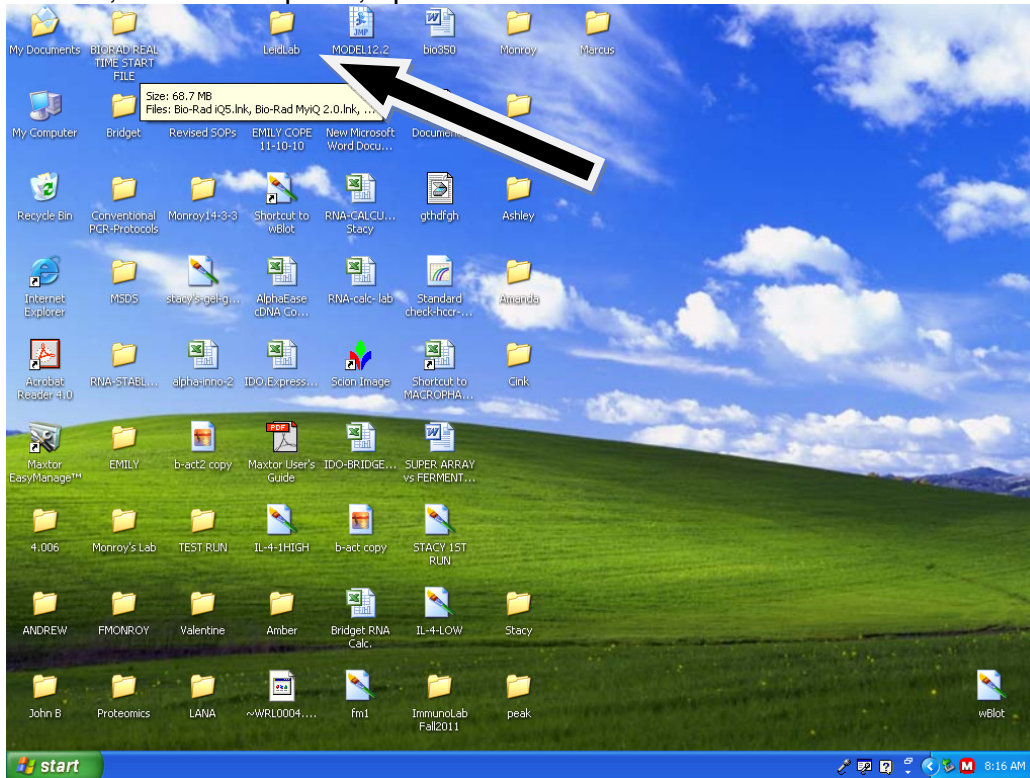
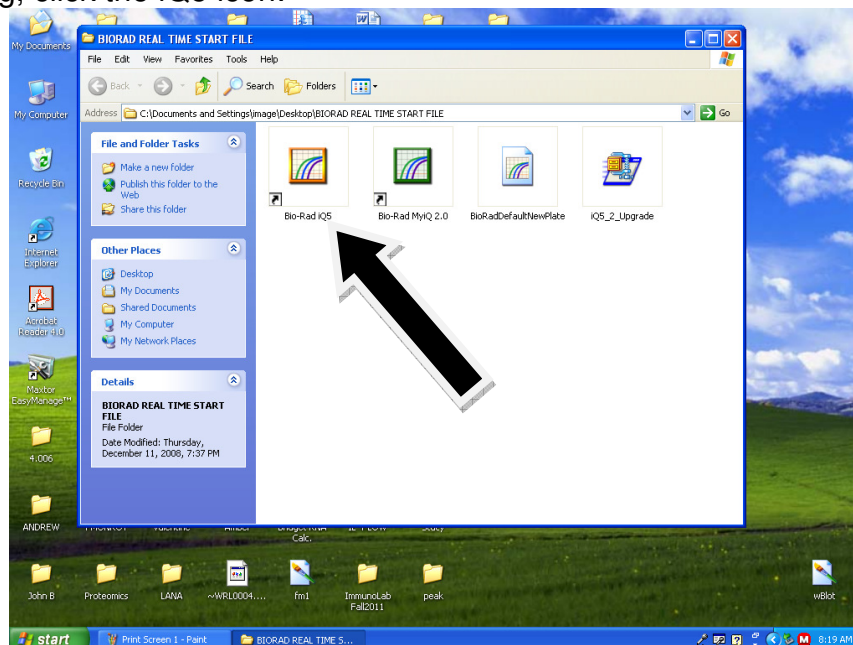


## Real-Time (qPCR) protocol:

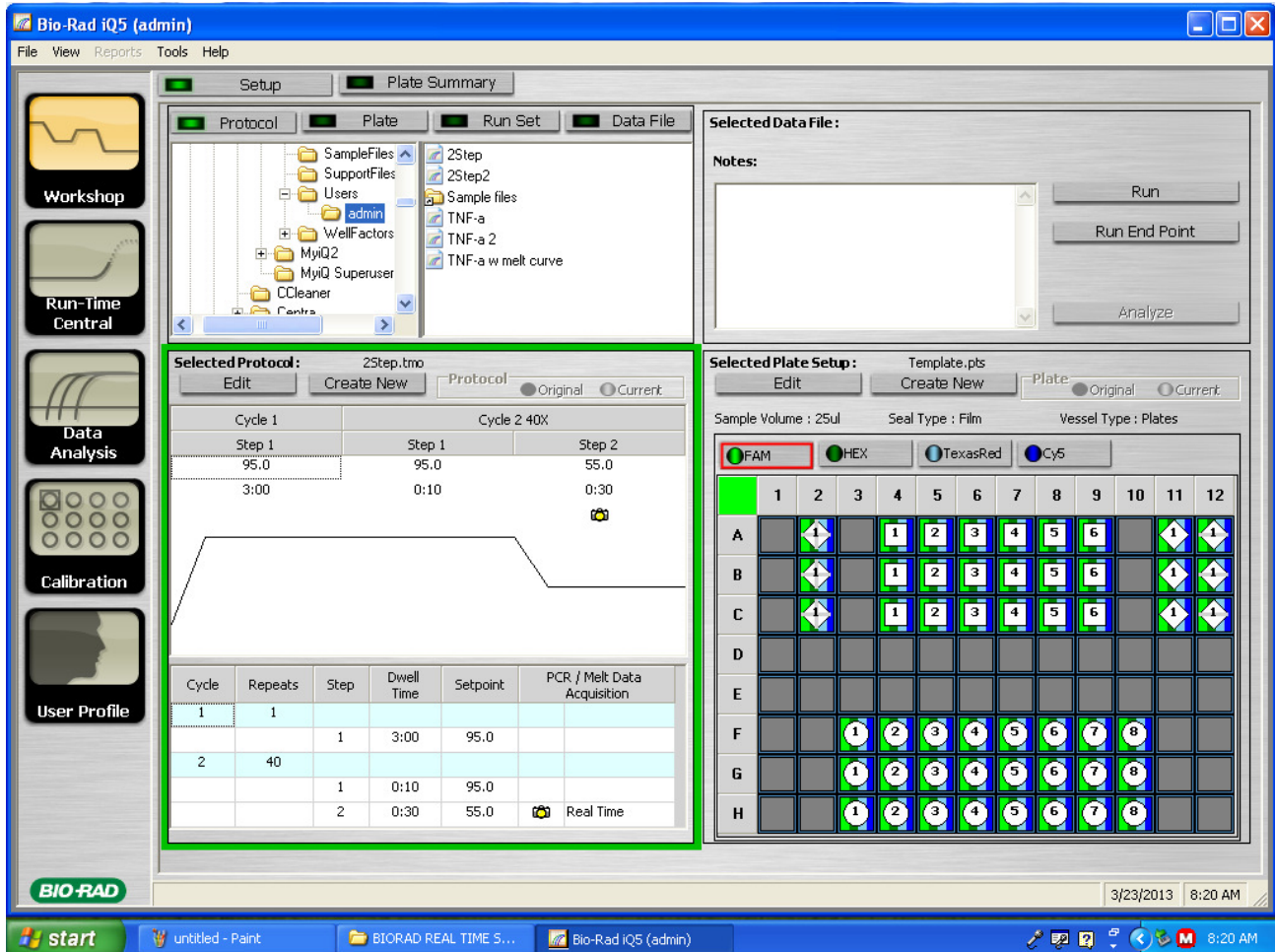
1. First, you'll want to turn on the qPCR machine itself before using the computer. Turn on the machine (**bottom switch**) and the camera (**top switch**)
2. Next, on the computer, open the "BioRad Real Time Start File" folder icon:



