

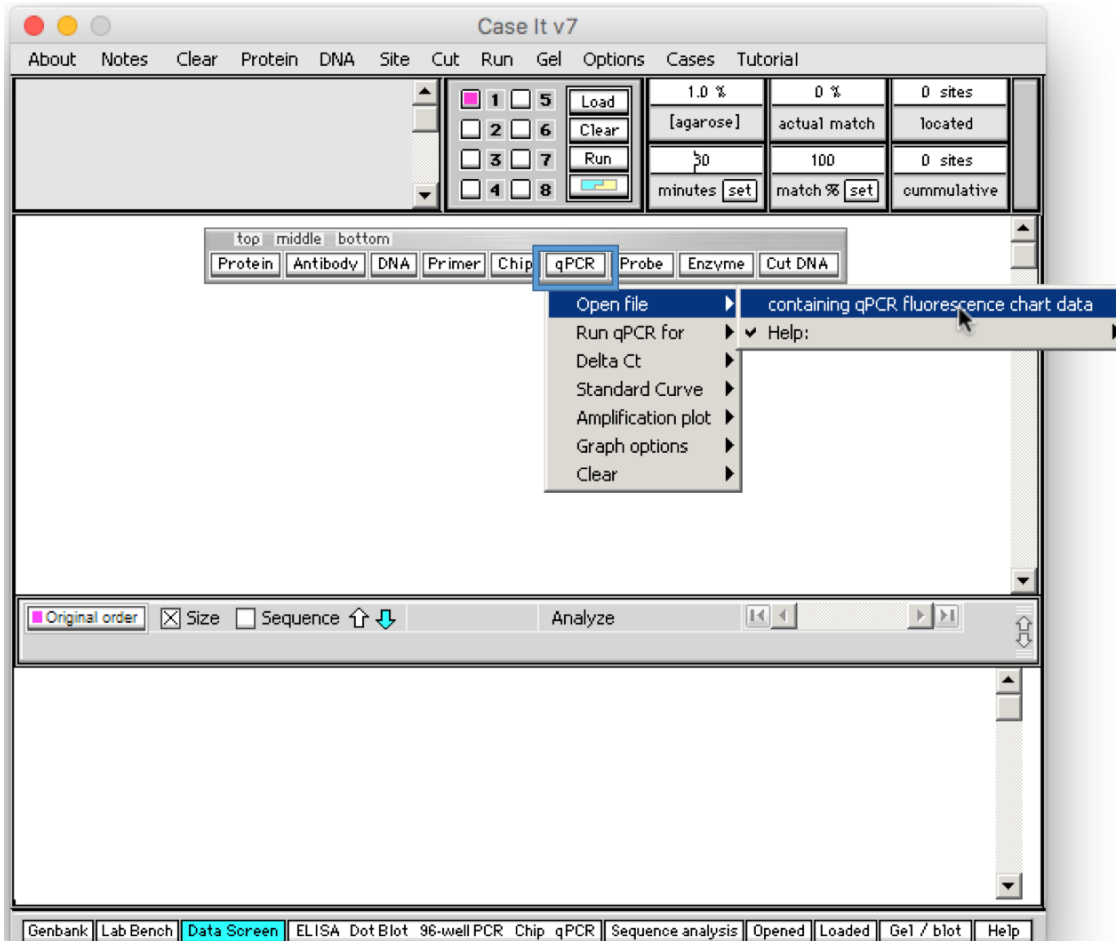
Tutorial for using Case It for qPCR analyses

[Basic procedure](#) for using Case It to display qPCR fluorescence values

Determining [Ct](#), [Delta Ct](#), and [fold change \(Delta-Delta Ct\)](#) method

Plotting the [standard curve](#) and determining [extrapolated copy values](#)

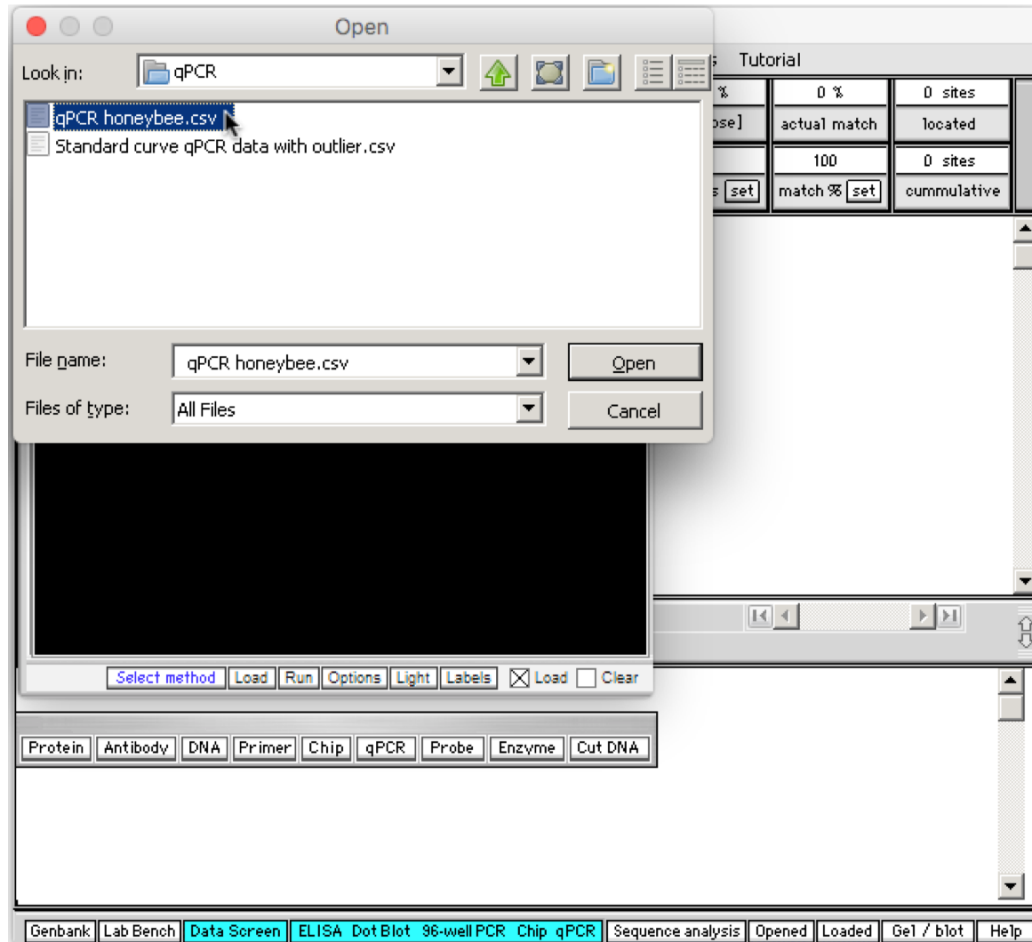
The first part of this tutorial shows how to use Case It v7 to display actual fluorescence data generated by a qPCR thermocycler. Click the **qPCR** button on the silver button bar, and select **Open file -> containing qPCR fluorescence data**.



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Open the file **qPCR honeybee.csv**(inside the folders Case It -> Cases -> qPCR -> qPCR honeybee.csv).

Note: The file is inside the qPCR folder, not the Honeybee cases folder.



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New windows appear when a qPCR file is opened. Click the **Load** button below the black region of the screen...

The screenshot displays the Case It v7 software interface. At the top, there is a menu bar with options: About, Notes, Clear, Protein, DNA, Site, Cut, Run, Gel, Options, Cases, Tutorial. Below the menu bar is a control panel with a grid of buttons (1-7) and a 'Load' button. A table below the control panel shows parameters: 1.0 %, 0 %, 0 sites; [agarose], actual match, located; 80, 100, 0 sites.

The main window is titled 'ELISA Dotblot 96-well PCR DNA chip qPCR'. It features a large black rectangular area on the left. Below this area, a text prompt reads 'Click the 'Load' button below'. A blue box highlights the 'Load' button in the control panel. Below the black area is a row of buttons: Select method, Load, Run, Options, Light, Labels, Load, Clear.

Below the buttons is a row of tabs: Protein, Antibody, DNA, Primer, Chip, qPCR, Probe, Enzyme, Cut DNA. At the bottom left, there is a 'Back to beginning' link.

On the right side, there is a 96-well plate grid with columns 1-9 and rows A-H. Below the grid is a qPCR graph. The graph has a y-axis labeled 'ΔCt' ranging from -5.0 to 20.0. The x-axis is labeled 'S1' through 'S11'. The graph shows two data points: a blue square at S2 (ΔCt ≈ 20.0) and a red square at S6 (ΔCt ≈ 20.0). Above the graph is a row of buttons: Show, Hide, Delta Ct, Fold change, Standard Curve.

...and the 96 wells turn orange in color, indicating that they have been loaded. Labels indicate names of the samples loaded in each well. To see all the labels we'll move the window to the left...

The screenshot shows the Case It v7 software interface. At the top, there is a menu bar with options: About, Notes, Clear, Protein, DNA, Site, Cut, Run, Gel, Options, Cases, Tutorial. Below the menu bar, there are control buttons for Load, Clear, and Run, along with a grid of checkboxes for wells 1-7. A table on the right shows parameters: 1.0 % [agarose], 0 % actual match, 0 sites located, and 60, 100, 0 sites.

The main window displays a 96-well plate grid with columns 1-12 and rows A-H. The wells are orange, indicating they are loaded. Below the grid, it says "Use the 'qPCR' button to run qPCR".

Below the grid, there are buttons: Select method, Load, Run, Options, Light, Labels, Load, Clear. A "Probe:" field is also present.

At the bottom, there is a search bar and a list of categories: Protein, Antibody, DNA, Primer, Chip, qPCR, Probe, Enzyme, Cut DNA. Below this, there are tabs: Genbank, Lab Bench, Data Screen, ELISA, Dot Blot, 96-well PCR, Chip, qPCR, Sequencing.

On the right side, there is a graph showing ΔCt values. The y-axis ranges from -5.0 to 20.0. The x-axis shows samples S1 through S11. Two data points are visible: a blue square at S2 and a red square at S6, both with ΔCt values around 20.0.

	1	2	3	4	5	6	7	8	9
A	1-Hive actin*	1-Hive actin*	1-Hive actin*	1-Hive DwV	1-Hive DwV	1-Hive DwV	1-Hive BQCV	1-Hive BQCV	1-Hive BQCV
B	2-Hive actin*	2-Hive actin*	2-Hive actin*	2-Hive DwV	2-Hive DwV	2-Hive DwV	2-Hive BQCV	2-Hive BQCV	2-Hive BQCV
C	3-Hive actin*	3-Hive actin*	3-Hive actin*	3-Hive DwV	3-Hive DwV	3-Hive DwV	3-Hive BQCV	3-Hive BQCV	3-Hive BQCV
D	4-Hive actin*	4-Hive actin*	4-Hive actin*	4-Hive DwV	4-Hive DwV	4-Hive DwV	4-Hive BQCV	4-Hive BQCV	4-Hive BQCV
E	5-Hive actin*	5-Hive actin*	5-Hive actin*	5-Hive DwV	5-Hive DwV	5-Hive DwV	5-Hive BQCV	5-Hive BQCV	5-Hive BQCV
F	6-Hive actin*	6-Hive actin*	6-Hive actin*	6-Hive DwV	6-Hive DwV	6-Hive DwV	6-Hive BQCV	6-Hive BQCV	6-Hive BQCV
G	7-Hive actin*	7-Hive actin*	7-Hive actin*	7-Hive DwV	7-Hive DwV	7-Hive DwV	7-Hive BQCV	7-Hive BQCV	7-Hive BQCV
H	8-Hive actin*	8-Hive actin*	8-Hive actin*	8-Hive DwV	8-Hive DwV	8-Hive DwV	8-Hive BQCV	8-Hive BQCV	8-Hive BQCV

... and all 12 label columns are visible. DWV stands for “Deformed Wing Virus”, and BQCV stands for “Black Queen Cell Virus”. An asterisk following a label name indicates a reference gene (e.g. actin*). Note that for this data set, 10 bee hives have been sampled for DWV and BQCV.

