAYS USE GLOVES WHEN TOUCHING THE REAL-TIME PCR MACHINE**

Setup Reaction Guidelines

- We recommend using excel or some other spreadsheet software to setup your real time plate and master-mix reaction amounts prior to using the machine. This will make the setup and your analysis easier.



- We use a total reaction volume of 20 μ l per well. This includes a total master-mix amount of 18.8 μ l (6.8 μ l of water, 1 μ l of .5 μ M forward primer, 1 μ l of .5 μ M reverse primer and 10 μ l of SsoFast EvaGreen Supermix with Low ROX (Bio-Rad)). We then add 1.2 μ l of cDNA sample to each of their respective wells.
- When setting up the plate, we always duplicate each sample to allow for averaging the cycle threshold value that is generated to compensate for pipetting error.
- Included a negative control from your reverse transcription procedure and a water sample.

Starting up the software

- The Real-time PCR machine has its own dedicated desktop computer that is currently running Microsoft 7. Login information and password are located on the top of the computer. Also at this time, turn on the CFX96 machine next to the computer.

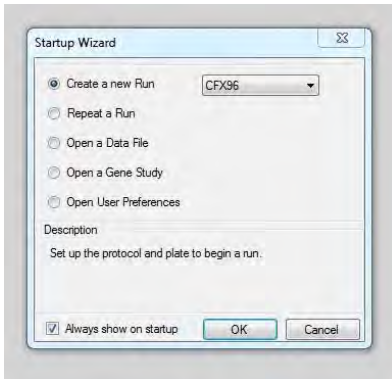


- Double-click on the icon labeled Bio-Rad CFX Manager.

- Login to the software. If you do not have a user ID please see Luciano Bonilla or Kyle Dobbs



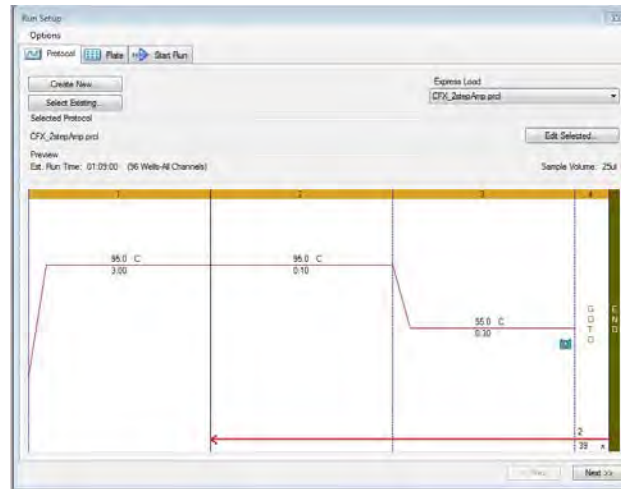
Setup Protocol on the Software



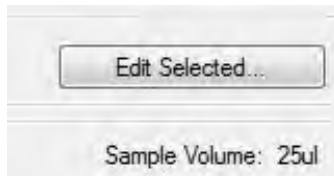
- When first starting the software, it will prompt you to create a new file, repeat a file or do other things that will be discussed later. Click on “Create a new file.”



- After clicking on “Create a new file”, you will need to create the PCR program that the machine will use. Initially, the software using a default program, but you will need to change this in order to use the real-time capabilities of the machine. After doing this once, you will not need to do this step again for future PCR reactions.



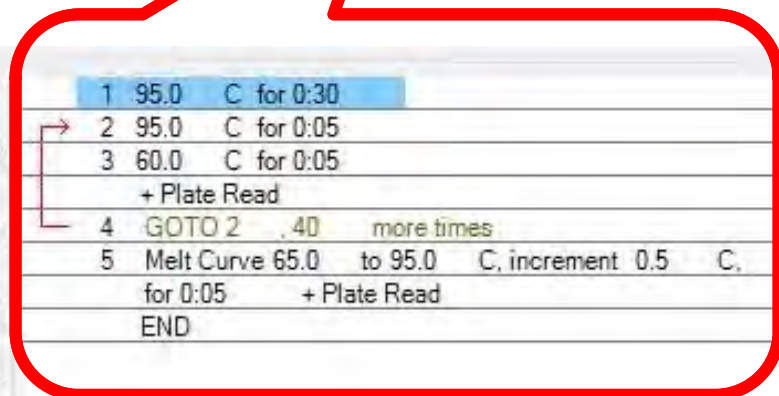
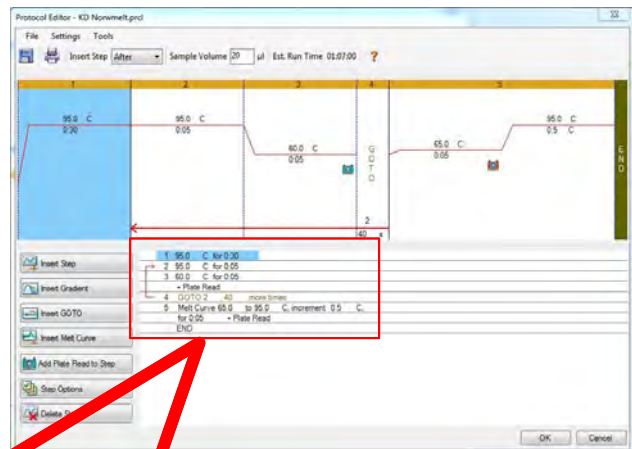
Setup Protocol on the Software



- To change the program protocol, click on the “Edit Selected” button in the top left of the window



- You will need to change the program to what is pictured on the right.