3. After opening, click the iQ5 icon:



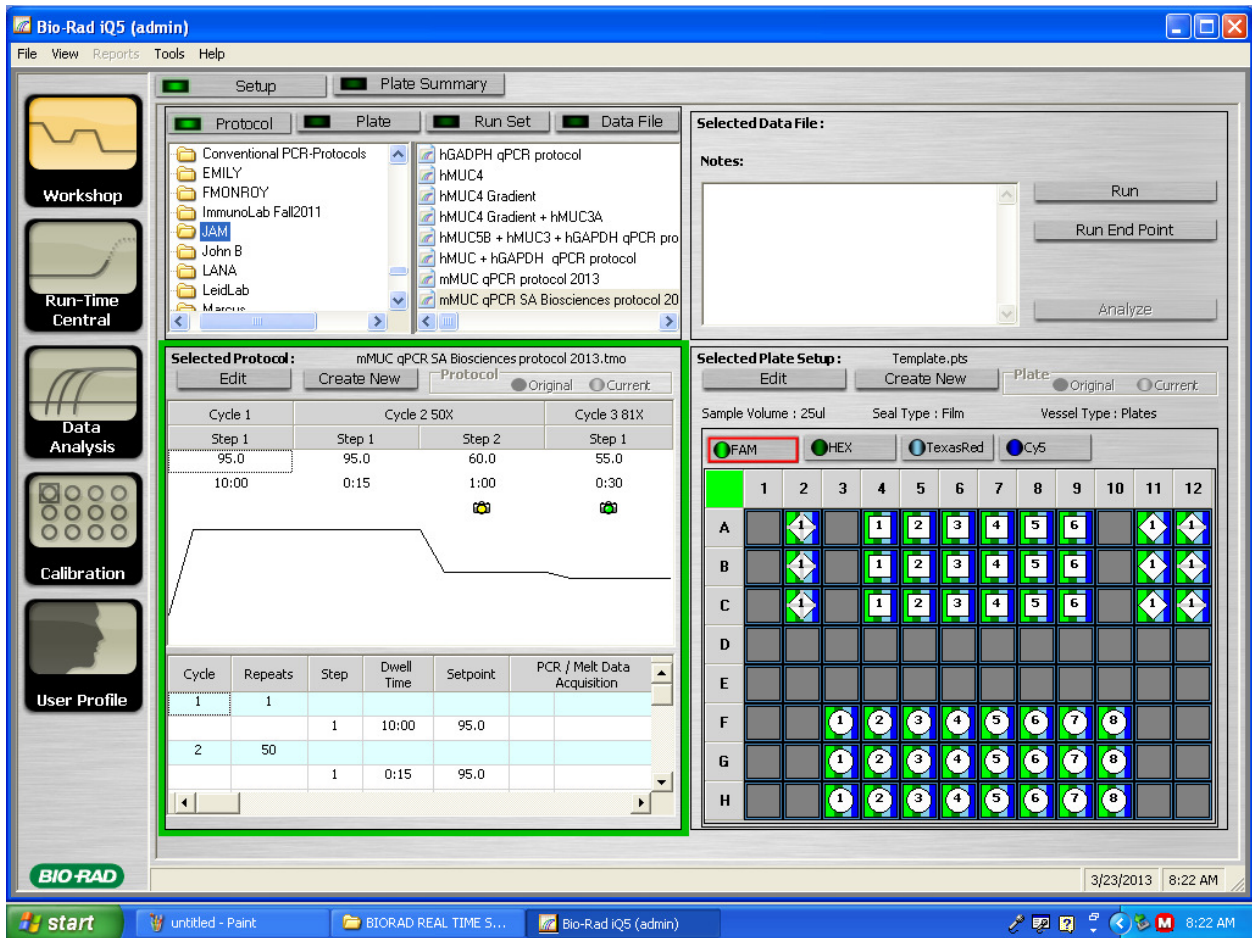
4. After opening the Bio-Rad iQ5 icon, you will be taken to the default (**Workshop**) screen:



5. There are 4 main windows in the workshop. The “folder/saved programs” window (**top-left**), the “protocol” window (**bottom left**), the uneventful “run” window (**top-right**) and the “plate setup” window (**bottom right**). By default, the program currently is viewing the **admin folder**. However, you will likely not save any of your protocols or plates in this folder. If you have a folder on this computer, you can find it by scrolling down in the “folder/saved programs” window.

-Also note that currently the folder is showing **protocols**, but you can view previously saved **plate setups** by clicking on “plate” in this window.