Case It v7

About Notes Clear Protein DNA Site Cut Run Gel Options Cases Tutorial

1 2 3 4 5 6 7 Load Clear Run

1.0 % 0 % 0 sites
[agarose] actual match located
60 100 0 sites

ELISA Dotblot 96-well PCR DNA chip qPCR

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-Hive actin*	1-Hive actin*	1-Hive actin*	1-Hive DWV	1-Hive DWV	1-Hive DWV	1-Hive BQCV	1-Hive BQCV	1-Hive BQCV	9-Hive actin*	9-Hive actin*	9-Hive actin*
B	2-Hive actin*	2-Hive actin*	2-Hive actin*	2-Hive DWV	2-Hive DWV	2-Hive DWV	2-Hive BQCV	2-Hive BQCV	2-Hive BQCV	9-Hive DWV	9-Hive DWV	9-Hive DWV
C	3-Hive actin*	3-Hive actin*	3-Hive actin*	3-Hive DWV	3-Hive DWV	3-Hive DWV	3-Hive BQCV	3-Hive BQCV	3-Hive BQCV	9-Hive BQCV	9-Hive BQCV	9-Hive BQCV
D	4-Hive actin*	4-Hive actin*	4-Hive actin*	4-Hive DWV	4-Hive DWV	4-Hive DWV	4-Hive BQCV	4-Hive BQCV	4-Hive BQCV	10-Hive actin*	10-Hive actin*	10-Hive actin*
E	5-Hive actin*	5-Hive actin*	5-Hive actin*	5-Hive DWV	5-Hive DWV	5-Hive DWV	5-Hive BQCV	5-Hive BQCV	5-Hive BQCV	10-Hive DWV	10-Hive DWV	10-Hive DWV
F	6-Hive actin*	6-Hive actin*	6-Hive actin*	6-Hive DWV	6-Hive DWV	6-Hive DWV	6-Hive BQCV	6-Hive BQCV	6-Hive BQCV	10-Hive BQCV	10-Hive BQCV	10-Hive BQCV
G	7-Hive actin*	7-Hive actin*	7-Hive actin*	7-Hive DWV	7-Hive DWV	7-Hive DWV	7-Hive BQCV	7-Hive BQCV	7-Hive BQCV	blank blank	blank blank	blank blank
H	8-Hive actin*	8-Hive actin*	8-Hive actin*	8-Hive DWV	8-Hive DWV	8-Hive DWV	8-Hive BQCV	8-Hive BQCV	8-Hive BQCV	blank blank	blank blank	blank blank

Labels Load Clear

Enzyme Cut DNA

Genbank Lab Bench Data Screen ELISA Dot Blot 96-well PCR Chip qPCR Sequencing

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Show Hide Delta Ct Fold change Standard Curve

Click the qPCR button and select **Run qPCR for -> orange wells only**. Note: the Run button next to the Load button has been grayed out, indicating that it is not used to run qPCR (it is used to run other types of analyses involving a 96-well plate, such as ELISA).

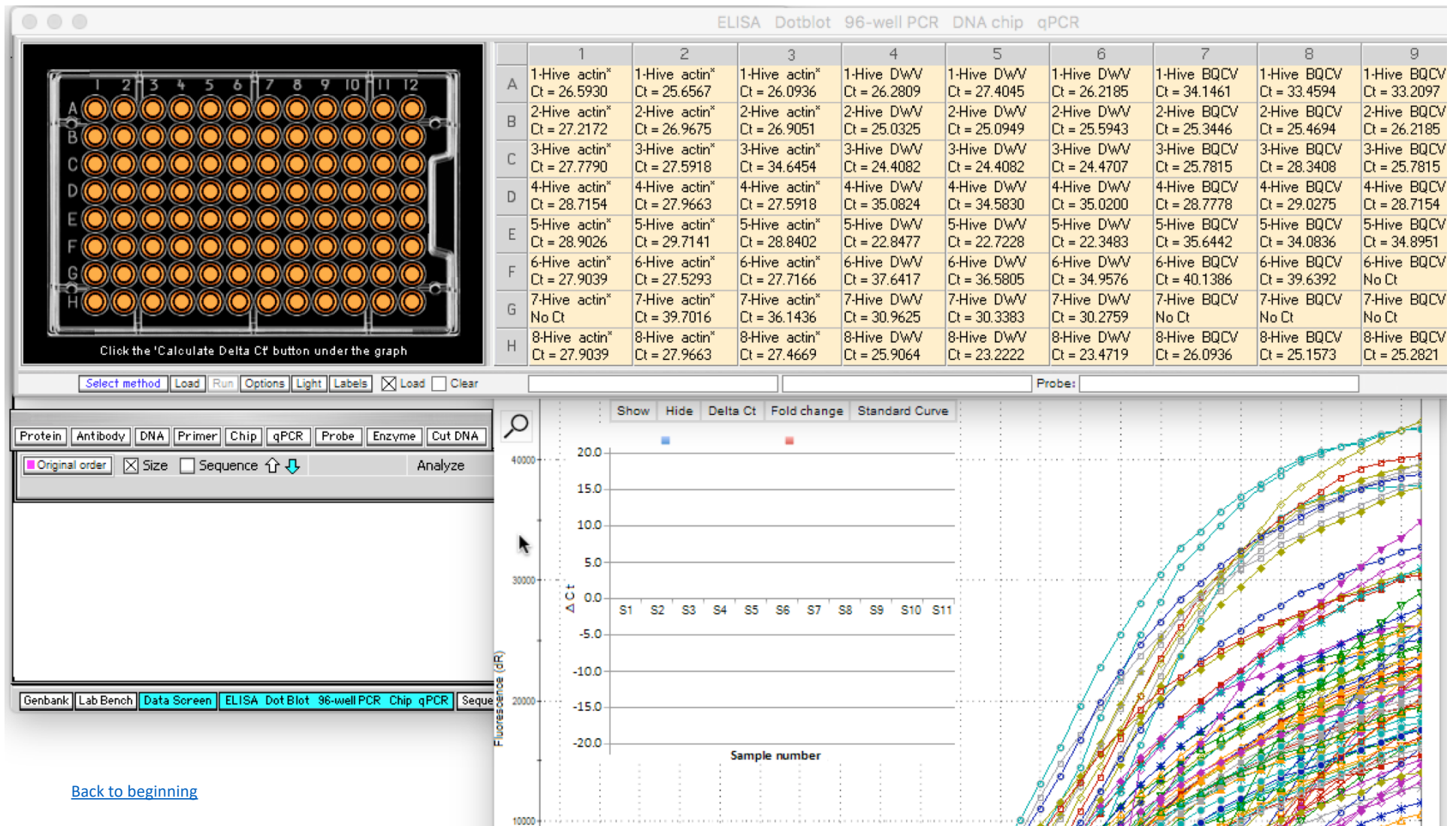
The software interface displays a 96-well plate layout with columns 1-12 and rows A-H. The plate content is as follows:

	1	2	3	4	5	6	7	8	9
A	1-Hive actin*	1-Hive actin*	1-Hive actin*	1-Hive DwV	1-Hive DwV	1-Hive DwV	1-Hive BQCV	1-Hive BQCV	1-Hive BQCV
B	2-Hive actin*	2-Hive actin*	2-Hive actin*	2-Hive DwV	2-Hive DwV	2-Hive DwV	2-Hive BQCV	2-Hive BQCV	2-Hive BQCV
C	3-Hive actin*	3-Hive actin*	3-Hive actin*	3-Hive DwV	3-Hive DwV	3-Hive DwV	3-Hive BQCV	3-Hive BQCV	3-Hive BQCV
D	4-Hive actin*	4-Hive actin*	4-Hive actin*	4-Hive DwV	4-Hive DwV	4-Hive DwV	4-Hive BQCV	4-Hive BQCV	4-Hive BQCV
E	5-Hive actin*	5-Hive actin*	5-Hive actin*	5-Hive DwV	5-Hive DwV	5-Hive DwV	5-Hive BQCV	5-Hive BQCV	5-Hive BQCV
F	6-Hive actin*	6-Hive actin*	6-Hive actin*	6-Hive DwV	6-Hive DwV	6-Hive DwV	6-Hive BQCV	6-Hive BQCV	6-Hive BQCV
G	7-Hive actin*	7-Hive actin*	7-Hive actin*	7-Hive DwV	7-Hive DwV	7-Hive DwV	7-Hive BQCV	7-Hive BQCV	7-Hive BQCV
H	8-Hive actin*	8-Hive actin*	8-Hive actin*	8-Hive DwV	8-Hive DwV	8-Hive DwV	8-Hive BQCV	8-Hive BQCV	8-Hive BQCV

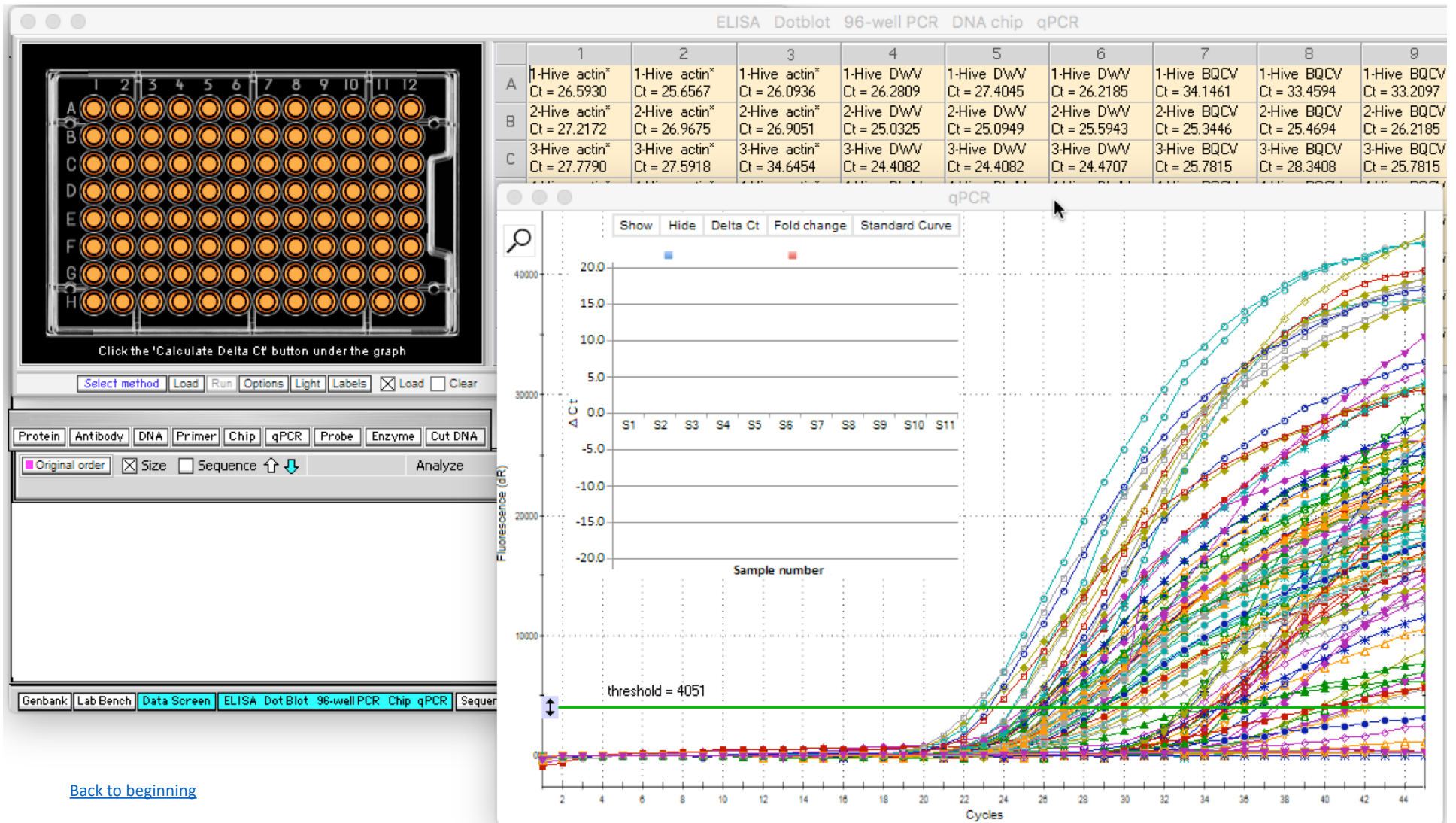
The control panel includes buttons for 'Select method', 'Load', 'Run', 'Options', 'Light', 'Labels', 'Load', and 'Clear'. A dropdown menu for 'Run qPCR for' is open, showing options: 'orange wells only', 'purple wells only', and 'Help:'. The graph shows fluorescence (dR) on the y-axis (10000 to 40000) and sample number (S1-S11) on the x-axis. A Delta Ct axis is also visible. The 'Run' button is grayed out.