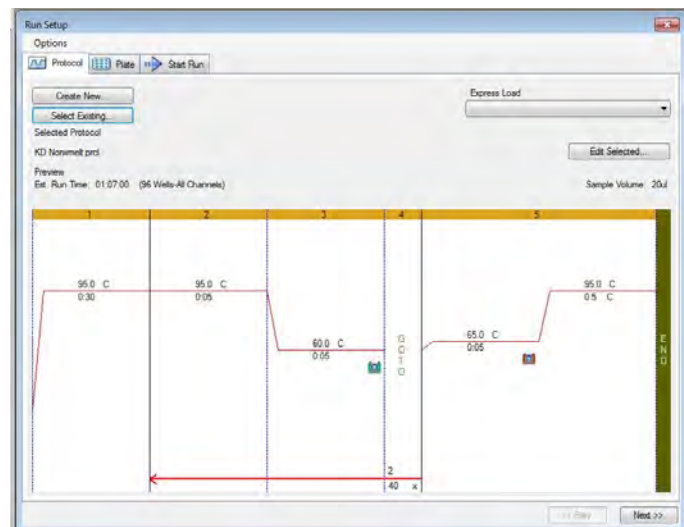


Setup Protocol on the Software

- Once the program has been changed, click OK. The software will ask for to save the file, do this as you can use this again by clicking on “Select Existing” the next time you need to run a Real-time PCR.

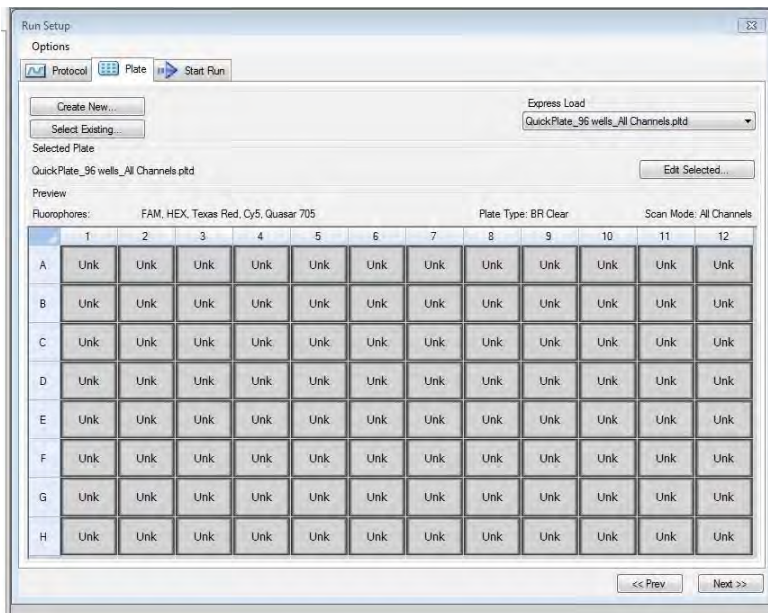


- You should now have a screen that looks exactly like the picture on the right. You are now ready to proceed to the plate setup



Setup Plate on Software

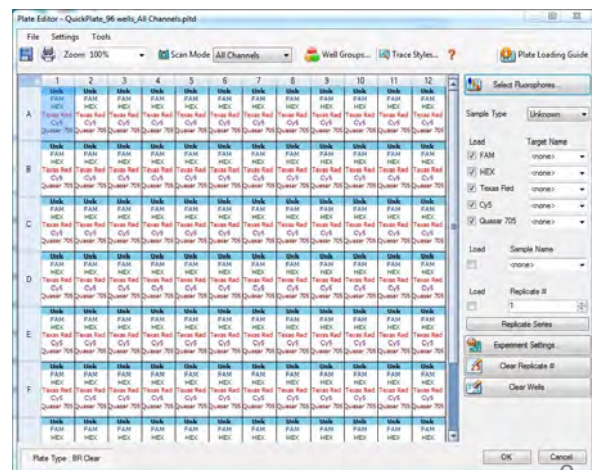
- Using the Excel spreadsheet you created earlier, you will now insert the same information into the software. *Note: The software is not as “intuitive” as Excel, i.e., you cannot copy and paste, etc.



- After clicking on the “Plate” tab located next to the “Protocol”, the image on the left should appear. This is the default plate setup that the software gives you. We will change this to mirror what you have on your Excel spreadsheet.

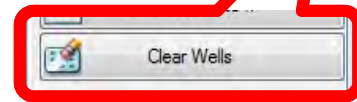
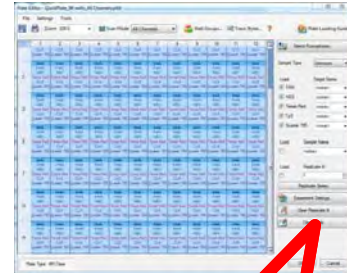


- Click on the “Edit Selected” button in the top right of the window displayed above. You will then see the window displayed to the right. As mentioned before, this is the default that the software gives you.