6. For example, we will open the **JAM** folder to see previously saved protocols. By clicking on one of these protocols, the program automatically displays the protocol in the “protocol window”.



7. It is important to note here that *the machine will not function/run your qPCR (for whatever reason) if you create a completely new protocol or plate without editing a previously saved template.* Yes, we know this is odd, but we play by these rules. So, what if you want to edit you qPCR protocol? *Good question.* Simply click edit in the protocol window, and you will be taken to the window below:

**Editing Protocol: mMUC qPCR SA Biosciences protocol 2013.tmo**

Buttons: Save & Exit Protocol Editing, Cancel & Exit Protocol Editing

Cycle 1	Cycle 2 50X		Cycle 3 81X
Step 1	Step 1	Step 2	Step 1
95.0	95.0	60.0	55.0
10:00	0:15	1:00	0:30

**Show Options**

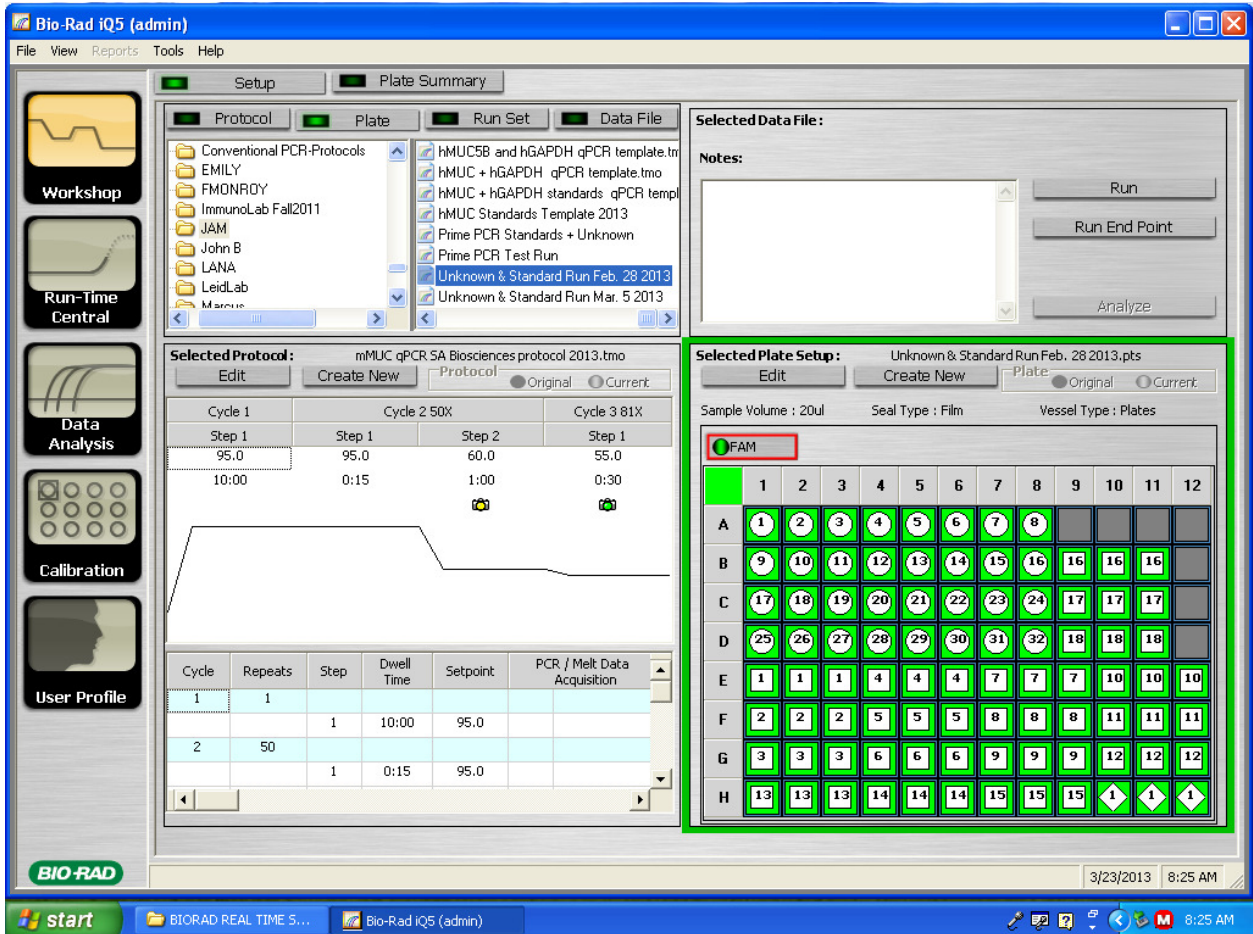
SHOW ALL, Infinite Hold, Temperature Change, Cycle Description, Gradient, Ramp Rate, Time Change, Step Process

Options	Insert	Delete	Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition	Temperature Change	End Temperature	Begin Repeat	How Often?
...	+	X	1	1								
...	+	X	2	50	1	10:00	95.0					
...	+	X	2		1	0:15	95.0					
...	+	X	2		2	1:00	60.0	Real Time				
...	+	X	3	81								
...	+	X			1	0:30	55.0	Melt Curve	0.5	95.0		

System tray: 3/23/2013 8:24 AM

- Here, you can modify whatever features/PCR protocols you would like to attempt in your next run. In this protocol, a 10:00 min. “warm-up/denaturation” step was performed to properly activate the SA Biosciences Taq polymerase (according to the manufacturer’s protocol).
- Then, a short two-step PCR cycle was implemented, and data was captured during the annealing/extension step (**Step 2 of “Cycle 2 50X”**). 50 cycles (**repeats**) was a bit excessive (40 cycles is a good standard to follow), but this ensured the amplification of product.
- Note the yellow camera icon under this step. To select a step to capture data, simply open the drop-down bar under the PCR/Melt Data Acquisition tab (shown by arrow).
- A **Melt Curve** step can be generated in a protocol. This is usually the last step performed to obtain data about non-specific PCR amplification. Note the 0.5°C increases every 30 seconds for 81 cycles. This gradient ensures enough time for potential products to be denatured at a specific temperature. If a single product is obtained, all PCR products should be denatured at **one temperature** (displayed as a single “peak” in melt curve data). The “end temperature” of 95°C is a good standard to follow; most PCR products beneath 400bp or so should melt at this temperature.