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After qPCR runs, Ct values will appear in the labels for each well, for example a Ct value of 24.0337 for well A1. Curves on the graph represent changes in the amount of DNA for each well for 45 qPCR cycles (bottom axis of graph). Click on the graph to bring it forward...

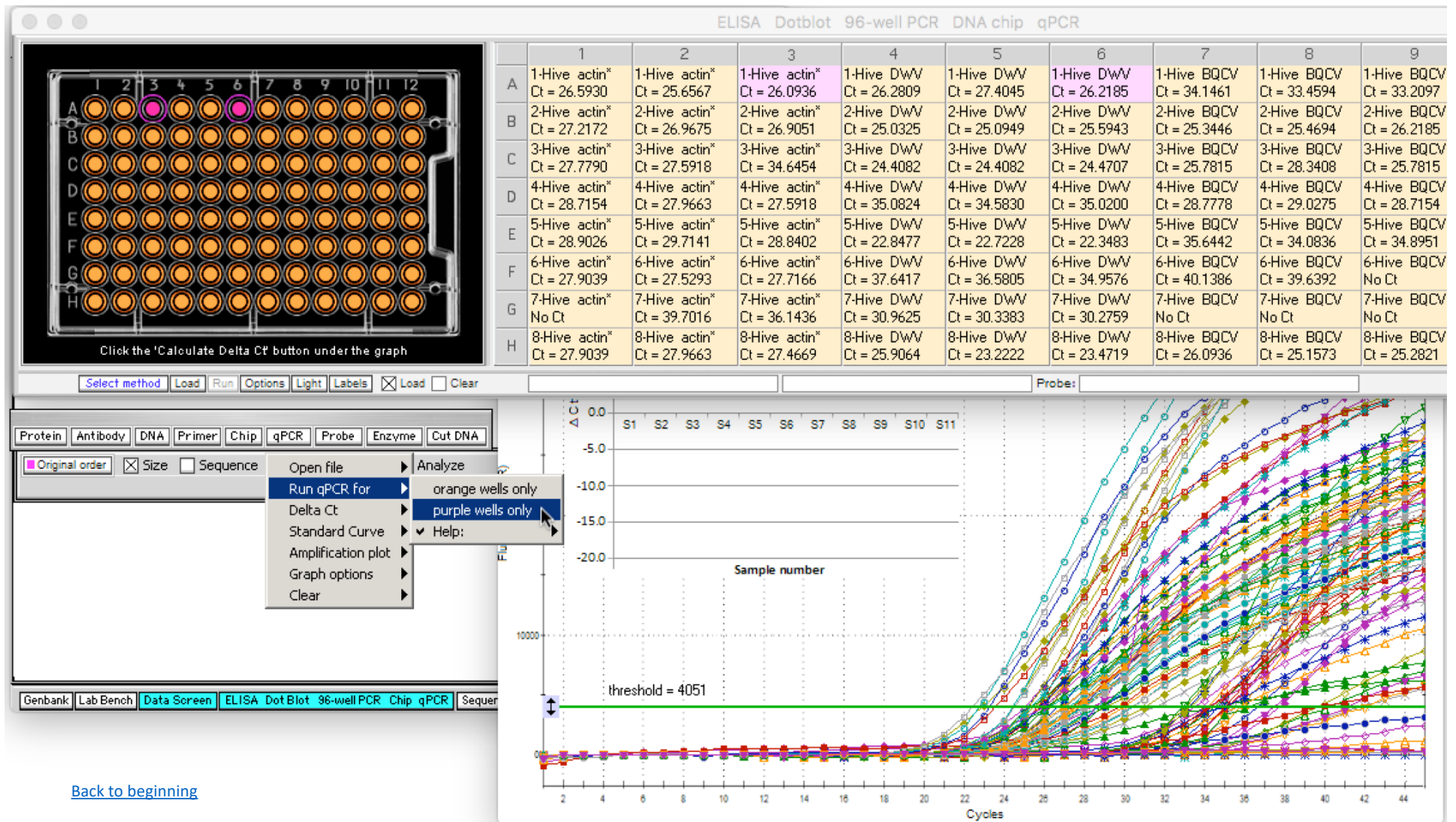


...and all 96 curves can be seen, each with a unique color/symbol combination. The next series of slides will explain how the Ct values were determined from the graph curves.



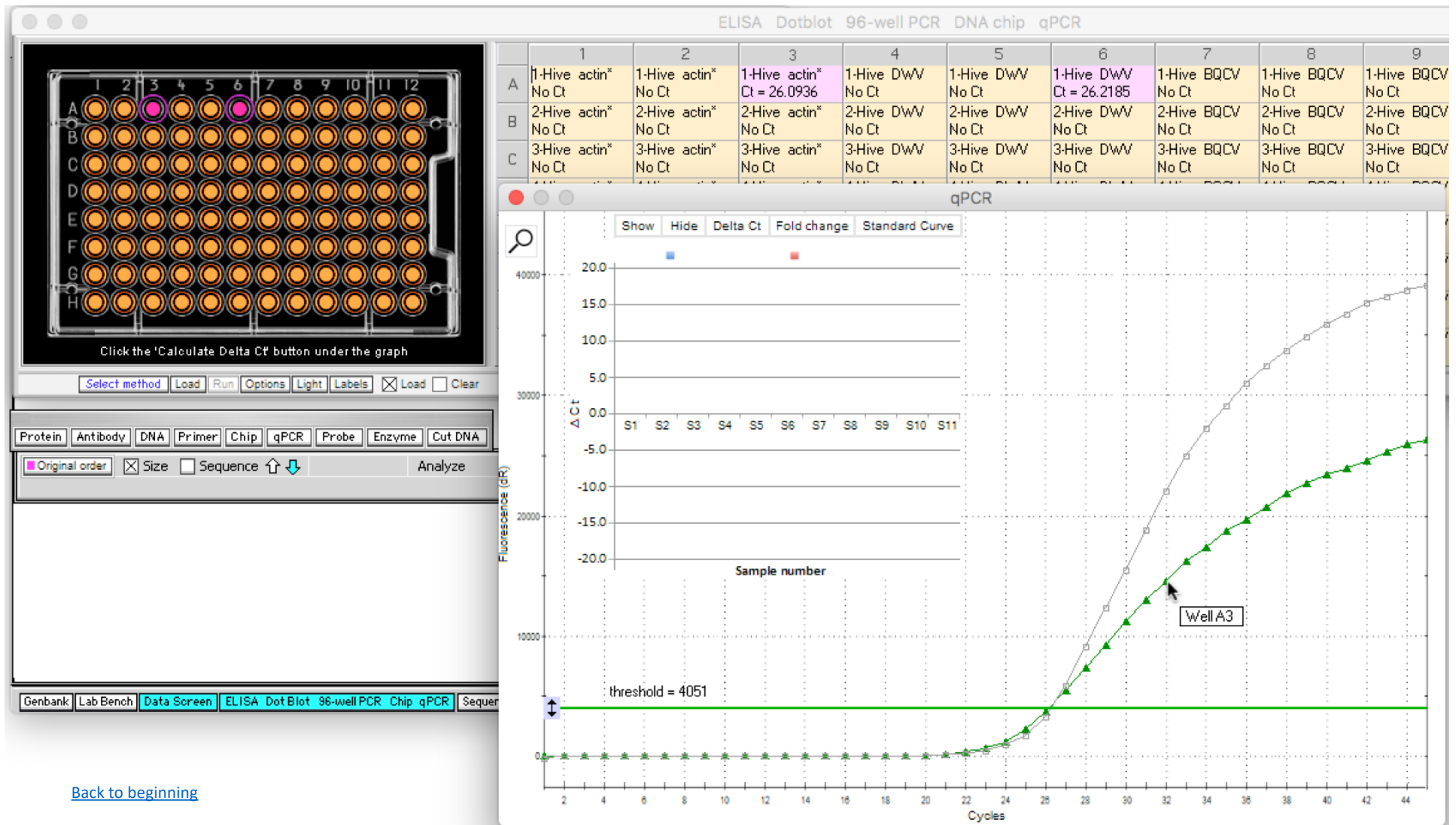
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First, **click two** of the 96 wells (**A3** and **A6**, for example). Note that the wells turn purple in color, as do the corresponding labels. Then, use the **qPCR** button and select **Run qPCR for -> purple wells only**.



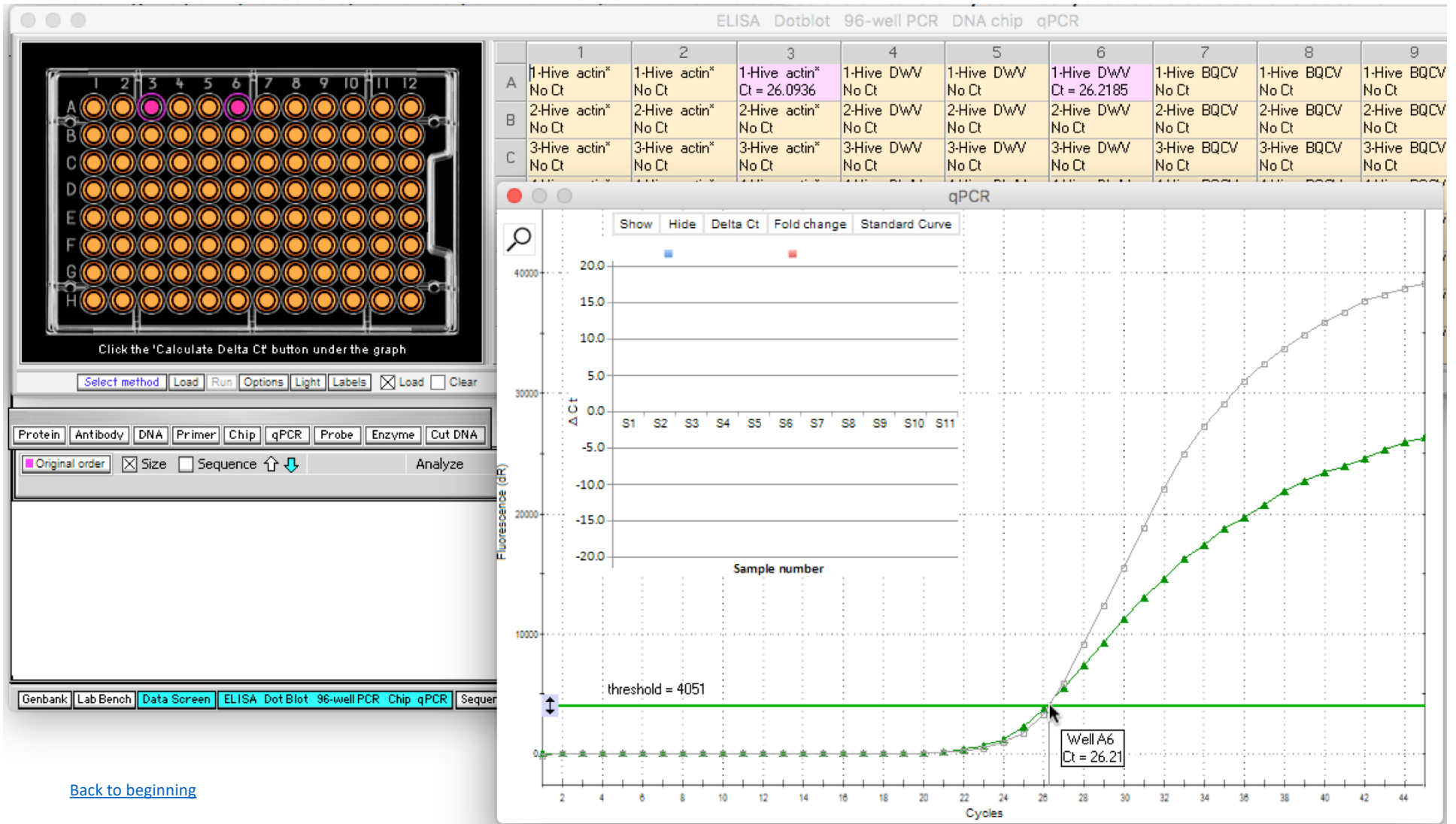
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Only two of the 96 curves now appear, along with corresponding Ct values for wells A3 and A6. If the cursor is held over any point on a curve, a box pops up indicating which well the curve is associated with (A3 green, A6 gray).