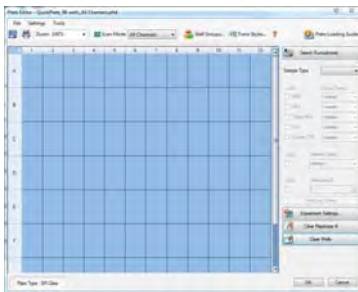


Setup Plate on Software

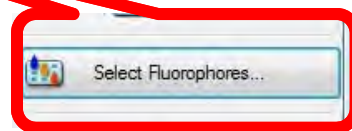
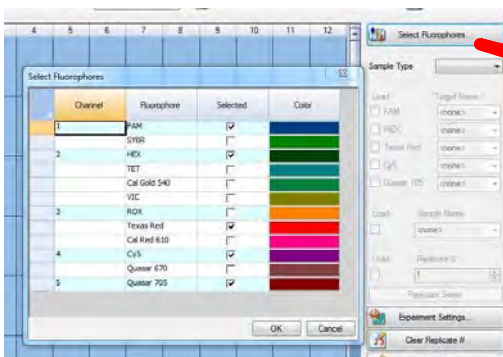
- Highlight all of the wells and click on the clear wells button.



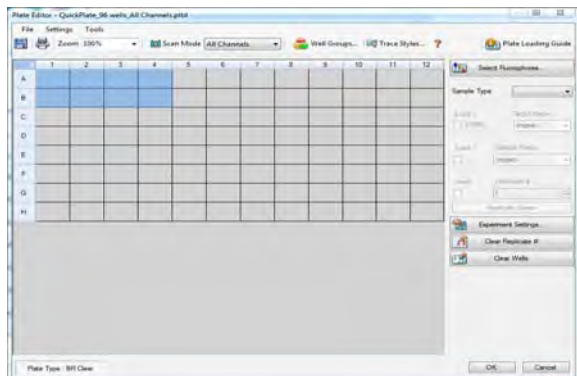
- All of the wells are now clear of the default settings.



- We use Sybr green with low ROX as our Master-mix, and we therefore only use one fluorophore. You can click on the “Select Fluorophore” button and deselect all fluorophores except Sybr. The software does not care which fluorophore you select so could just as easily select ROX, but we only need one.



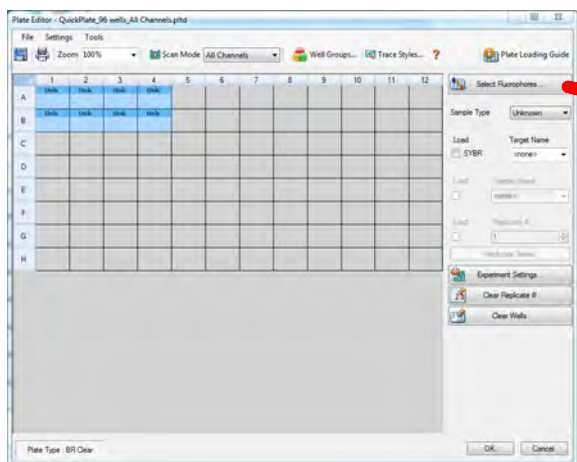
Setup Plate on Software



- Now, you can begin the replication of your Excel file onto the software. First highlight the area on the plate where you are going to be placing all of your samples.

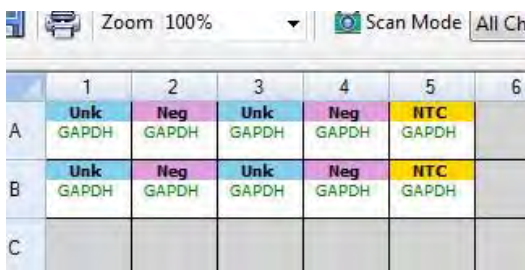
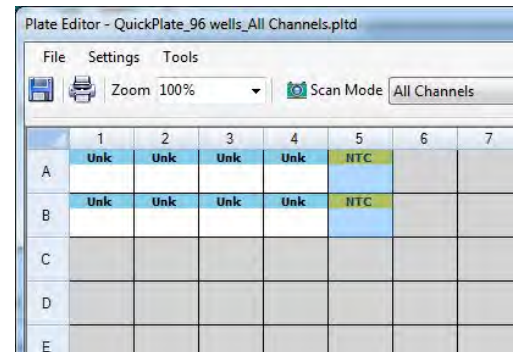


- Once highlighted, you can now select what those wells are to become. This is done by clicking on the “Select Type” button on the left. Scroll down until you find what type of sample you are inserting.



Setup Plate on Software

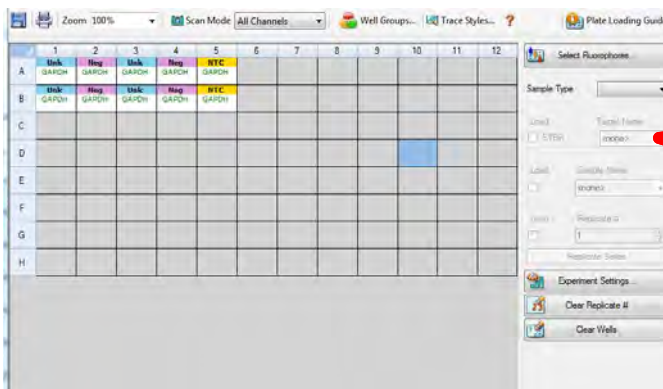
- Repeat the previous step for the water samples on your plate by highlighting those wells and selecting “NTC.”