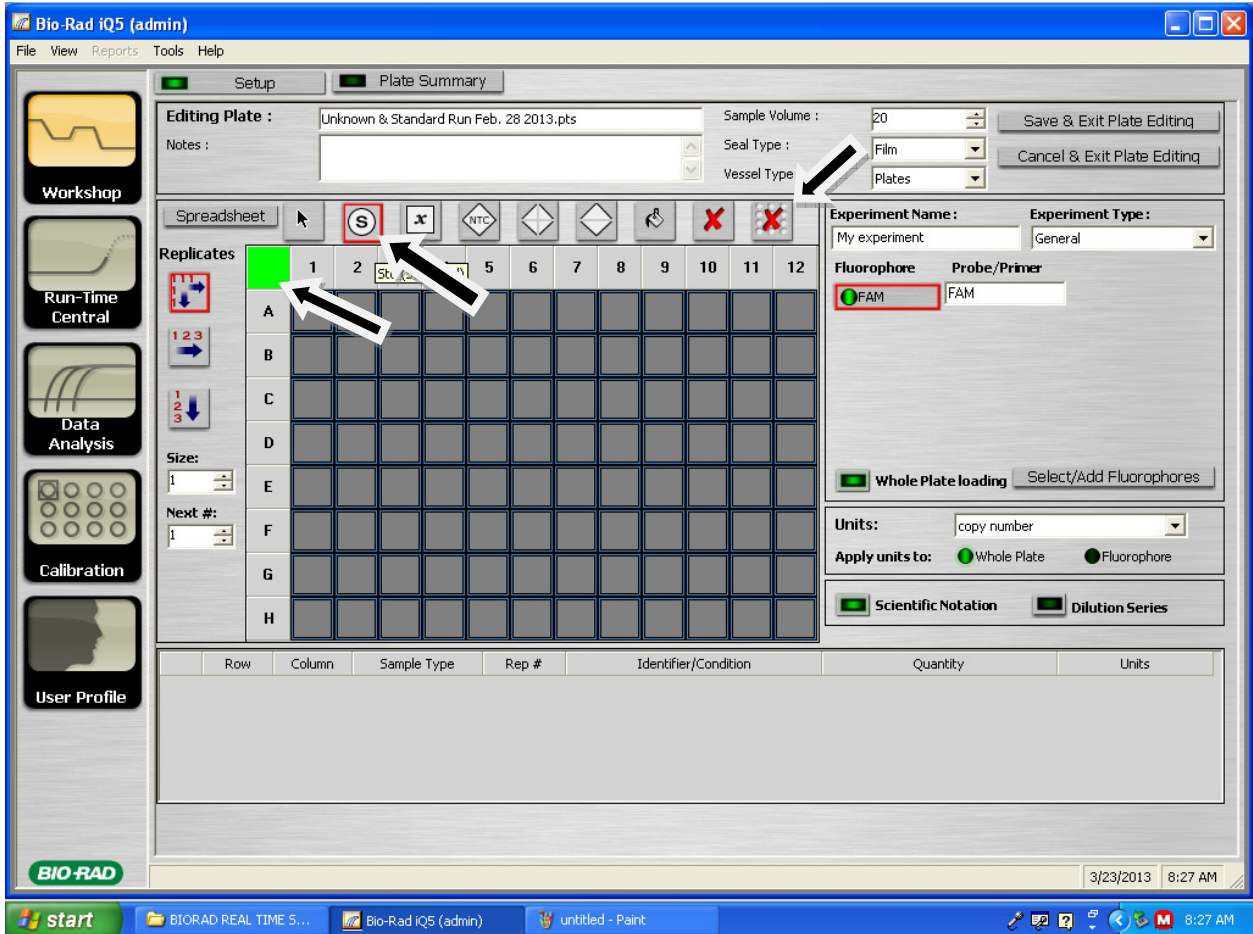
8. After you are satisfied with your ingenious protocol, you can “Save & Exit” Your protocol (see arrow above). Remember to save your file *in your folder* for convenience. Next, you can open a **plate template** by clicking on plate (as previously mentioned) in the “folder/saved programs” window. Here, we are looking at a plate template file from the **JAM** folder again. Again, note that the program automatically displays the plate template upon clicking the file.



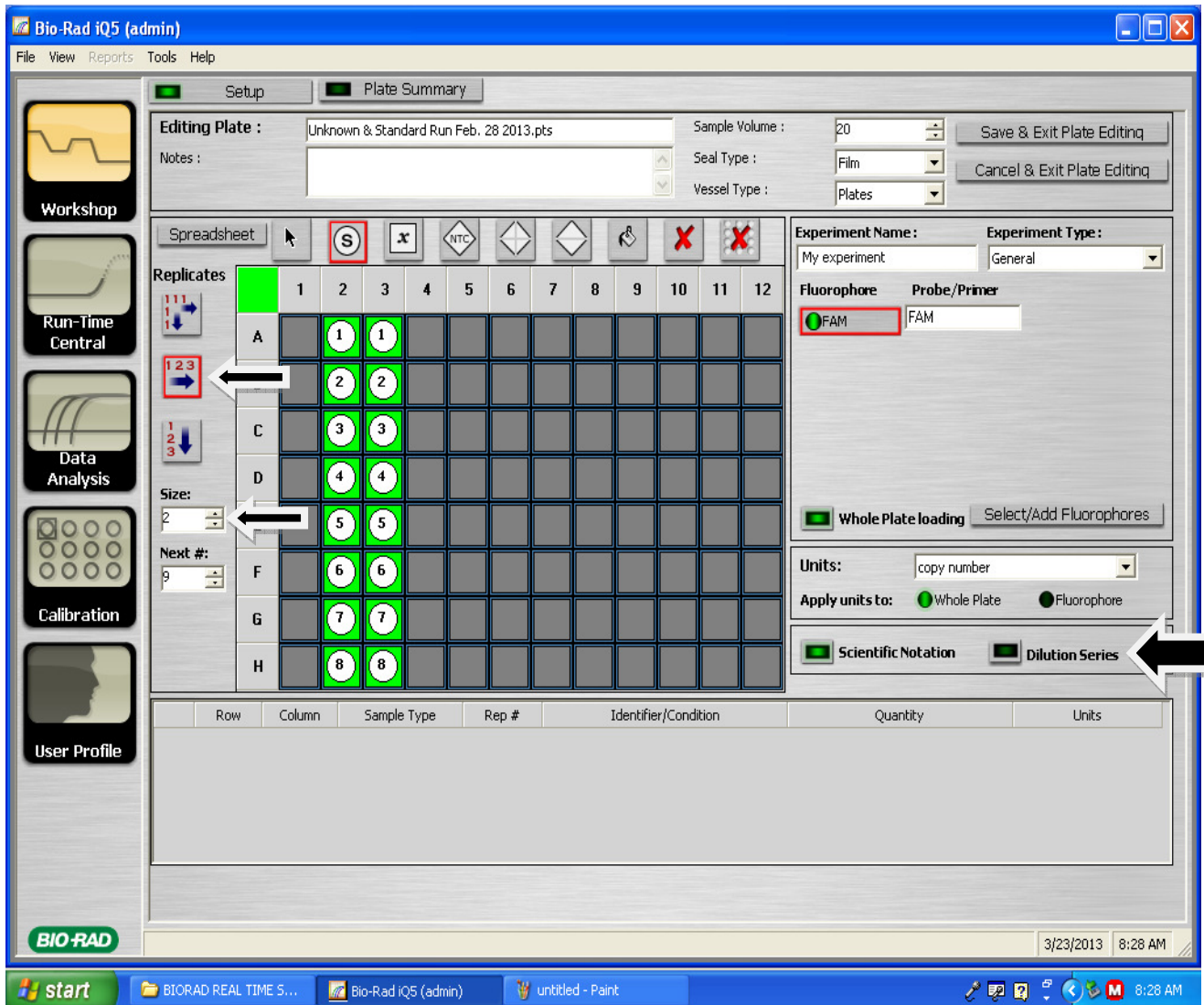
9. To modify the plate template, simply click on **Edit** in the plate-setup window.