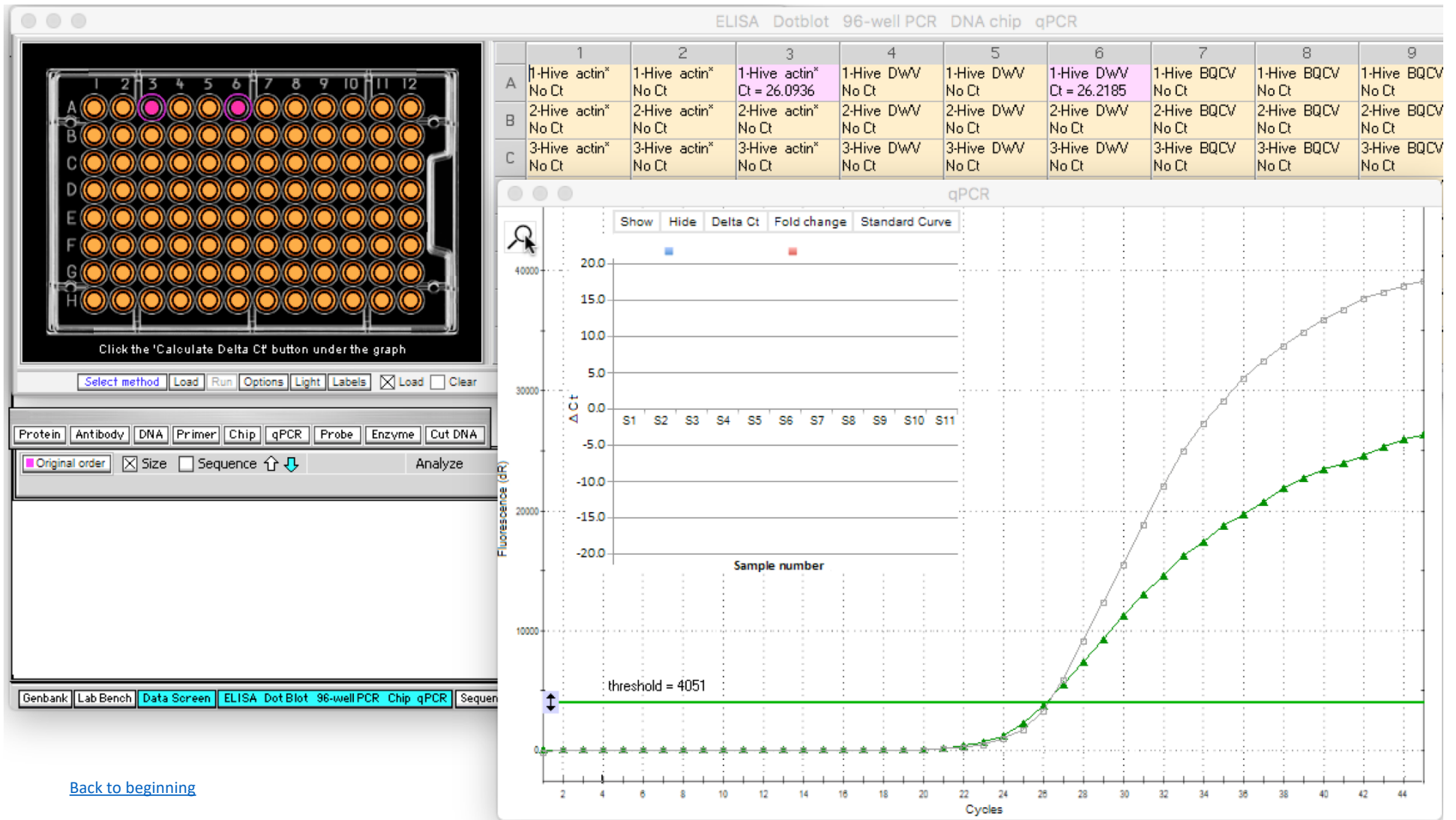
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If the cursor is held over the area where a curve intersects the green threshold line, a vertical line is drawn from that point to the bottom axis, indicating the point at which that particular curve crossed the threshold. By definition, that is the Ct value for that curve (26.21 in this case). For this example, both curves have similar Ct values.



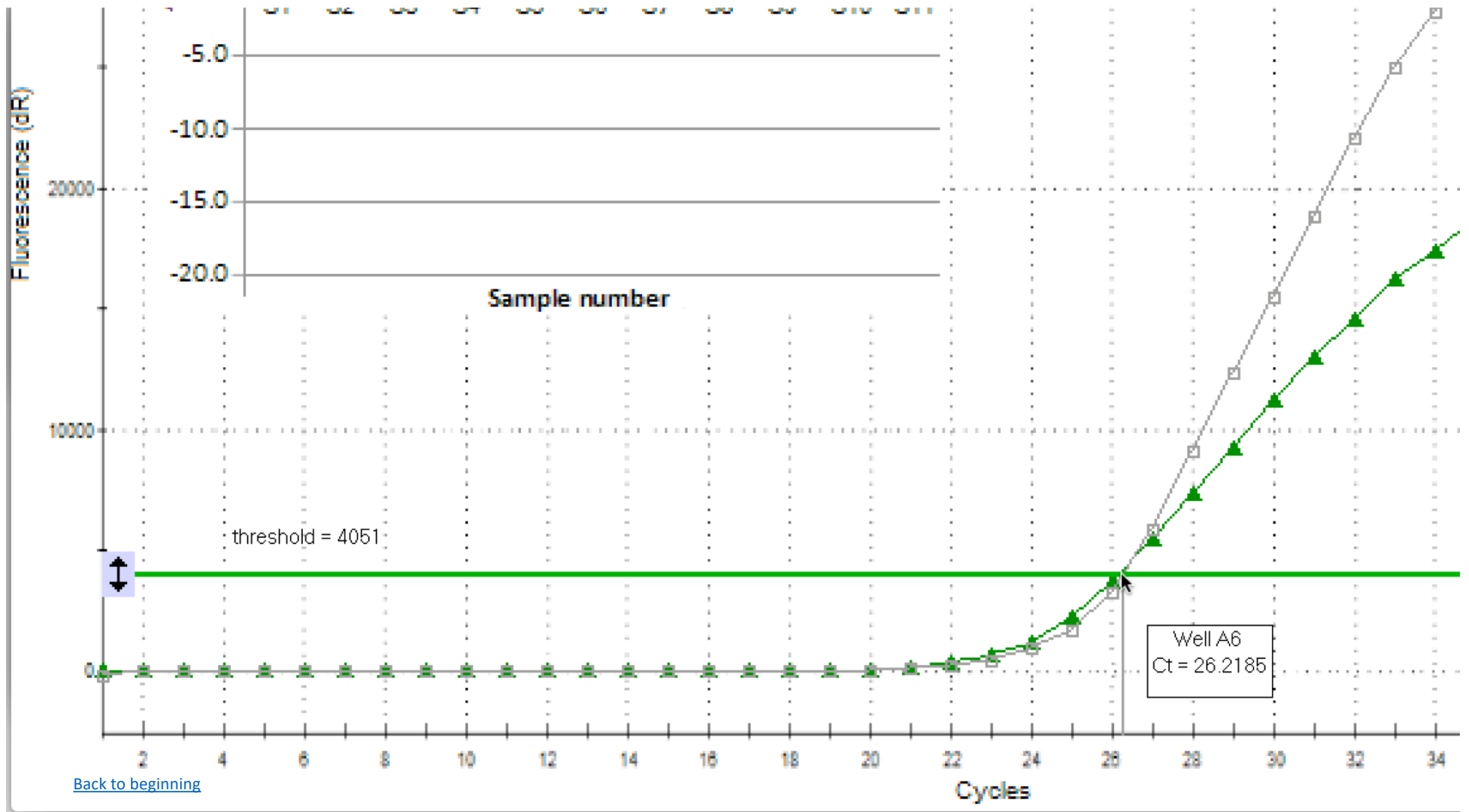
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For easier viewing, the graph can be magnified by clicking the magnifying glass icon in the upper left-hand corner of the graph...

