Gene Expression Profiling with qPCR – plate set-up

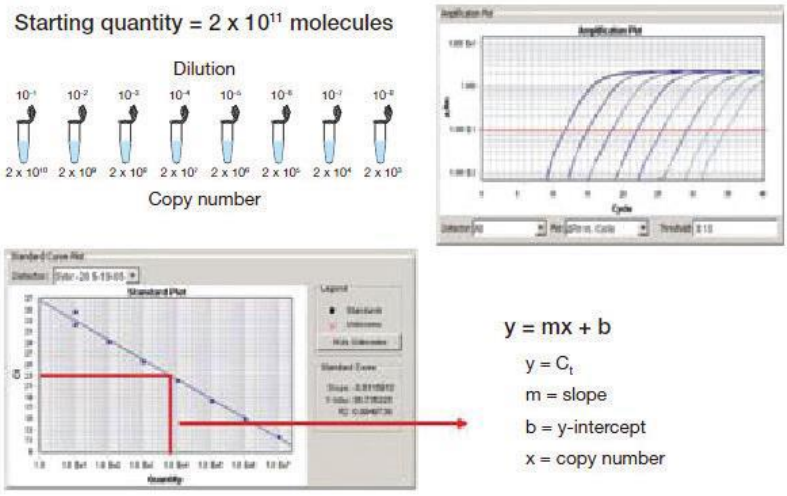
Most qPCRs at our Institute are performed in 10 µL, or 5 µL reaction volumes. It is very important that the cDNA and reaction volumes remain constant across all samples that need to be compared. When using the 384 well plates, your assay volume per well will be **10 µL** MAX and it is important to note the following:

- **ALWAYS USE ELECTRONIC PIPETTES FOR 384 WELL qPCR PLATES (found in molecular core room 553. Please sign then out!)**
- Before setting up a plate for your assay, it is necessary to determine the exact number of wells you will need for each target gene of interest, including unknowns, standards (for standard curves) and no template controls (NTC) so you can prepare your master mixes accordingly
- Adjust the volume of primer added to give you the optimized final concentration (determined during primer validation)
- Once you know your total sample number, you can perform the calculations for your master mix for all genes as in the example below and prepare master mixes including **10-20% extra volume** to account for pipetting error
- **ALWAYS** perform a minimum of triplicate reactions per sample

Reagent	Stock Concentration	Volume per single reaction (µl)		No. of samples	Vol. Of Master Mix cocktail needed (µl)	Final Reaction concentration
SYBR Green PCR Master Mix	2X	5.00	X	5	**25.0**	1X
Forward Primer	10 µM	0.15	X	5	**0.8**	150 nM
Reverse Primer	10 µM	0.15	X	5	**0.8**	150 nM
Sterile Rnase/Dnase free water	N/A	2.70	X	5	**13.5**	N/A
	***Total:**	**8.0**		**Total:**	**40.0**	

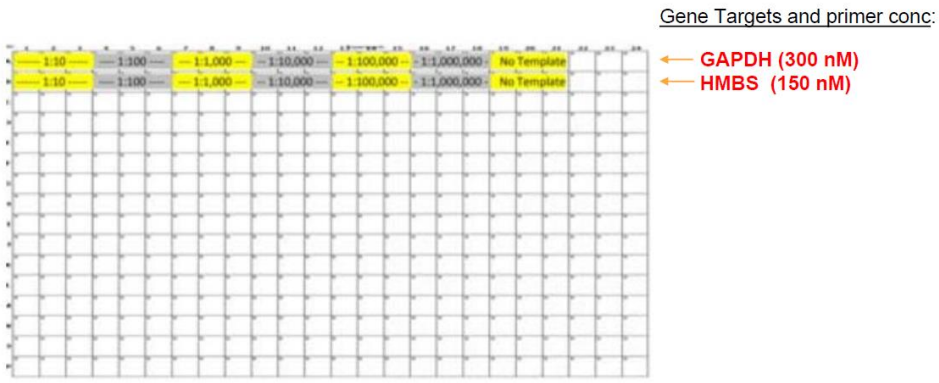
Master Mix Template II 384 well

- If you are doing absolute quantification using a standard curve, standards of known copy number quantity will also need to be prepared. This is done by making serial dilutions from a stock of plasmid DNA. Genomic DNA (gDNA) is also commonly used:



- NOTE: Because plasmid (and to a lesser extent gDNA) sequences are highly abundant, they can be sources of PCR contamination. Extreme caution must be exercised when working with these DNAs to prevent their exposure to stock PCR reagents, solvents, used for the dilution of PCR reagents and laboratory equipment and surfaces. USE THE RNA CLEAN ROOM FOR SET UP!