10. Below, the plate was cleared of all samples by clicking the **X** icon, and the green box in the top right corner of the plate (see arrows below).

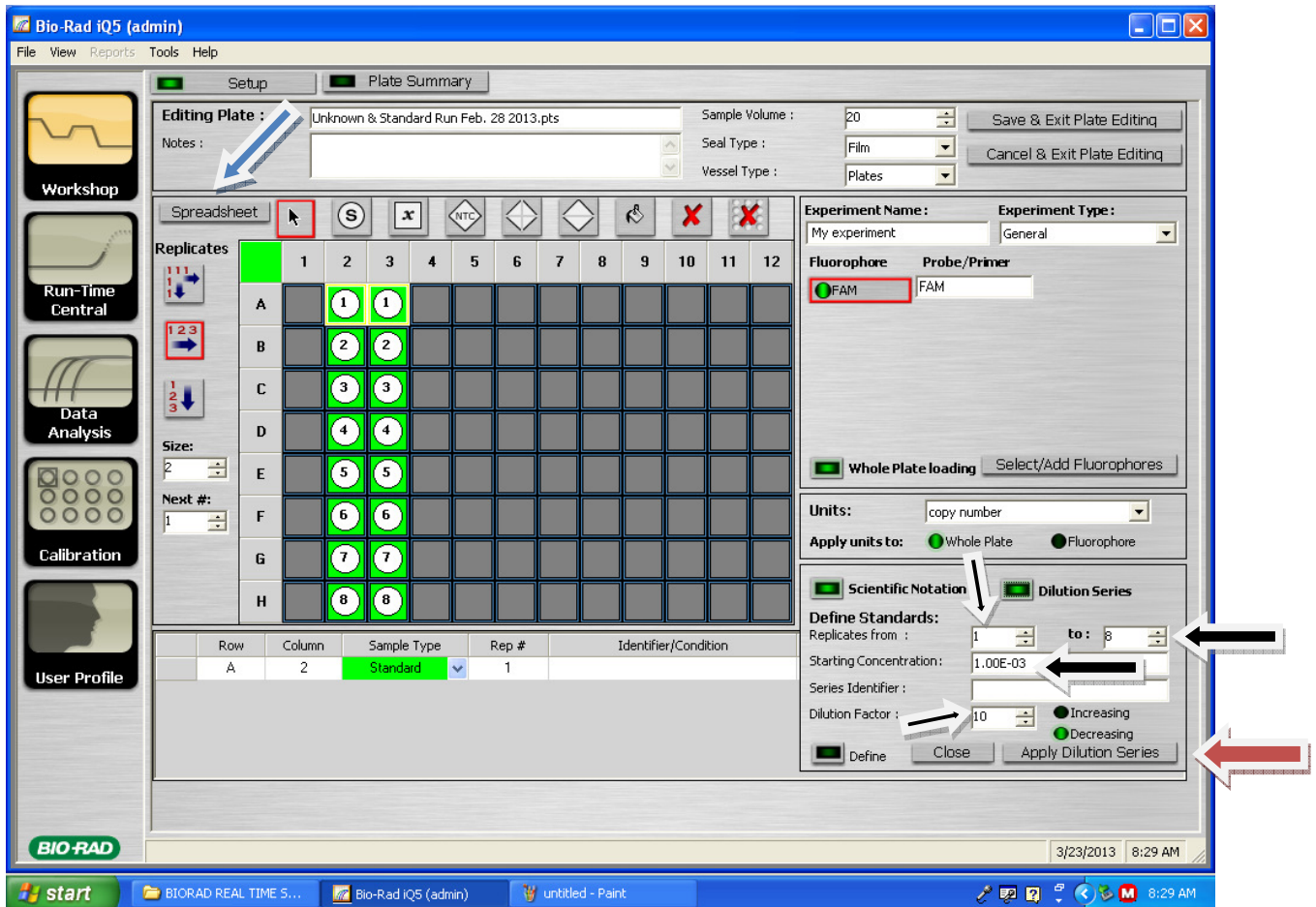
- Now, when performing a qPCR, you will likely be running a standard of one of your genes. To inform the machine that you will be running standards on your plate, click on the standard icon (see arrow below). Next, you can place your standard samples wherever you wish on the plate.
- When possible, it is best to avoid the edges of the plate. However, in previous experience, this does not seem to affect results very much.



- Unless you are confident in your technique, it is advisable to run **duplicates** of your standards. Duplicates can be indicated in the plate template by the left column on the window under “replicates”. Note that the arrows indicate how the replicates will be laid out on the plate. (See arrows below). You can change the amount of “replicates” you have by adjusting the value under the “size” designation (see arrows).