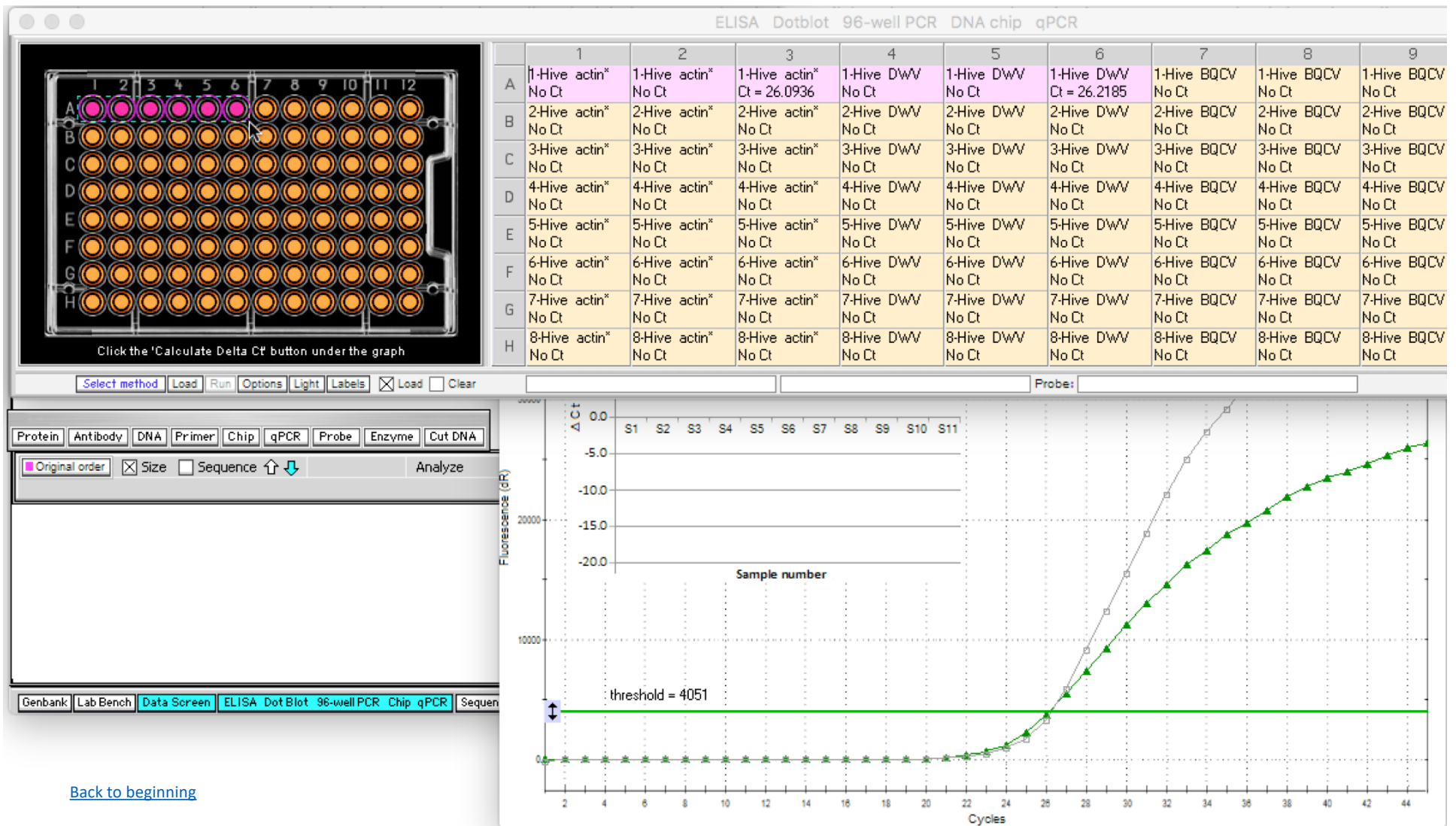


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...which makes the intersection of the threshold and curve easier to see. Clicking the magnifying glass icon repeatedly cycles between small, medium, and large views of the graph (note that the icon is not shown in this screen shot).

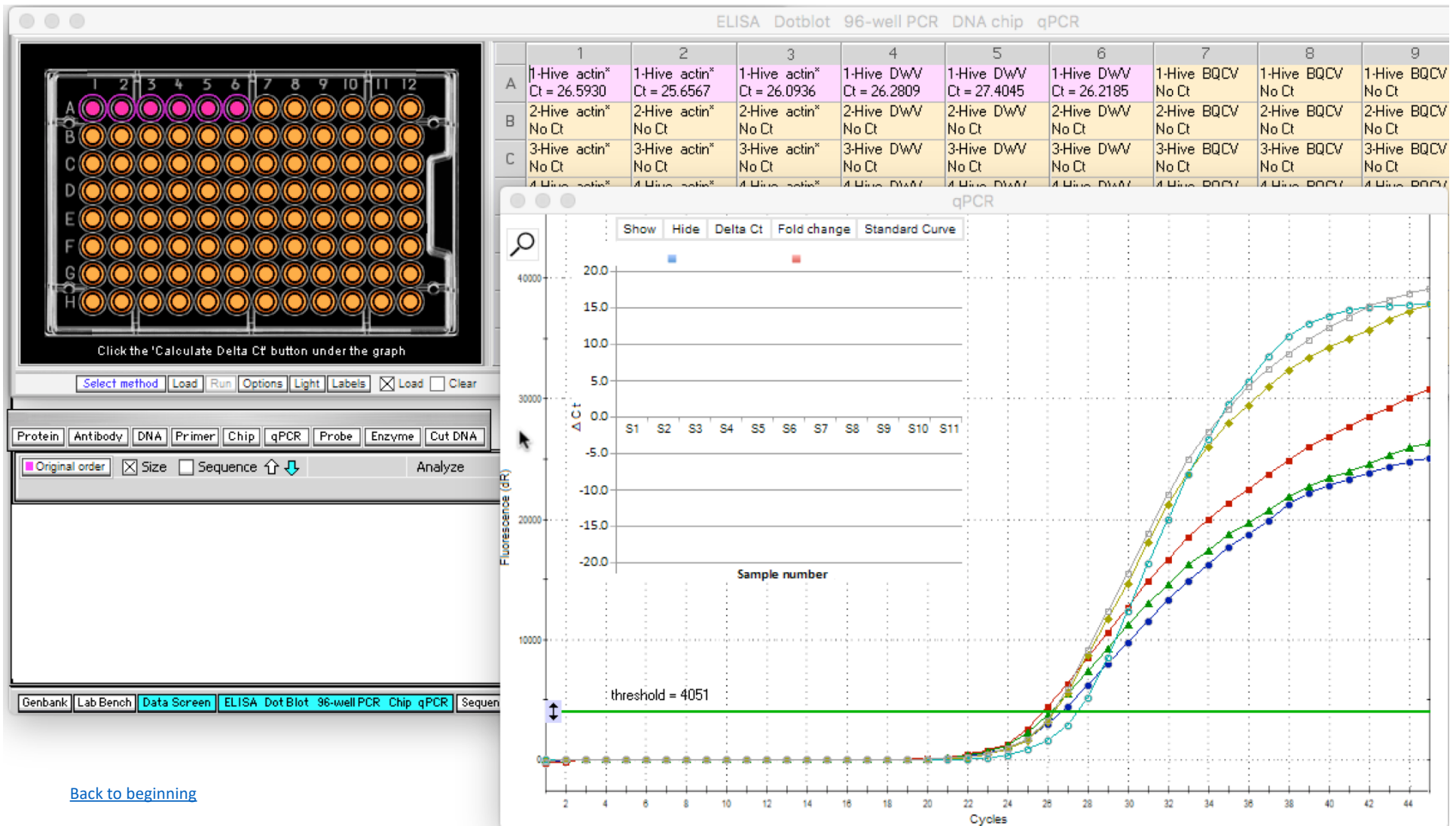


To increase accuracy, qPCR samples are usually loaded in triplicates. **Drag over or click wells as shown below so that wells A1 through A6 are purple.** Note that you can make wells purple by clicking either the well or the label associated with that well, but that you can only make them orange again by clicking the well, or by reloading the file.



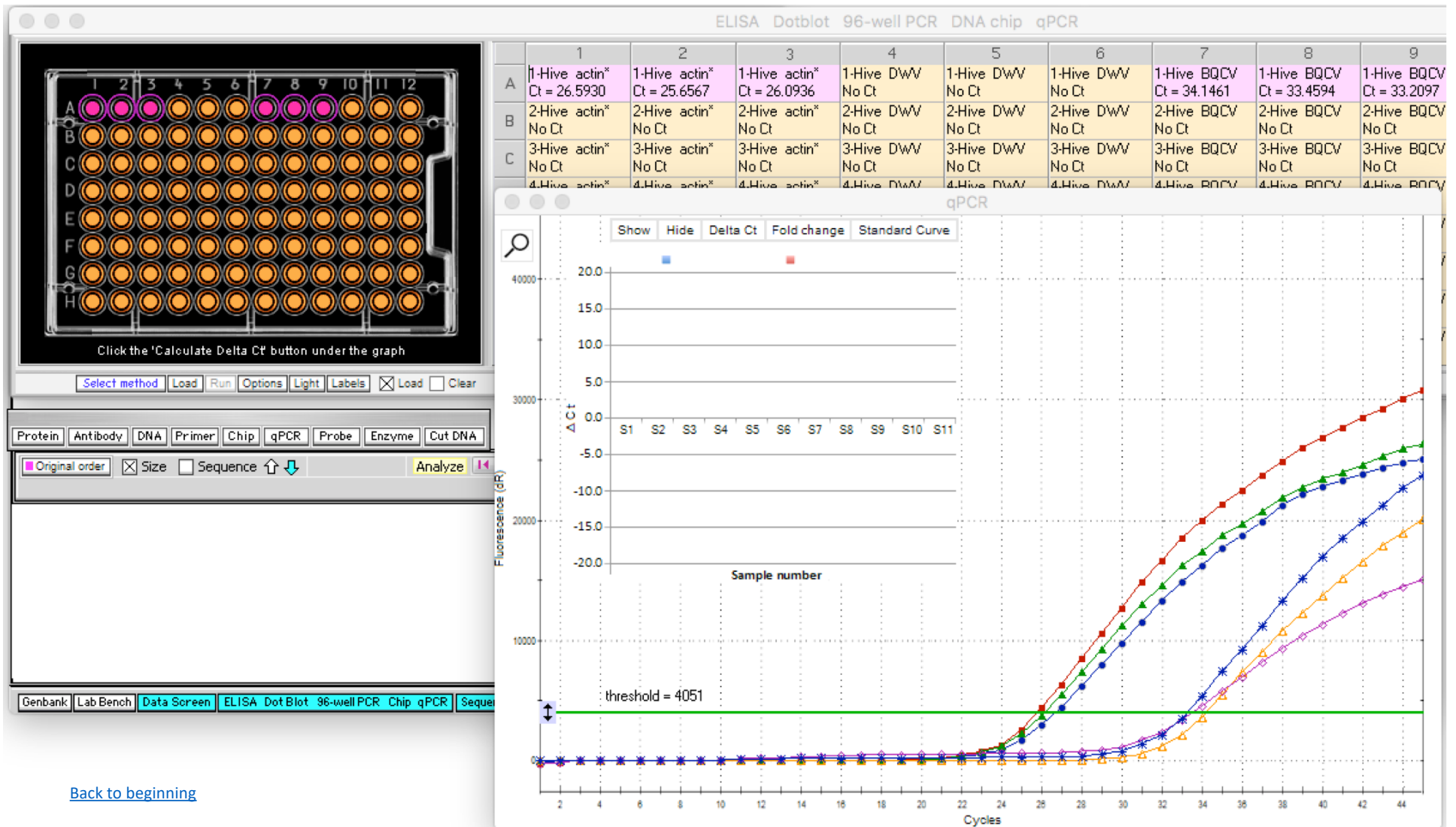
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Run qPCR for purple wells only, using **qPCR button** -> **Purple wells only**. Note that for this example, the three actin curves and the three DWV curves cross the threshold at similar locations, resulting in similar Ct values for samples associated with wells A1 through A6.