- At this point, have a template filled in designating your wells for NTC, standard dilutions and unknown sample etc.. Fill in as many details about your experiment for your records, EG:



y.

NOTE: Good pipetting technique is a must throughout such a sensitive assay as real-time PCR, so if necessary, practice using the electronic pipette using water before loading your actual master mix and samples

➢Using the 384 well template you made for the assay, load the plate starting with the master mixes for all genes

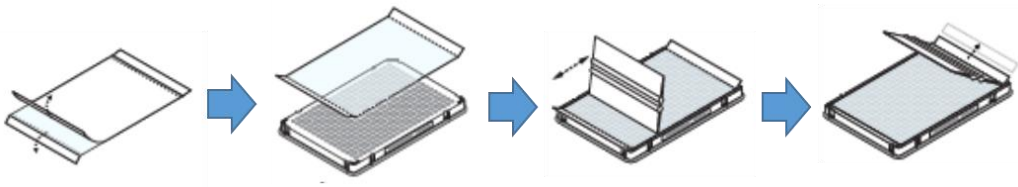
➢Holding the pipette at an angle, touch the pipette tip to the side of the well and close to the bottom of it. The master mix will stick to the side of the well by surface tension. Repeat for each well being used

➢Once the master mixes are all loaded on the plate, it is time to load your unknowns, standards and NTC, again using the plate template as a guide and spot your cDNA to the other side of the well

NOTE: It is easy to get distracted and lose one's place/forget what has or hasn't been added to a well, so your complete and utter attention while pipetting is a must! If need be, use a highlighted guide but try not to mark the plate with a sharpie (can auto-fluoresce in the machine)

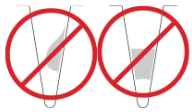
- After you have loaded all mastermixes and samples on the plate, CENTRIFUGE PLATE FIRST WITHOUT A SEAL (1000 xg for 2 minutes) to ensure all samples are at the bottom of the well

- AFTER the spin, seal with the MicroAmp™ optical adhesive film (AB Cat. # 4311971). Handle covers by the edges wearing powder free gloves. Ensuring that PCR plates have been sealed correctly (no bubbles and covering all wells) is a must

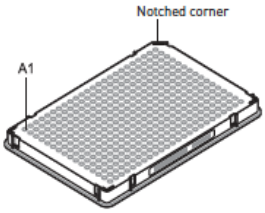


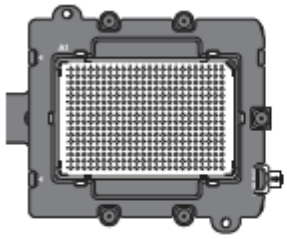
After optical films are placed, fingerprints and marks should be avoided on the top of the film! No Sharpie marks on plate. Use qPCR plate tool to remove air bubbles from under the surface and ensure white edges are ripped off before putting in the machine

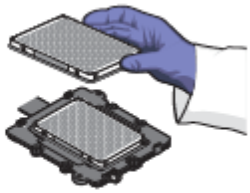
- Spin plate again (1000 xg for 2 minutes). After spinning, check plate from below to ensure that liquid is at the bottom of all wells as in diagram below:

Correct	Incorrect
 Liquid is at bottom of well	 • Not centrifuged with enough force, *or* • Not centrifuged for enough time