11. In the screenshot above, we have up to 8 standard dilutions. However, we'll wish to indicate to the machine the **factors** (usually ten-fold) of the dilutions. To do this click on the "dilution series" option on the right side of the window (arrow). Once you have selected this option, see the screenshot below:

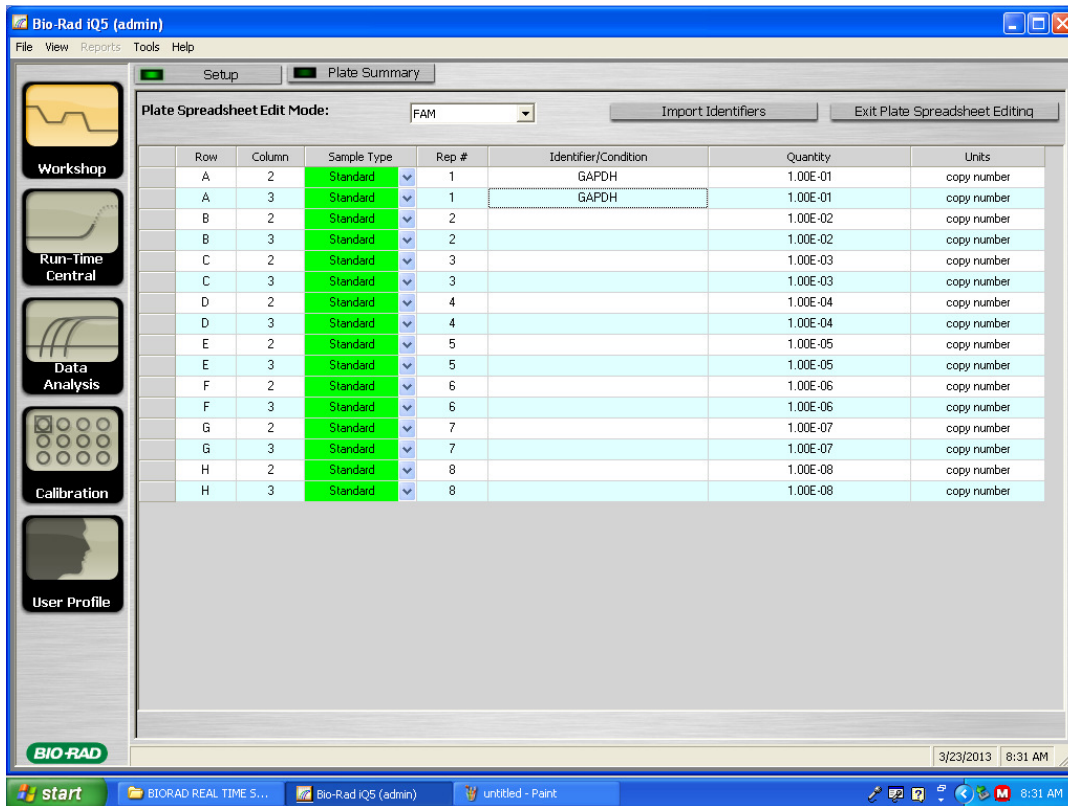


- Next, you'll want to adjust what standards to apply the dilution series to (**important when you have multiple standards on the plate, although not depicted in this protocol**). As shown by the arrows, indicate the range of your standards. Next, apply the starting concentration (the concentration of standards 1 in this case...if this sample is not your stock concentration of standard, then it would be a 1:10 dilution or 1.00E-1). Note the arrows above. Also note that the starting concentration should be 1.00E-1 as mentioned earlier. When you are done with your modifications, do not forget to hit **Apply dilution series** (see red arrow)...or all of your previous modifications will be in vain!

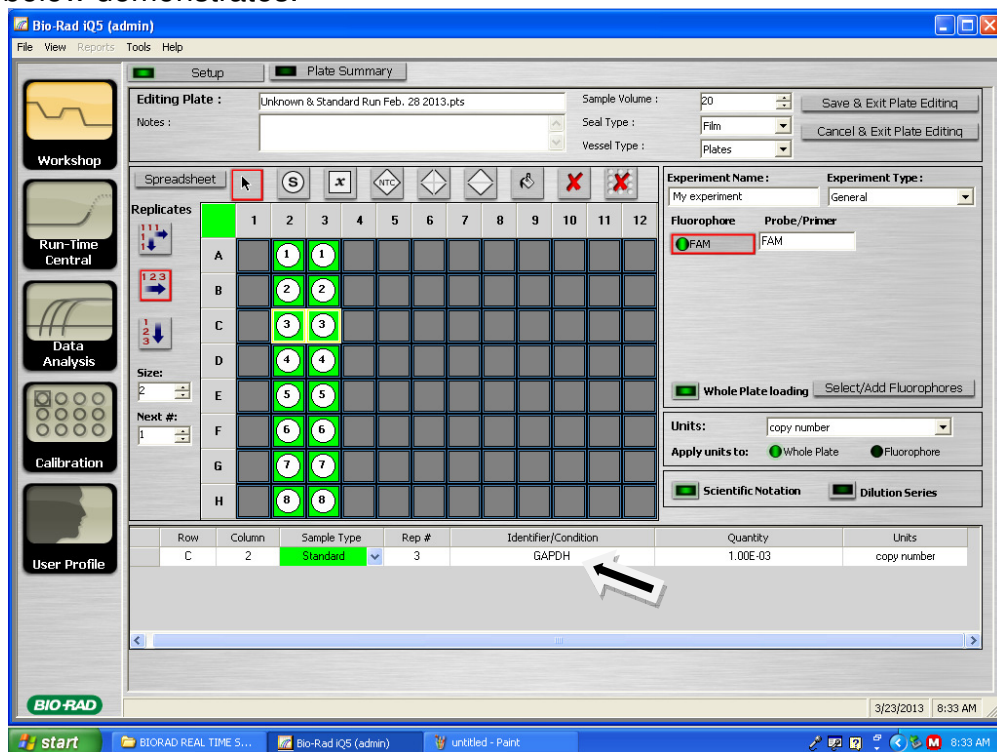
- Note that this dilution series will be applied to both duplicates. A handy detail, so that you won't need to modify the dilution series for the other duplicate.
- So, if there were another set of standards, say 9-16, we would want to apply the dilution series as we had for standards 1-8 above.