- Repeat the previous step for the water samples on your plate by highlighting those negative control from the reverse transcription reaction and selecting “Neg.”



- Next, highlight all of the wells that are using the same primer for analysis and click on the button located to the right labeled “Target Name.” *If your gene name or “Target” is not in the scroll down list, type your gene of interest into the box.



Setup Plate on Software

- Repeat this step for your different “Targets” until you are done. Once done, click the “OK” button and save the plate setup.

The screenshot displays a software interface for setting up a plate. The main area is a grid of wells, labeled A through H and 1 through 11. The wells are color-coded and contain text indicating the target name and color. The right-hand panel contains settings for fluorophores, sample type, target name, sample name, and replicate number. The bottom status bar shows 'Plate Type : BR Clear'.

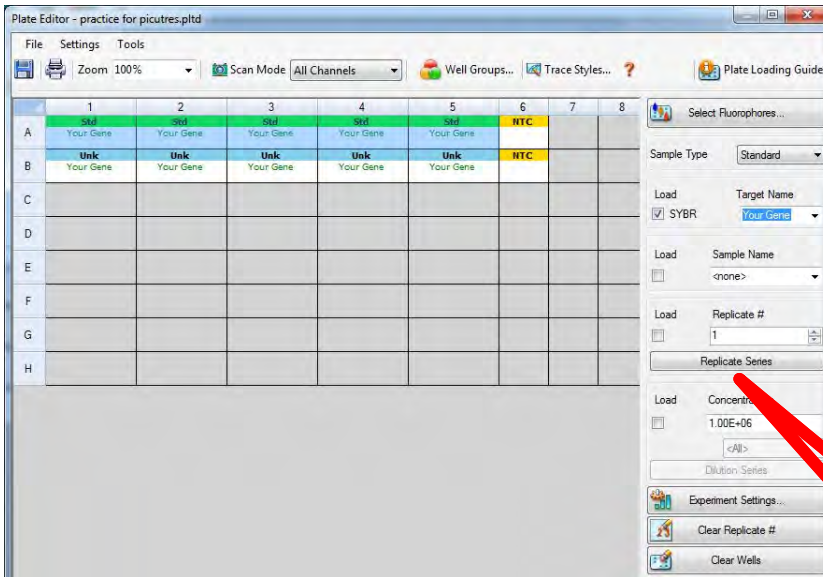
	1	2	3	4	5	6	7	8	9	10	11
A	Unk GAPDH	Neg GAPDH	Unk GAPDH	Neg GAPDH	NTC GAPDH						
B	Unk GAPDH	Neg GAPDH	Unk GAPDH	Neg GAPDH	NTC GAPDH						
C	Unk Your Gene	Neg Your Gene	Unk Your Gene	Neg Your Gene	NTC Your Gene						
D	Unk Your Gene	Neg Your Gene	Unk Your Gene	Neg Your Gene	NTC Your Gene						
E											
F											
G											
H											

Plate Type : BR Clear

Standard Curve Setup

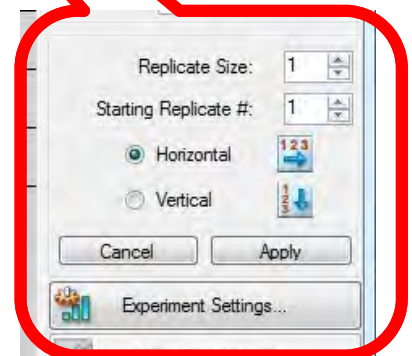
(skip if already determined)

- If you are using new primers, you need to determine their efficiency. More information on this is located on the Polymerase Chain Reaction protocol online while this portion is how set-up those wells on the Bio-Rad machine.



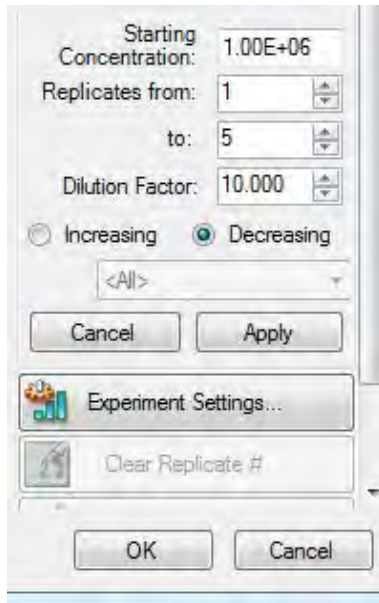
- Highlight the wells you want to use for standard curve analysis and click on Standard under the “Sample Type” button.

- Next, set the replicate size by clicking on the “Replicate Series” button. Assign the appropriate replicates



Standard Curve Setup

(skip if already determined)



- Next, assign your dilution values by first entering a starting concentration. Usually we use our nanodrop reading. The starting concentration is going to become your first standard or in this instance, well A1. You can also change your dilution factor from 10 to whatever dilution value you used. Normally, we make A1 the highest concentration, therefore A2-A5 decrease.