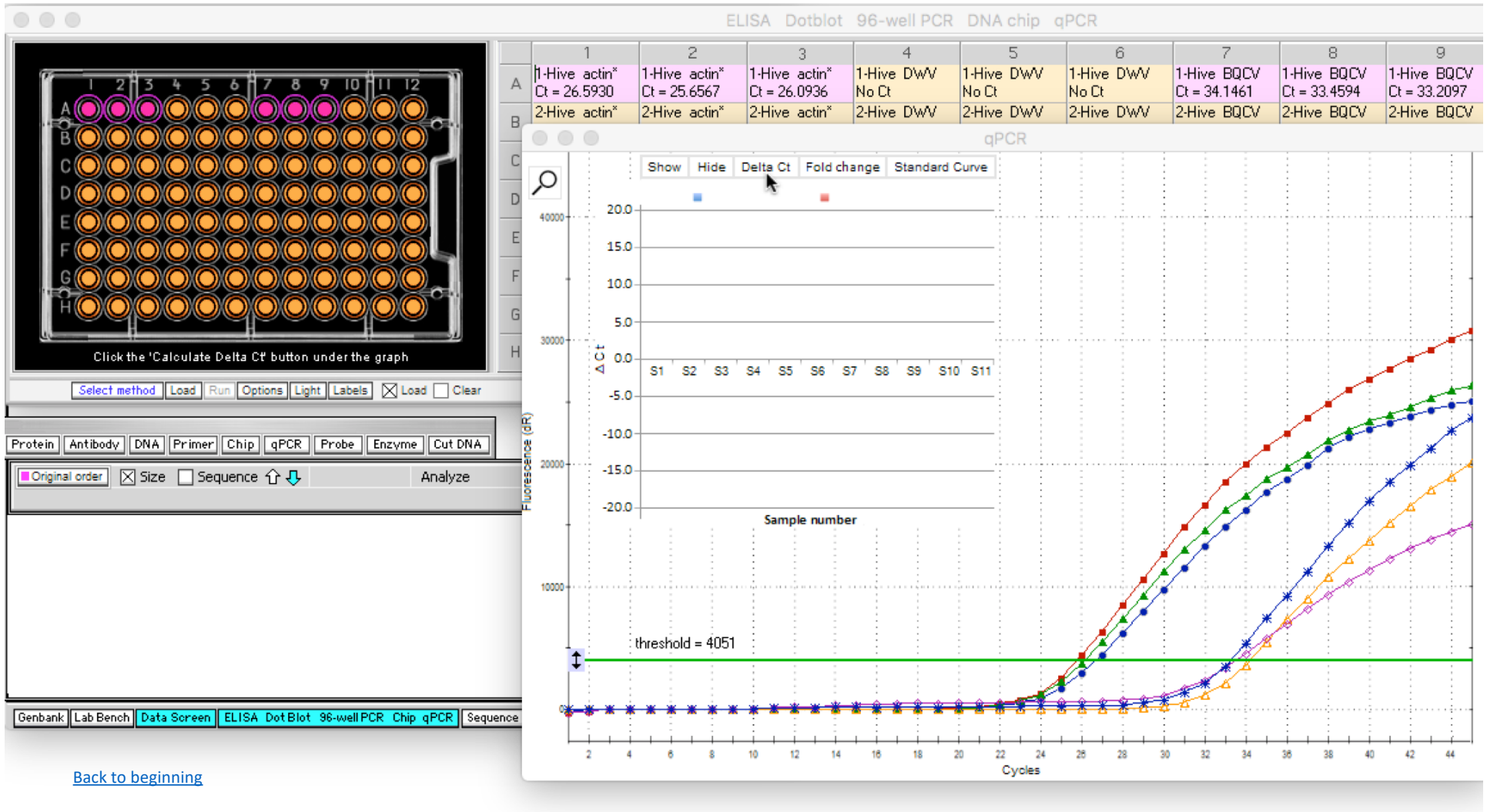
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Now click wells A3, A4 and A5 to make them orange again, and click or drag wells A7, A8 and A9 to make them purple. Re-run qPCR for purple wells only, using the same menu commands as before, and note that the three BQCV curves are separated from the three actin curves.



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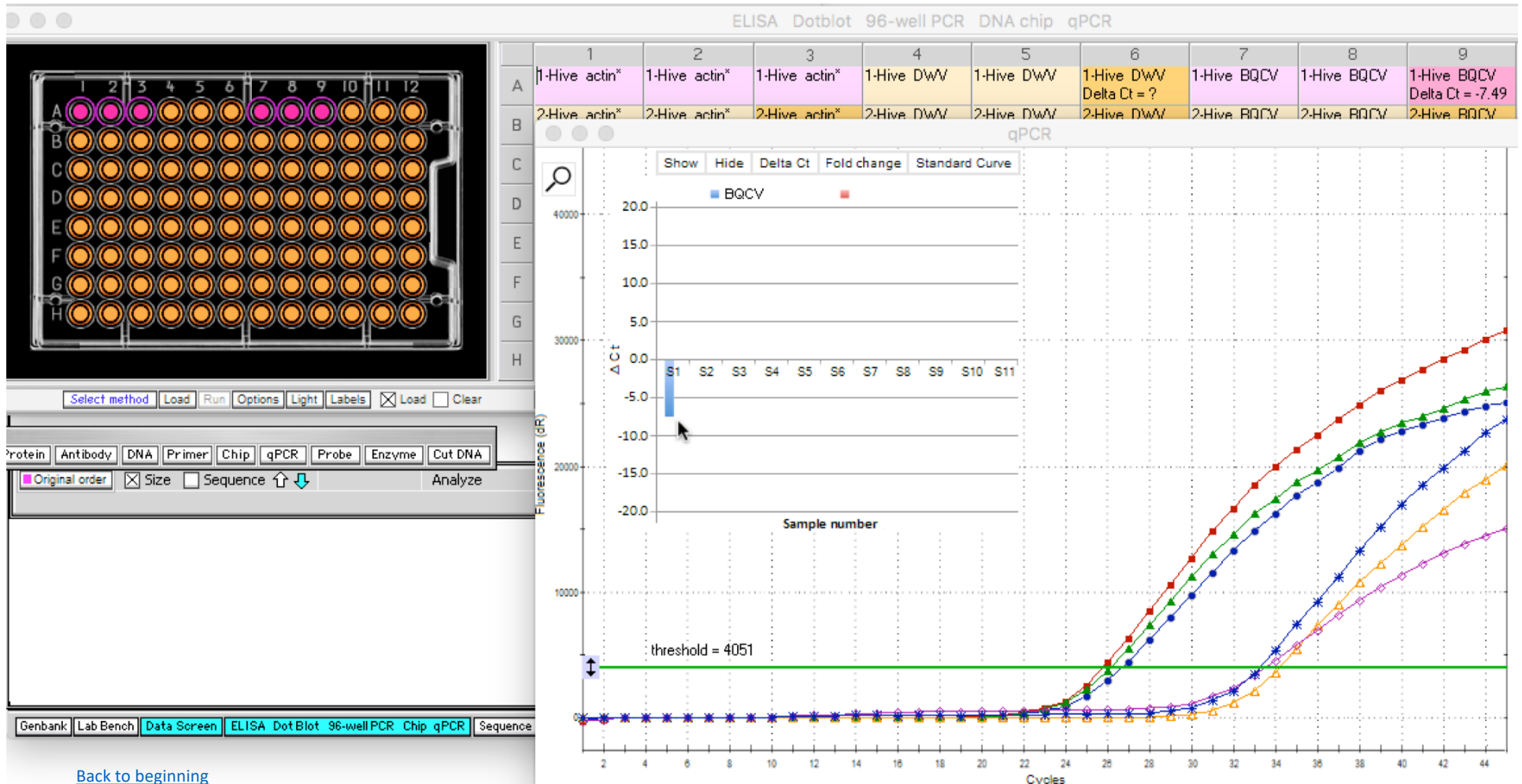
Subtracting the average of the three BQCV Ct values from the average of the three actin Ct values gives the Delta Ct value for this comparison. If you do this by hand for the six purple values below, the average for the actin values is 26.11, and the average for the BQCV values is 33.61, for a Delta Ct value of -7.49. To have the simulation make the calculation, click the **Delta Ct** button on the graph...



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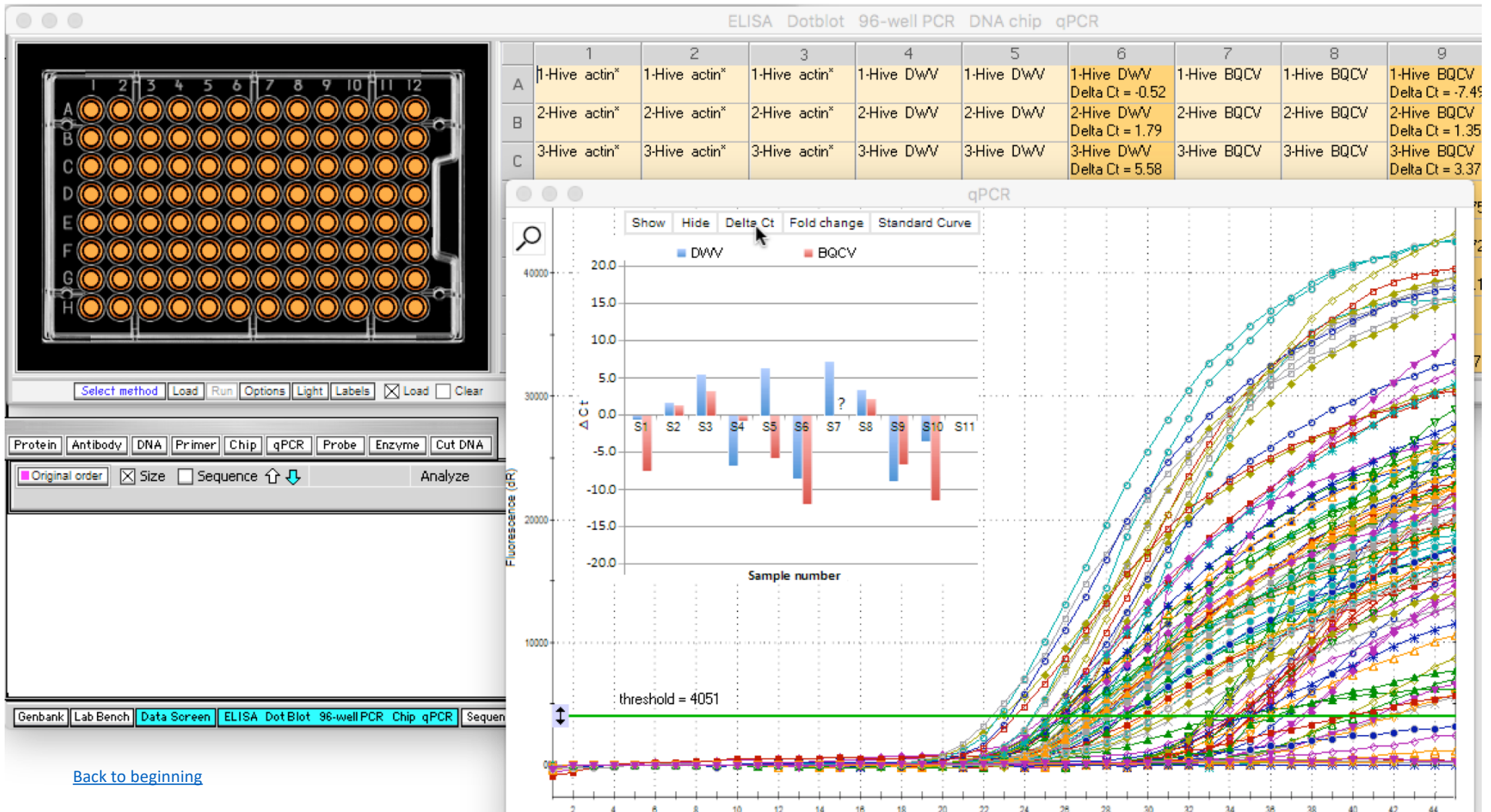
...and the **Delta Ct** value of -7.49 appears in label A9. Note that a blue bar appears representing this value, pointing downwards because it is represented by a “negative” number.

Note: The threshold is crossed more quickly when initial cDNA amounts are greater, so relatively small Ct values represent relatively large initial cDNA amounts. Thus, an average actin value of 26.11 represents relatively more initial cDNA than an average BQCV value of 33.61. That is why the BQCV value is subtracted from the actin value, resulting in a negative number.