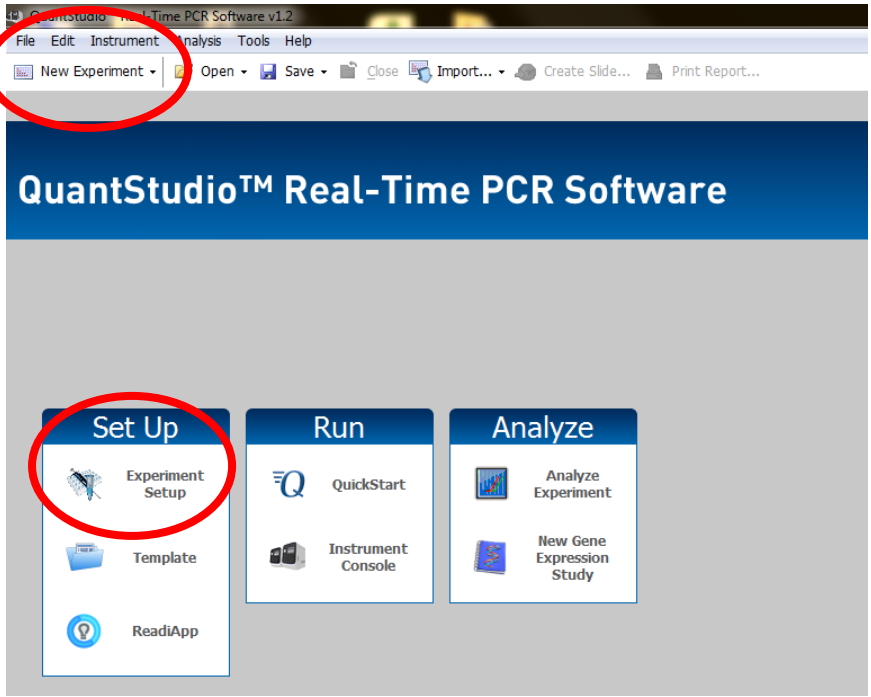
- The sealed plate is now ready to be loaded into the instrument and assay parameters assigned.
  NOTE: A1 is always in the top left corner and top left notch must line up in plate holder