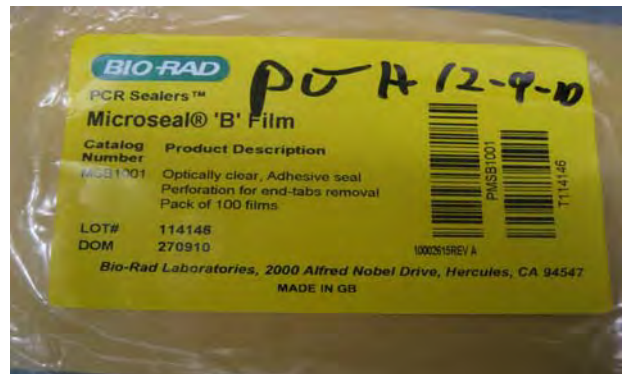


- You should see something similar to the picture below.

	1	2	3	4	5	6
A	Std-1 1.00E+06	Std-2 1.00E+05	Std-3 1.00E+04	Std-4 1.00E+03	Std-5 1.00E+02	NTC
B	Unk Your Gene	Unk Your Gene	Unk Your Gene	Unk Your Gene	Unk Your Gene	NTC
C						

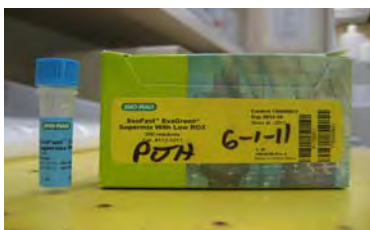
Setup Plate

- To see the reaction setup, please see the “Setup Reaction Guidelines” page. When using the Bio-Rad machine, you can ONLY use Bio-Rad plates, master-mix and plate covers. Failure to do so will result in undesired results. Plates and plate covers are located in the top shelf of the molecular biology bench



- Bio-Rad 96-well plates
- Bio-Rad 96-well plate clear covers

- EvaFast Master-Mix with low ROX is located in two places. The currently used Master-Mix is located in walk-in fridge in the “Molecular Biology” drawer while the stock tubes are located in the -20 freezer located in the lab.