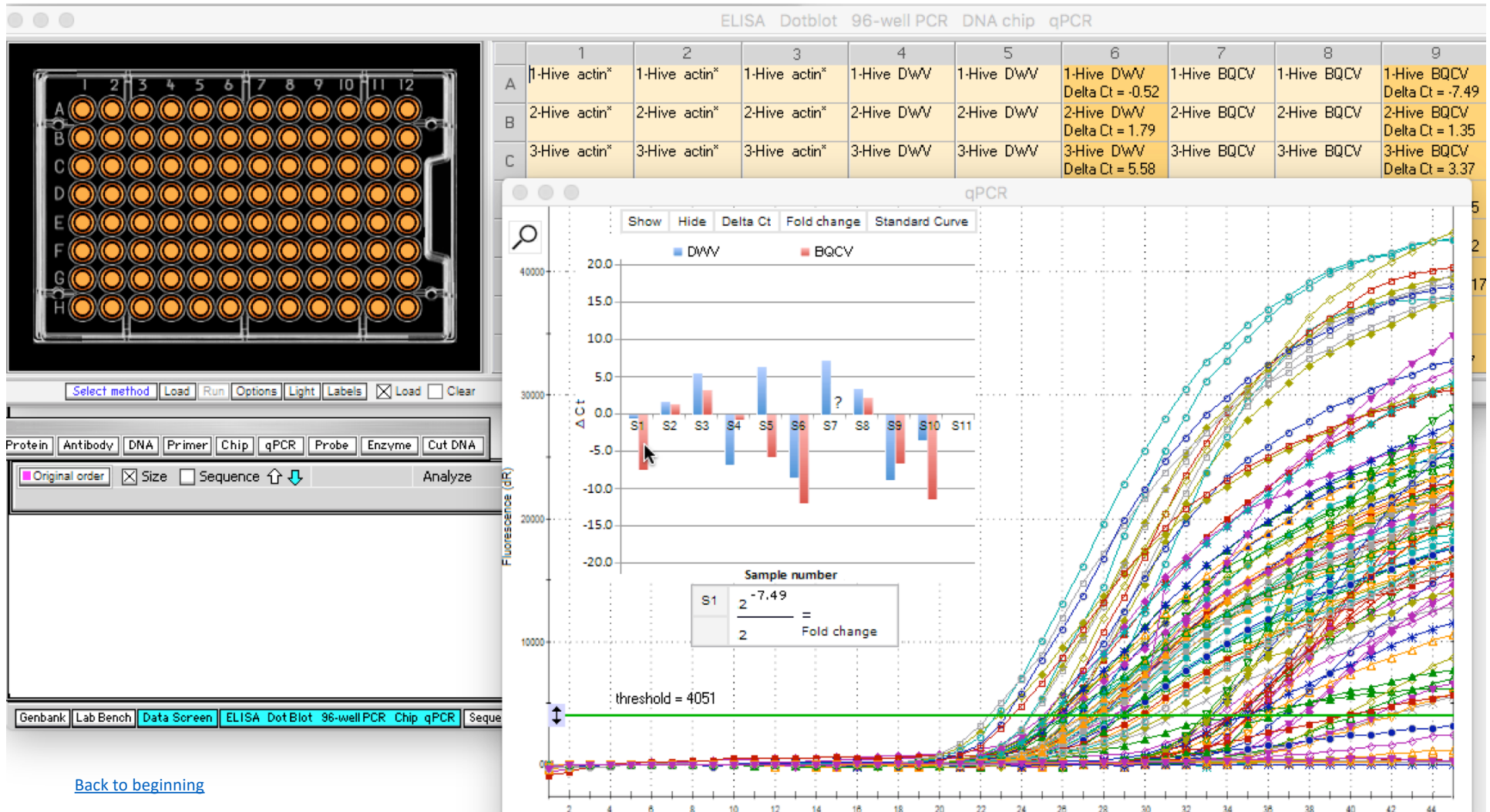


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To see the complete set of Delta Ct values, **first make all wells orange** (either by clicking on the purple wells or by re-opening the re-loading the file). Then **re-run the qPCR** procedure and click the **Delta Ct** button. The BQCV bars are now colored red, and the DWV bars are colored blue. Bars that point upwards represent relatively more initial DNA than the reference gene (actin), whereas bars that point downwards represent relatively less initial DNA than the reference gene. Not all columns are shown.

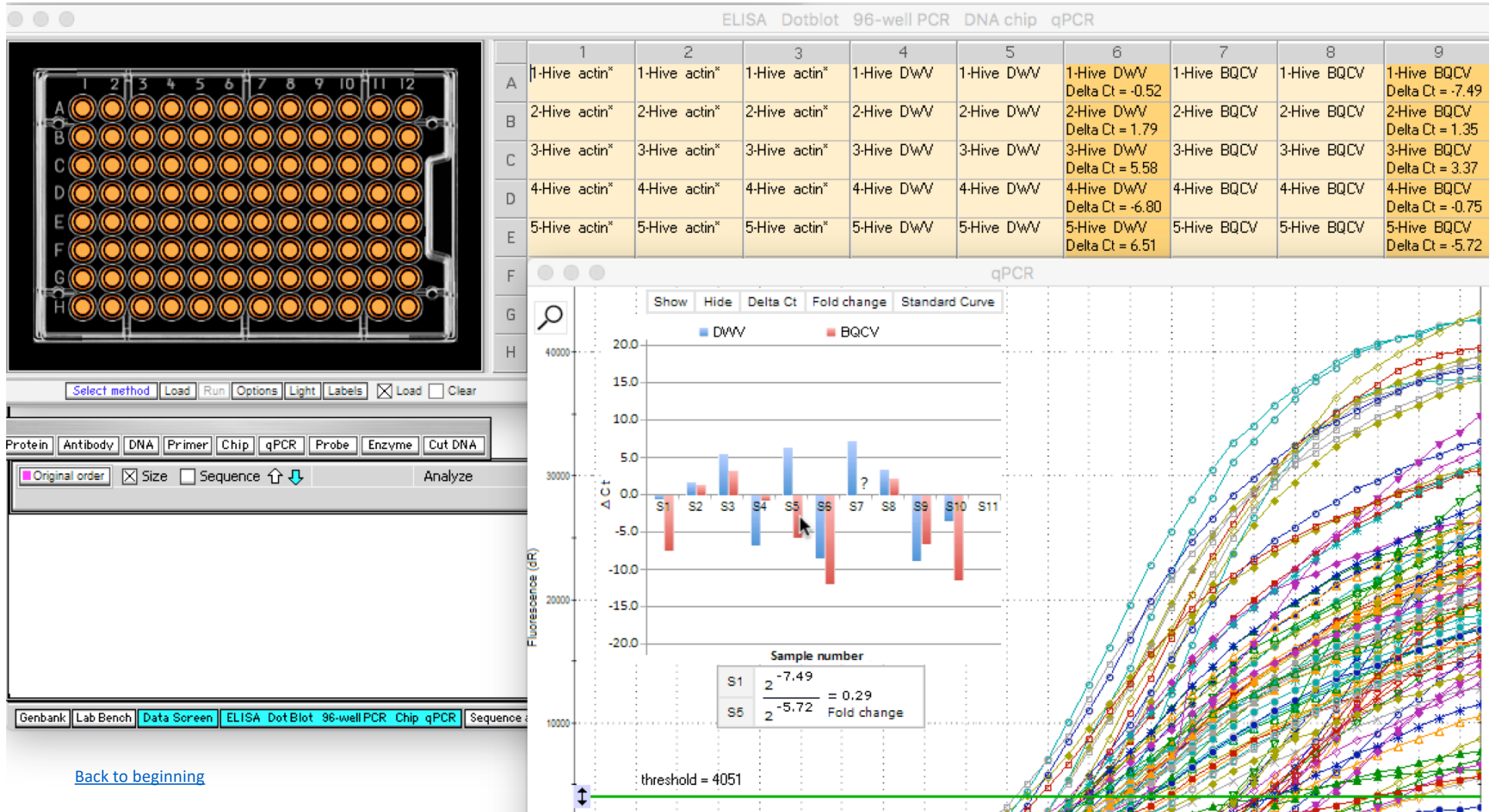


To calculate the fold change (Delta-Delta Ct method) between any two bars on the graph, first single-click on a bar (e.g. the red bar for S1) to put the associated Delta Ct value in the numerator. Note that the numerator of the formula for Delta-Delta Ct is 2 raised to the Ct value, or 2 raised to -7.49 in this case.



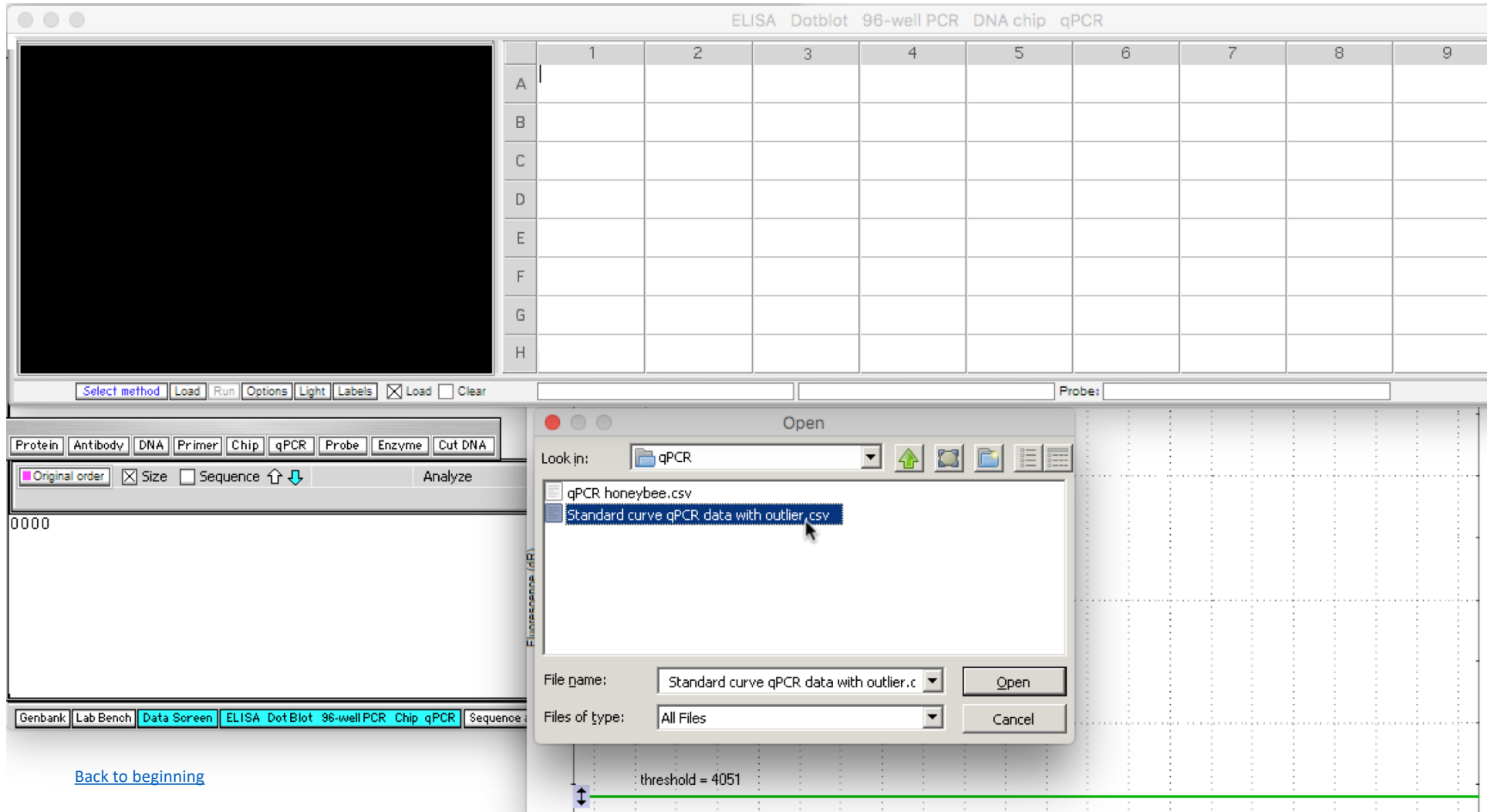
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Double-click on any bar to put that bar's associated Delta Ct value in the denominator of the formula. In this example, double-clicking on the red bar for S5 would put -5.72 in the denominator of the formula, with a resulting fold change of 0.29 between Hives 1 and 5 for BQCV cDNA levels.