12. After the dilution series has been applied, the "identifier/Condition" tab will still be blank, and if there are multiple standards on the plate, you'll certainly want to know which standards are for which gene. So, to label your standard samples, click on the spreadsheet tab in the upper right-hand corner of the plate (see blue arrow above). Clicking on this tab will bring you to the following window:

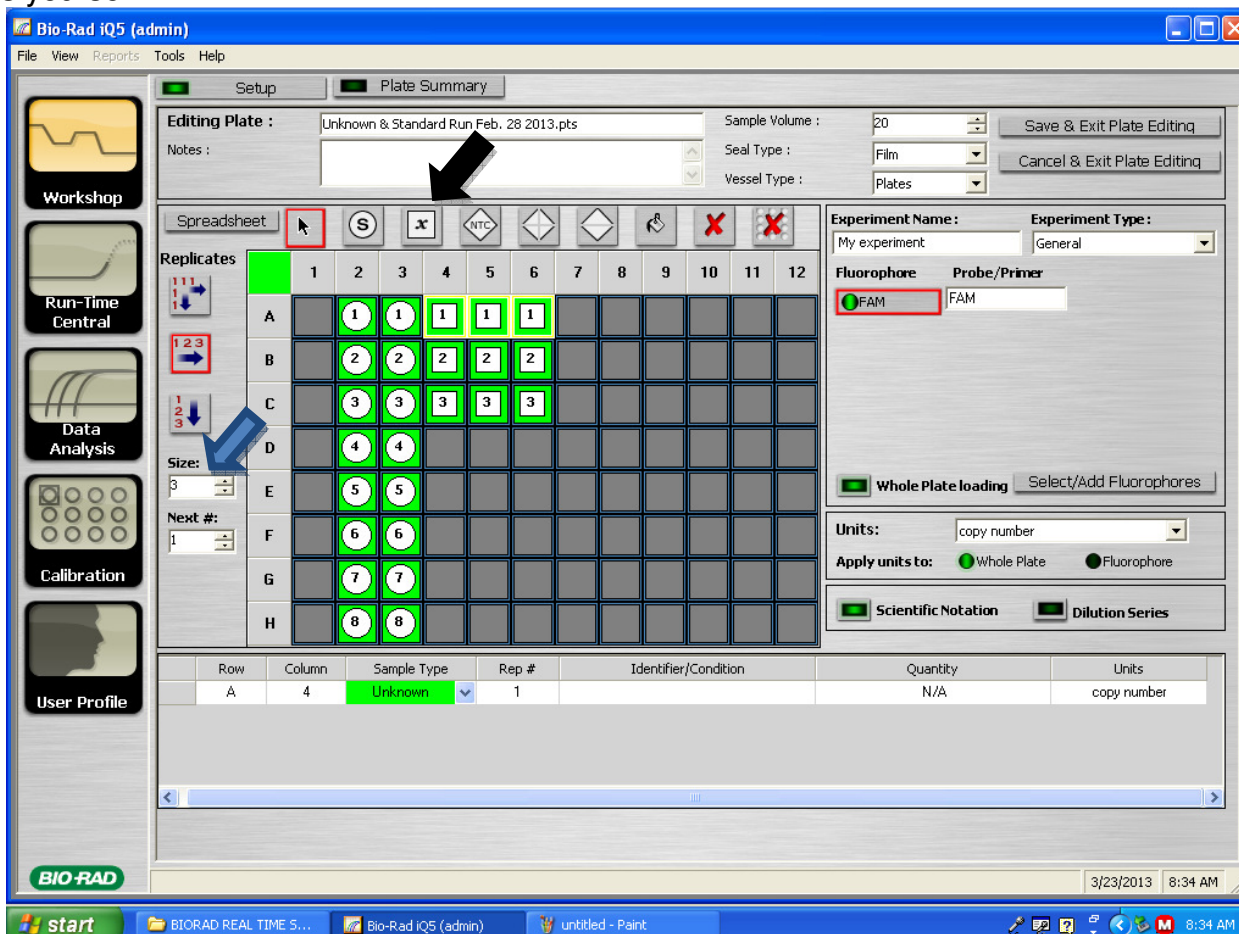




- Here, the standard sample can be labeled (GAPDH above) so that they can be instantly identified upon clicking on them in the regular plate setup window, as the image below demonstrates.



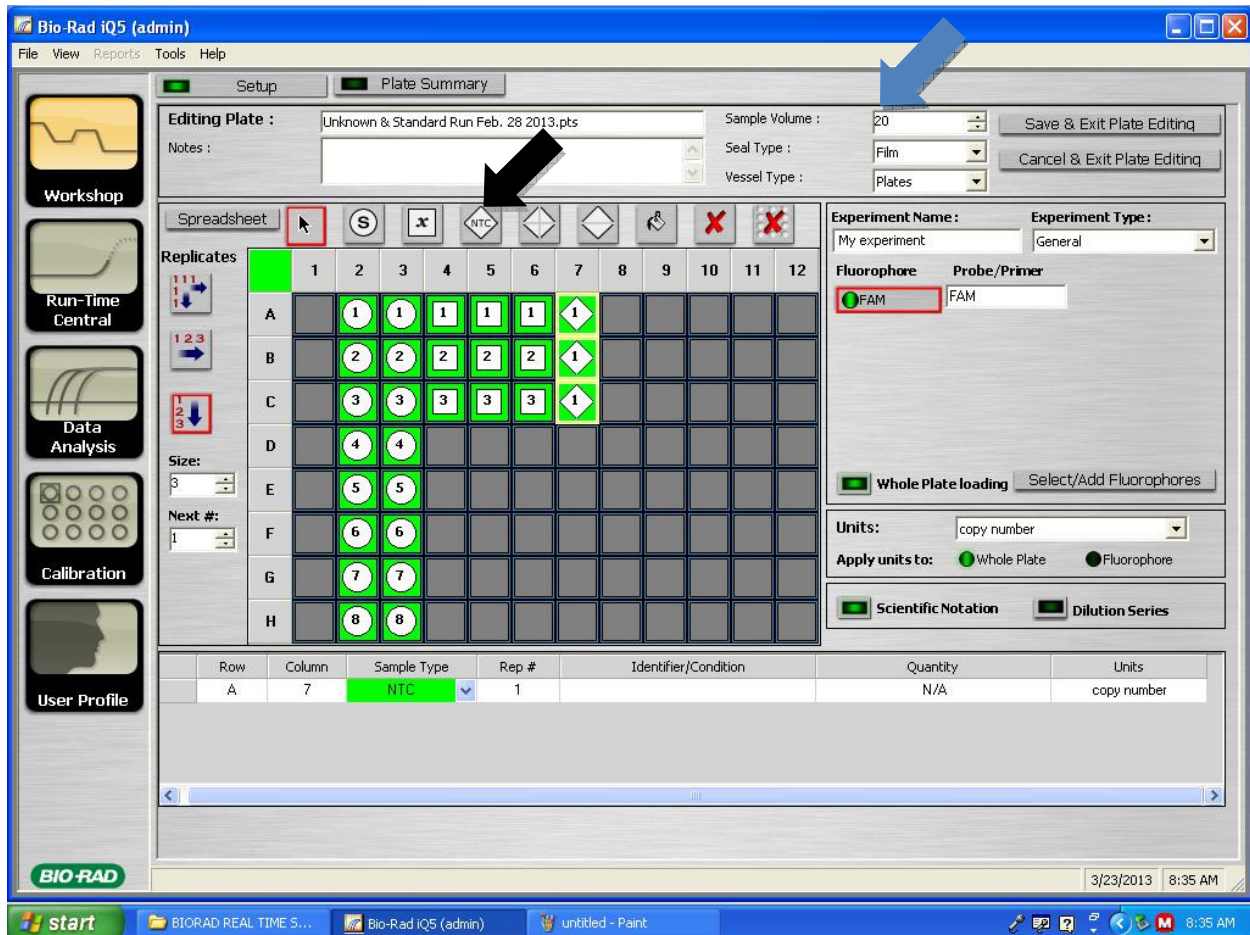
13. Next, unknowns will likely be incorporated into the qPCR run. Recall that unknowns are simply the treatment or control samples of your cDNA template (basically, not your standard dilutions). It is advisable to include **triplicates** of your unknowns, regardless of your technique. Unfortunately, the Taq polymerase or primers may not be as skilled as yourself.