- Bio-Rad Master-Mix



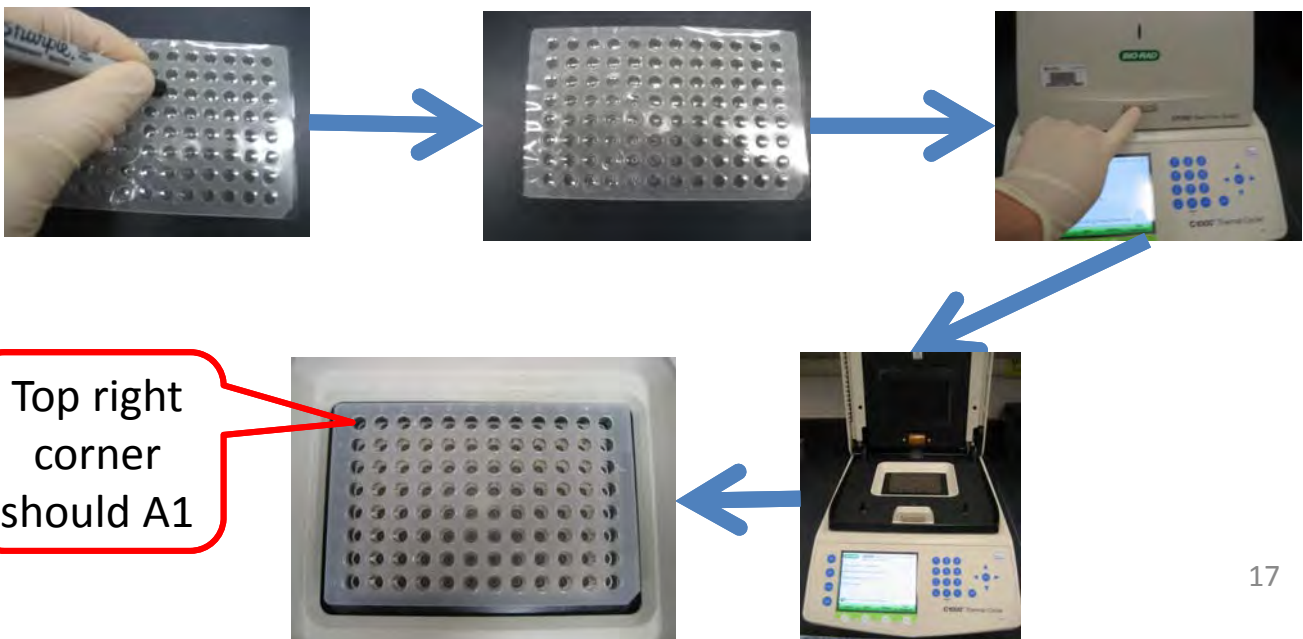
- Location of Stock



- Location of currently used

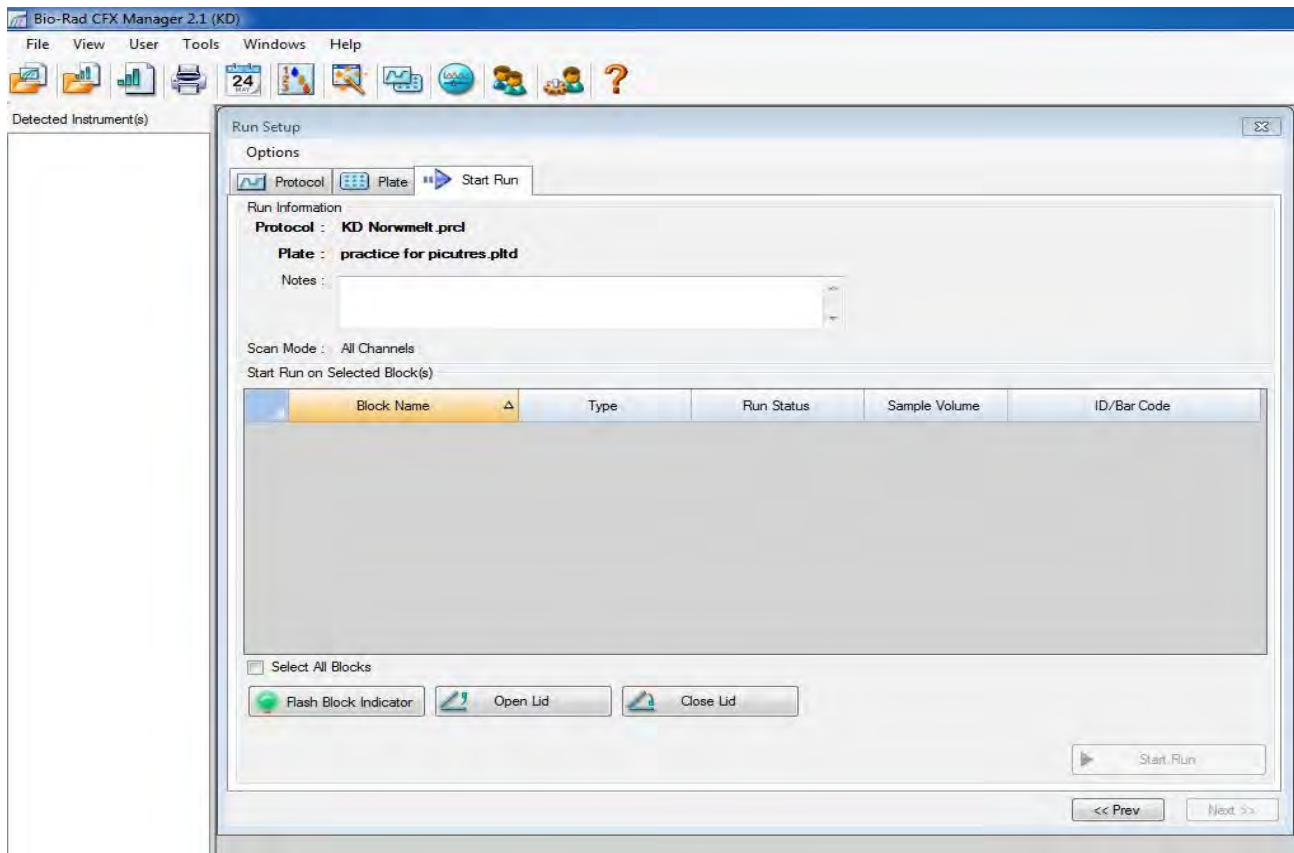
Setup Plate

- When making the master-mix for the wells, it is crucial to use DEPC treated water. We have a stock bottle located to the right of the molecular biology bench. PLEASE aliquot a small amount (~3-4 ml) into a 10 ml tube and label this with your initials. ONLY use your tube and do not share your tube with others. Be extremely careful not to contaminate the stock.
- When making your plate, use a new aerosol tip each time you pipette something.
- Once you are finished with your plate, you need to place a cover on the top of the plate. Be extremely careful not to shake or bump you plate while doing this. You can centrifuge the plate at this step if needed. We use the blunt end of a sharpie to seal the gaps between the wells. Failure to do so will allow the master-mix in each well (along with your sample) to evaporate during the real-time process.