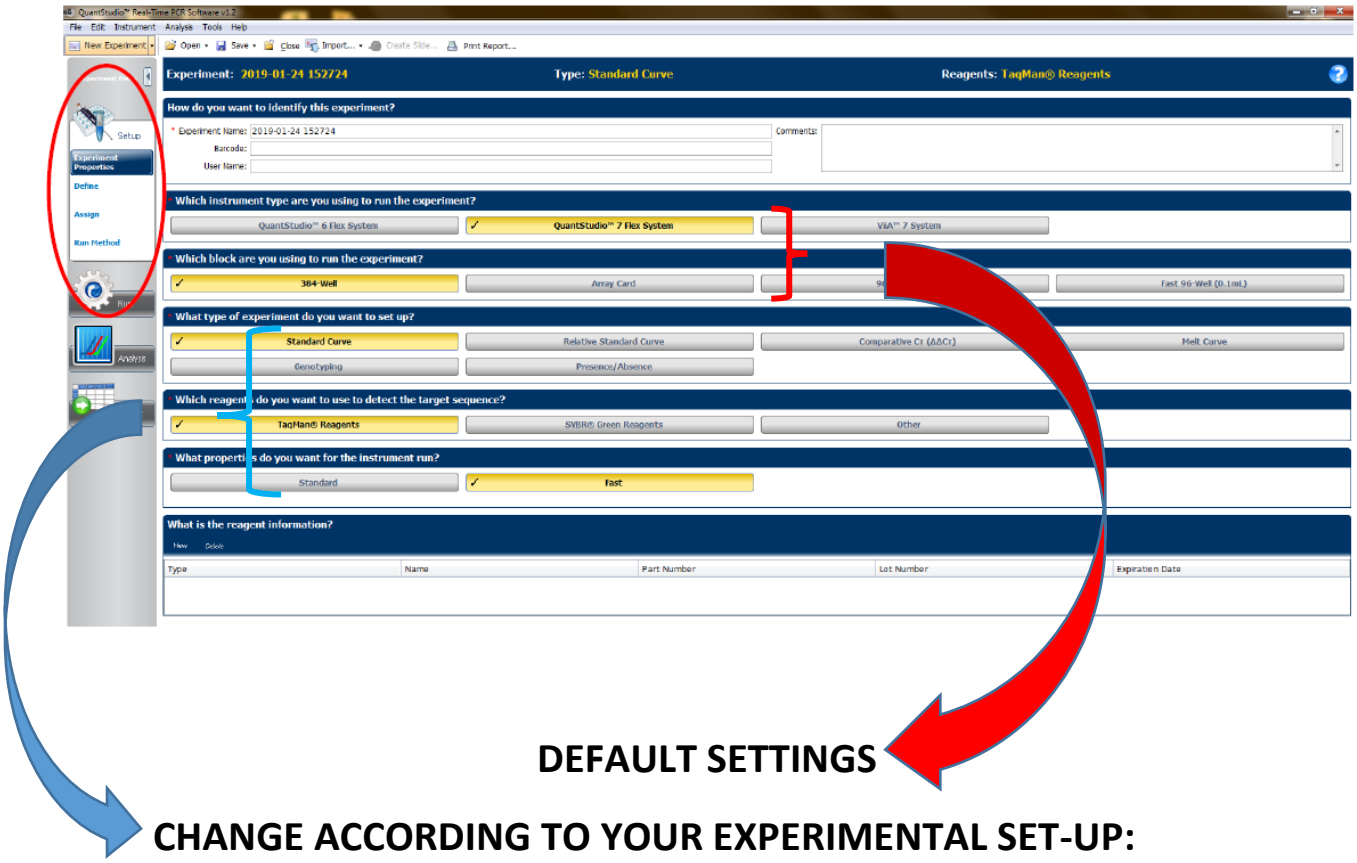


QUANTSTUDIO 7 SOFTWARE: Landing page



Setup Tab - Experimental Properties

Follow tabs on the left side of the screen, starting with defining experimental properties. Note that Fast blocks do not exist for 384 well (the only format on our QuantStudio 7 machines)

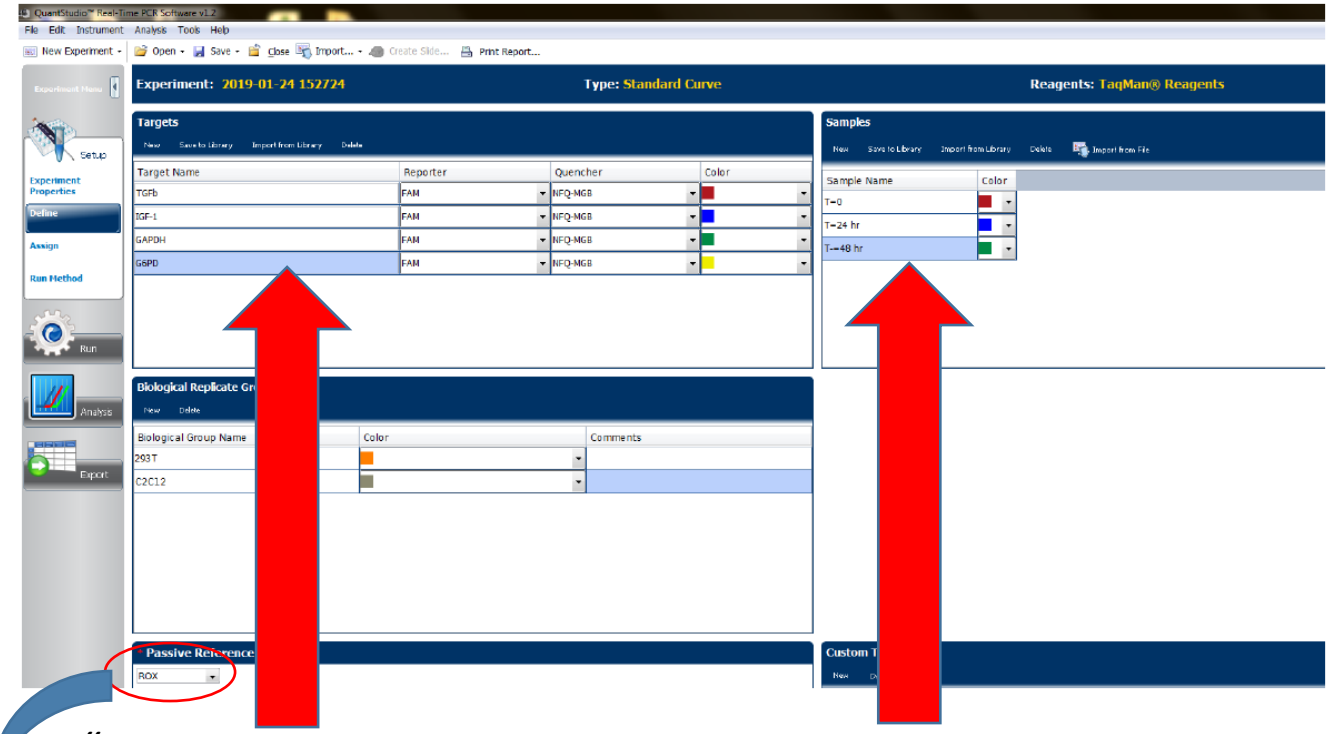


DEFAULT SETTINGS

CHANGE ACCORDING TO YOUR EXPERIMENTAL SET-UP:

- Standard curve for primer validation and absolute quantitation
- Comparative Ct for all other experiments
- TaqMan for probe chemistry
- SYBR for primer chemistry
- Standard setting always (for 384 well)

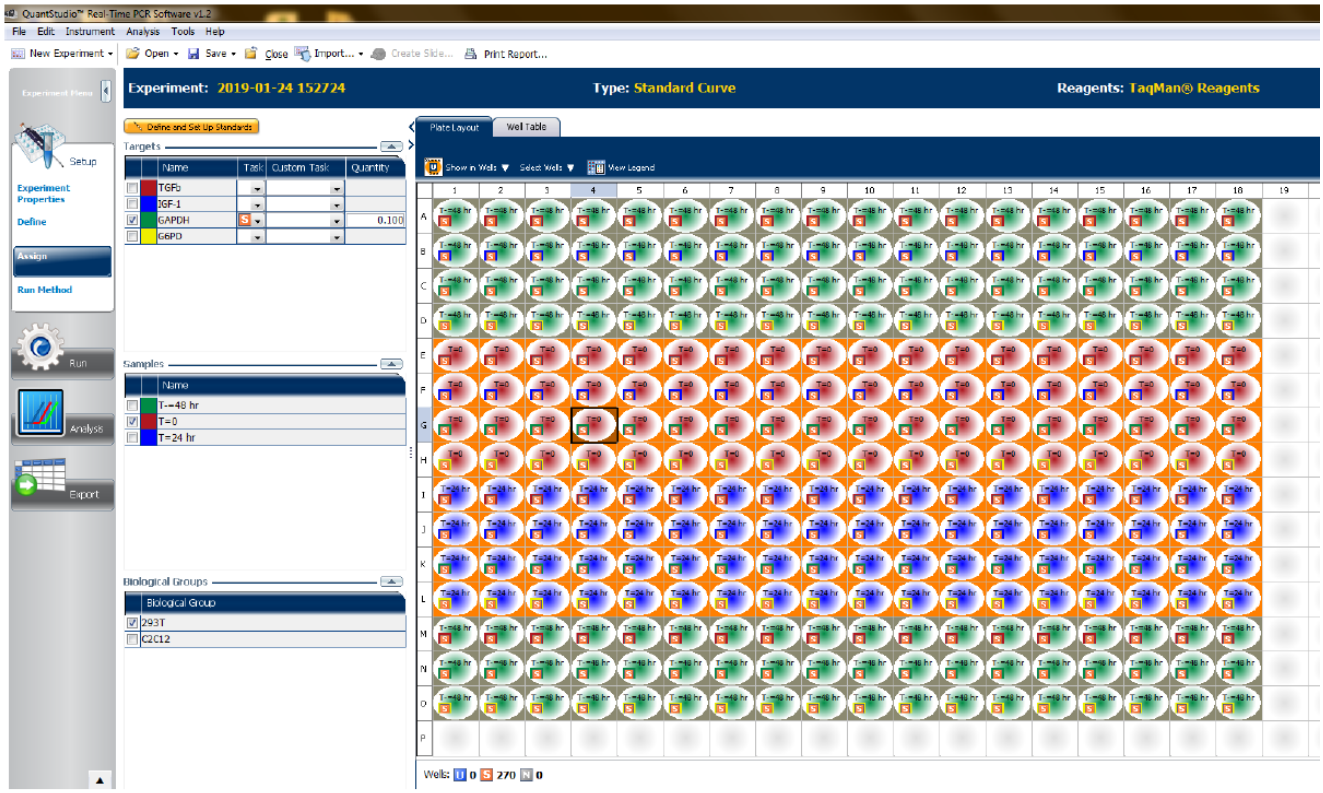
Setup Tab – Define



"Add "names of genes of interest and HKG and…. samples or distinct cDNAs

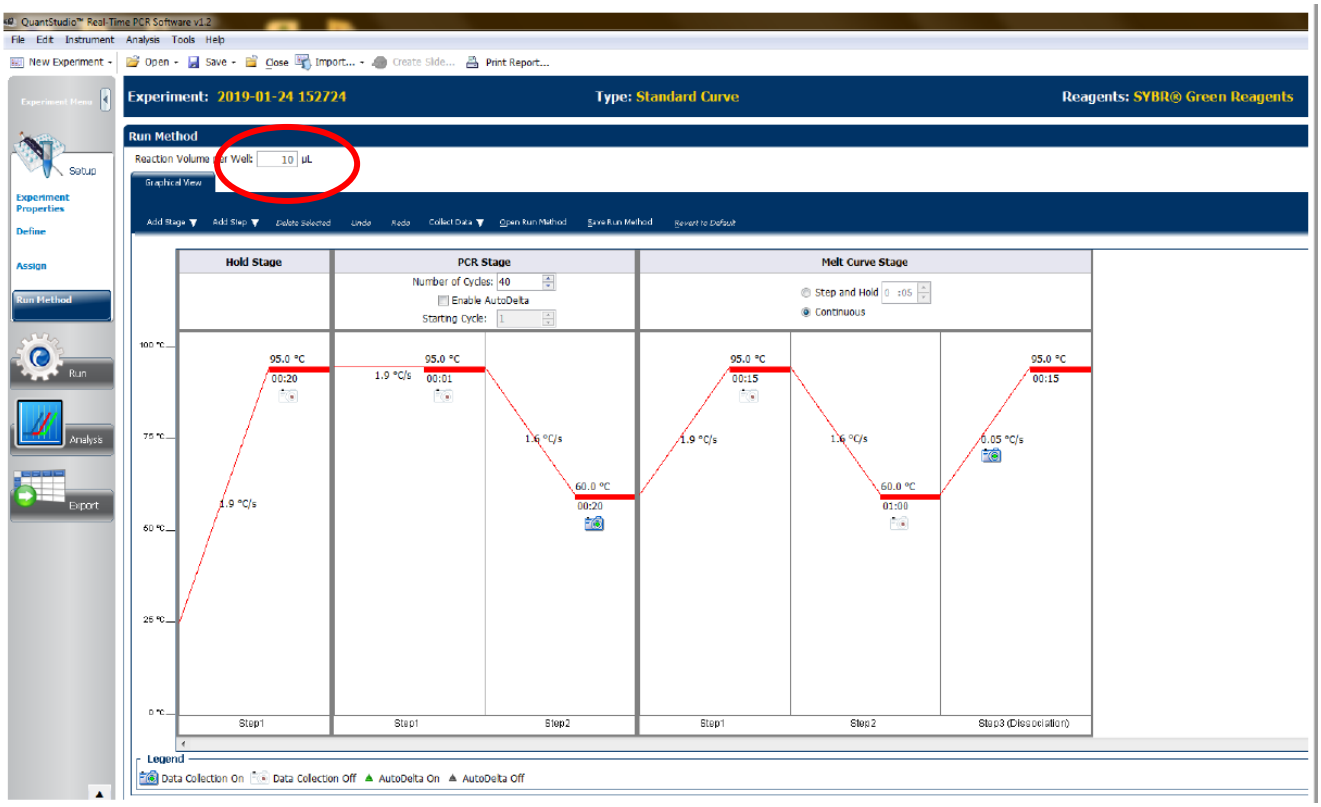
Ensure that correct passive reference dye is entered here. You can check the product literature for your master mix of choice to ensure which passive reference dye is used (if any) and whether it is compatible with the QuantStudio 7. The passive reference dye is used for normalizing the derivative reporter in your amplification plot. It accounts for variability in loading due to pipetting errors that may translate to differences in Ct values.

Setup Tab – Assign



- **Use "Define and set up standards" for standard curves. This is a useful tab as it will automatically input dilutions**
- **Select gene of interest and define action (unknown=U, standard=S, no template control=N) then highlight corresponding well on plate. Then select sample**
- **Use CNTL to select multiple wells simultaneously**
- **Plate can be re-defined after the run as well**

Setup Tab – Run Method



- **Ensure volume is changed to 5-10 ul for 384 well format**
- **Edit any temperature or time parameters by clicking on and using arrows to increase or decrease value**
- **Ensure picture of "camera" is selected at annealing/extension step and last ramp of melt curve. The camera has to be on (or visible) for data acquisition to occur. To delete step or stage, click to highlight and then press "delete selected"**
- **File must be saved as an "*.eds" prior to the run tab. Please do not save your experiments on the hard drive but rather your H:\ drive**
- **When run tab is pressed, click on green "START RUN" button and then click on the number that appears below the tab to engage the run**
- **Lock screen during run if desired and remove AND DISCARD your plate directly after run**