14. To include triplicates of your unknown, click on the unknown icon (arrow) and use the same configurations used for standards to incorporate triplicates unto the plate (blue arrow).

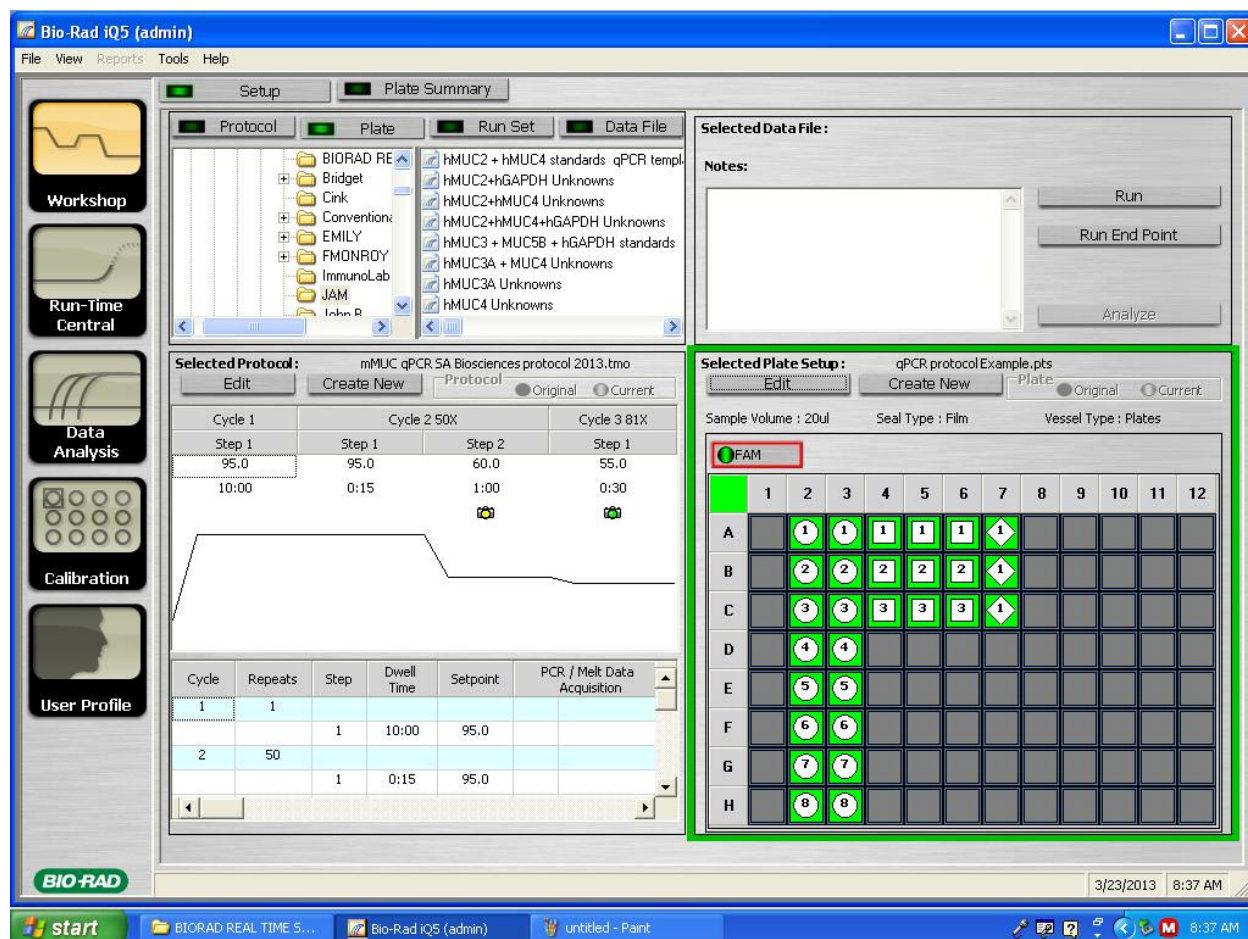
- Do not forget to label your unknown samples in the spreadsheet.

15. You will want to include "No Template Controls" (NTC) in your qPCR run. Recall that the NTC is every ingredient necessary for the qPCR reaction except for cDNA template. Instead, the amount of template used (usually 1uL) is replaced with the same amount of UP H<sub>2</sub>O. Typically, a triplicate of NTC's are adequate for a single qPCR run. To include NTC's, click on the NTC icon (arrow below) and add to the plate.



- It is worth mentioning that most qPCR reactions run are 20uL total. This is the default set by the qPCR program. However, if you are using a different amount, you may wish to modify this amount in the sample volume section of the plate-setup window (blue arrow above).
- Several other details before exiting the plate: Whether you are using a plate with a film cover, or qPCR strips with caps, you can leave the “seal and vessel type” features alone. In previous experience, they do not seem to alter data interpretation by the machine much. Also, unless you are using a different probe besides SYBR green, you can leave the “FAM” default fluorophore feature alone.
- After you are satisfied with your plate-setup, you can **save & exit plate editing**, and save the file in your folder.

16. After you have finally created the protocol and plate setup you wish to run, you can click on the run option (arrow below).



- After clicking on the run option, you will see several windows appear, all of which you should simply respond with “yes”. However, one of these windows will ask to “**save optical data**”. This feature allows you to save your data file in your folder of interest, as well as label the data file with date, unknowns and standards tested, among other labels (such as constellation and planetary alignments). **This data file is very important.** From this file, you can analyze your data for further processing.

This concludes the qPCR protocol; please notify Dr. Monroy if any of these steps are unclear.