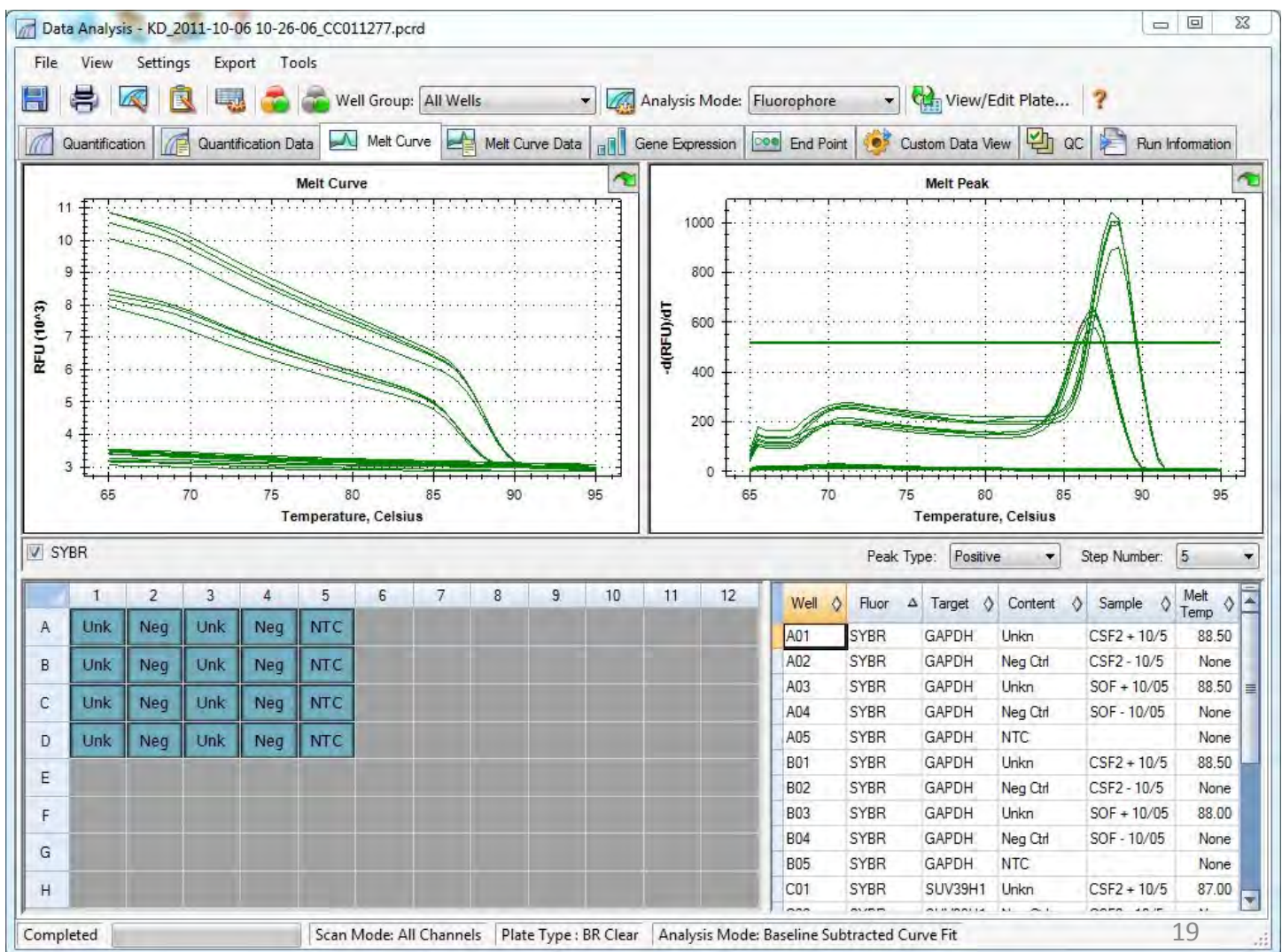
Start!

- After closing the lid on the Bio-Rad machine, the “Start Run” button will appear and the software is ready to start the run. After clicking the “Start Run” button, you will be prompted to save the data file. This will save the data generated by the run at each of the steps during the reaction.



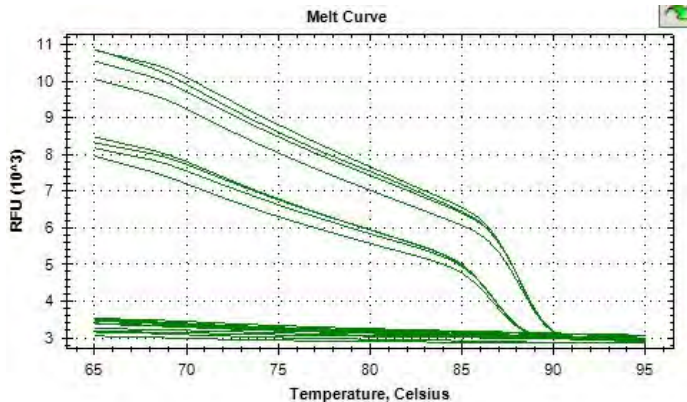
Data Analysis of Output

- Analysis of how to understand cycle threshold is explained in the Polymerase Chain Reaction protocol online. This will only explain how to read what the software gives.
- When the run has been completed, the picture below will be displayed. There are many features of this software that we do not use on a a daily basis, so we will cover only the basics. See the instruction manual for more information.



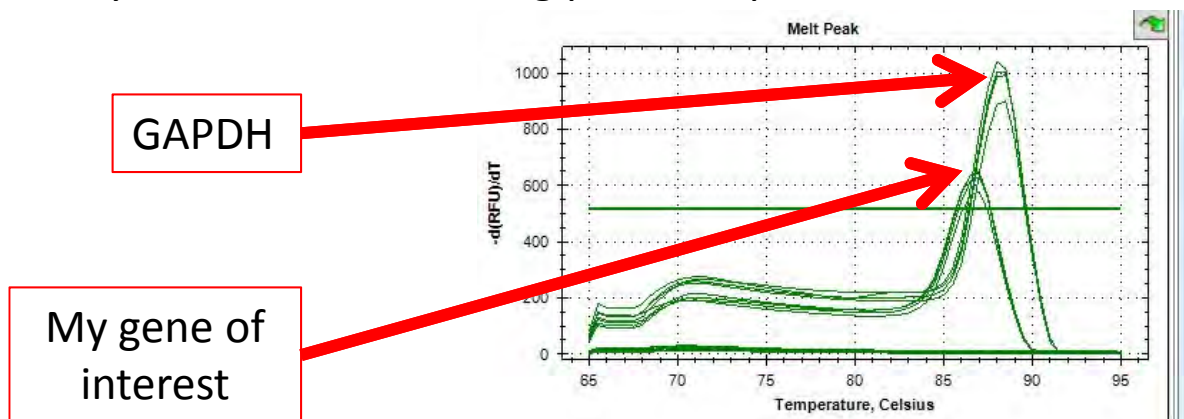
Data Analysis of Output

- There are many places to start the analysis of your data, but we will start with examining the Melt Curve.

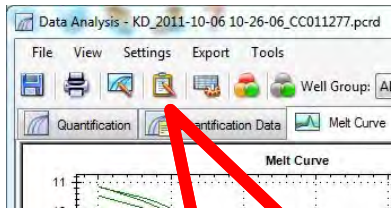


- It is critical to use Melting Curve analysis to determine that you are only amplifying one product. You can also run your sample on a agarose gel and have your sample sequenced to insure you are amplifying the correct product.

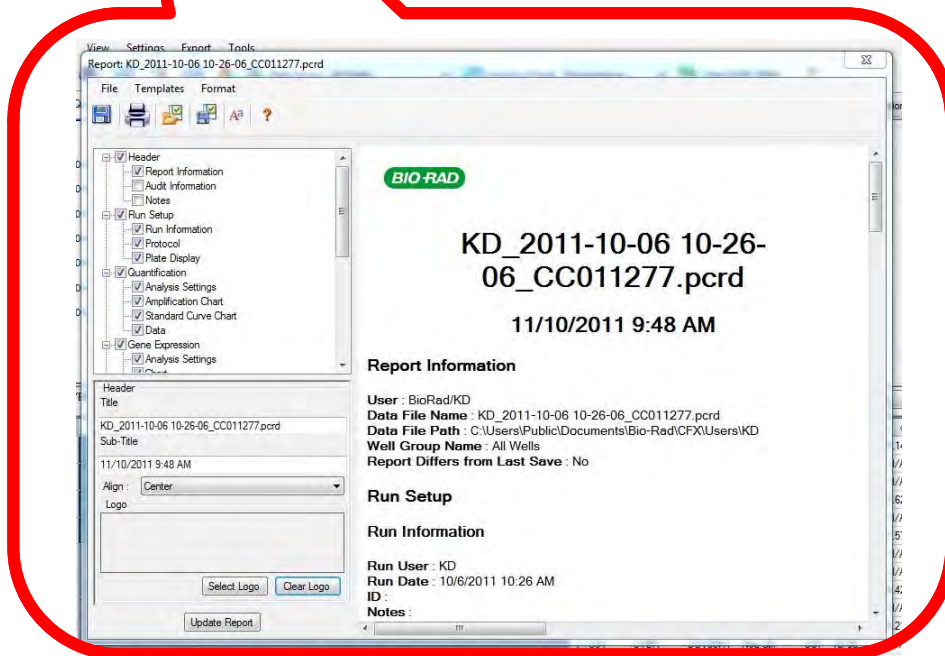
- Sybr green binds to all dsDNA and a melting curve allows you to see when that piece of dsDNA dissociates, including small pieces of possible primer dimers. By examining the the Melting Curve and seeing that there are only slopes for as many primers used, you can reassure yourself you are only analyzing the correct product. This is also shown in the Melt peaks, showing that for the two different primers used, there are only two different melting peak temperatures.



Data Analysis of Output



- A print out of all of the data that was generated can be obtained by clicking on the “Report” button. The file displayed below is a PDF which can also be save and used on a computer that does not have the Bio-Rad software.



- In this report, the cycle threshold values are display for each of the wells. From here you can calculate the fold differences between the genes.

Fold Change Calculations

- Before you calculate the fold change of your samples, you need to examine the ct values generated between duplicates. If the values have a large difference (~.5 ct or greater), you might not want to use them. Also, insure that your negative controls do not have a viable ct value. This means that as long as your negative controls have a ct that does not make sense or is a “N/A”, you are OK. The same goes for the water sample.

	A	B	C	D	E	F	G	H	I	J	K
1					Average CT	GAPDH Average CT					
2		Date	Sample	CT			Delta Ct	Delta-Delta Ct	Fold difference of relative to negative treated		
4		10/5/11	GAPDH CSF2+	27.08	27.04						
5		10/5/11	GAPDH CSF2+	27							
6		10/5/11	GAPDH SOF+	27.5	27.475						
7		10/5/11	GAPDH SOF+	27.45							
8		10/5/11	SUV39H1 CSF2+	31.57	31.685	27.04	4.645	-0.83	1.777685362		
9		10/5/11	SUV39H1 CSF2+	31.8							
10		10/5/11	SUV39H1 CSF2+	32.9	32.95	27.475	5.475	0	1		
11		10/5/11	SUV39H1 CSF2+	33							

- Fold change is calculated by first averaging the ct values for all samples. Following this, find the delta ct by taking the gene of interest average minus the GAPDH average. Do this for treated and not treated samples. After this, find the delta-delta ct by taking the treated delta ct value minus the non treated sample delta ct value. Determine the fold by taking the delta-delta ct value (x) and calculating 2^{-x} .