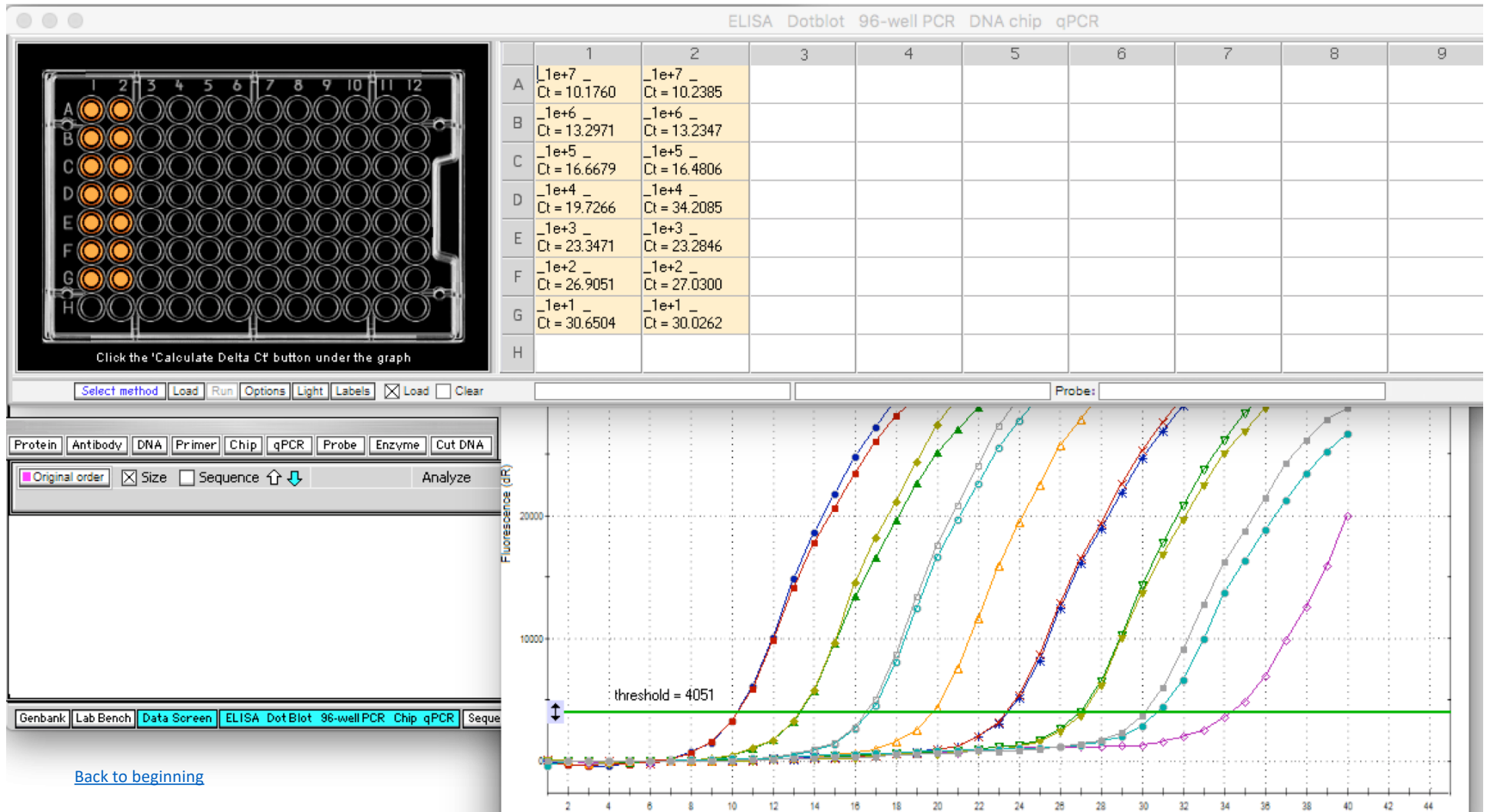
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Another feature of the simulation allows the user to determine standard curve values, used for absolute quantification of DNA quantities. To demonstrate this, open the file **Standard curve qPCR data with outlier.csv**, located inside Cases->qPCR.

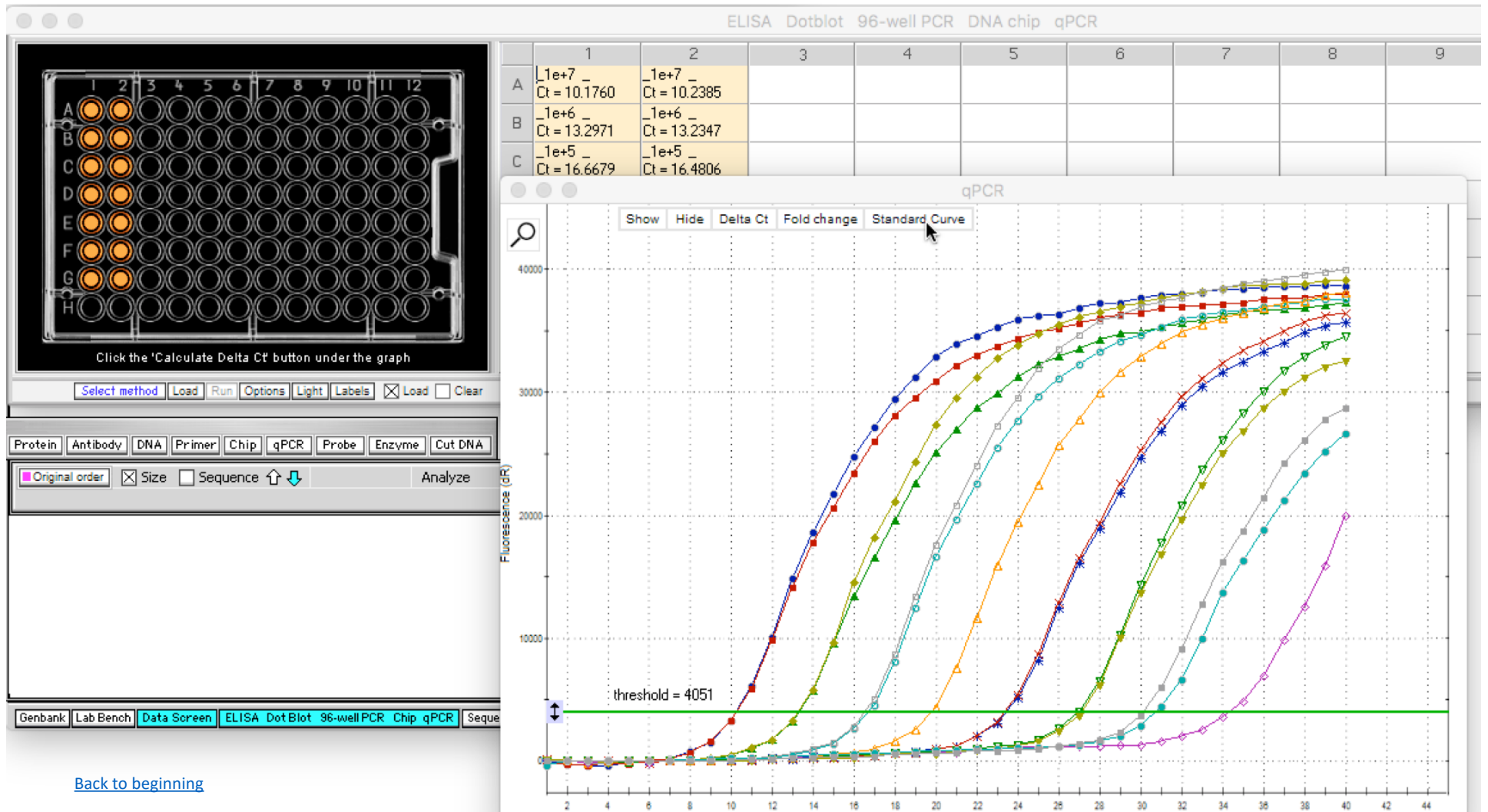


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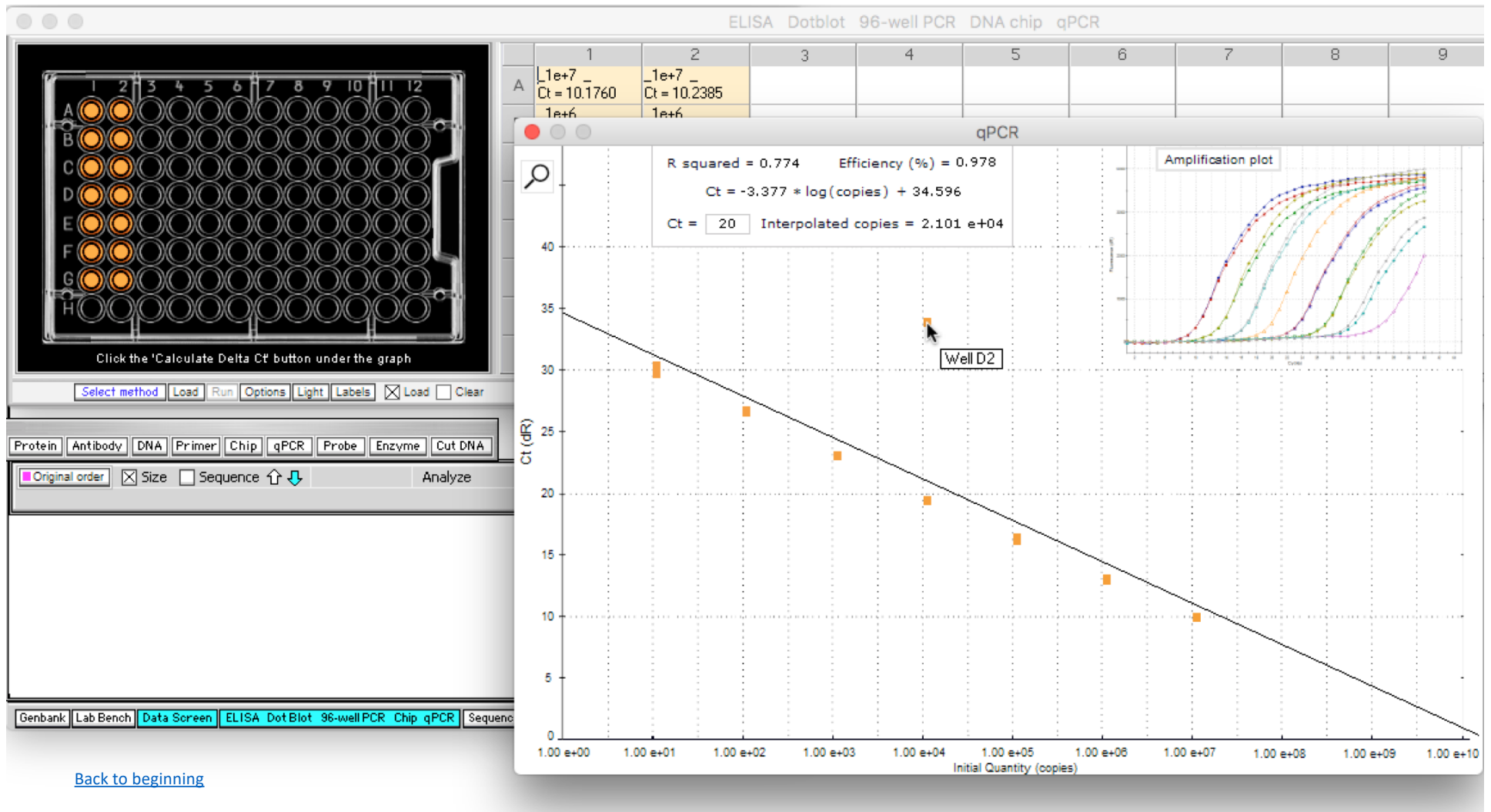
Click the **Load** button under the 96-well plate, then use the **qPCR** button and **Run qPCR for -> Orange wells** only. Standard curve data will appear as shown below. To create a standard curve, known quantities of DNA are amplified and Ct values determined. Note that due to the large numbers involved, **e notation** is used to display the results (for example, $1e+7 = 1.0 \times 10^7 = 10000000$ DNA copies).



To generate the standard curve from these data, click the Standard Curve button at the top of the graph...

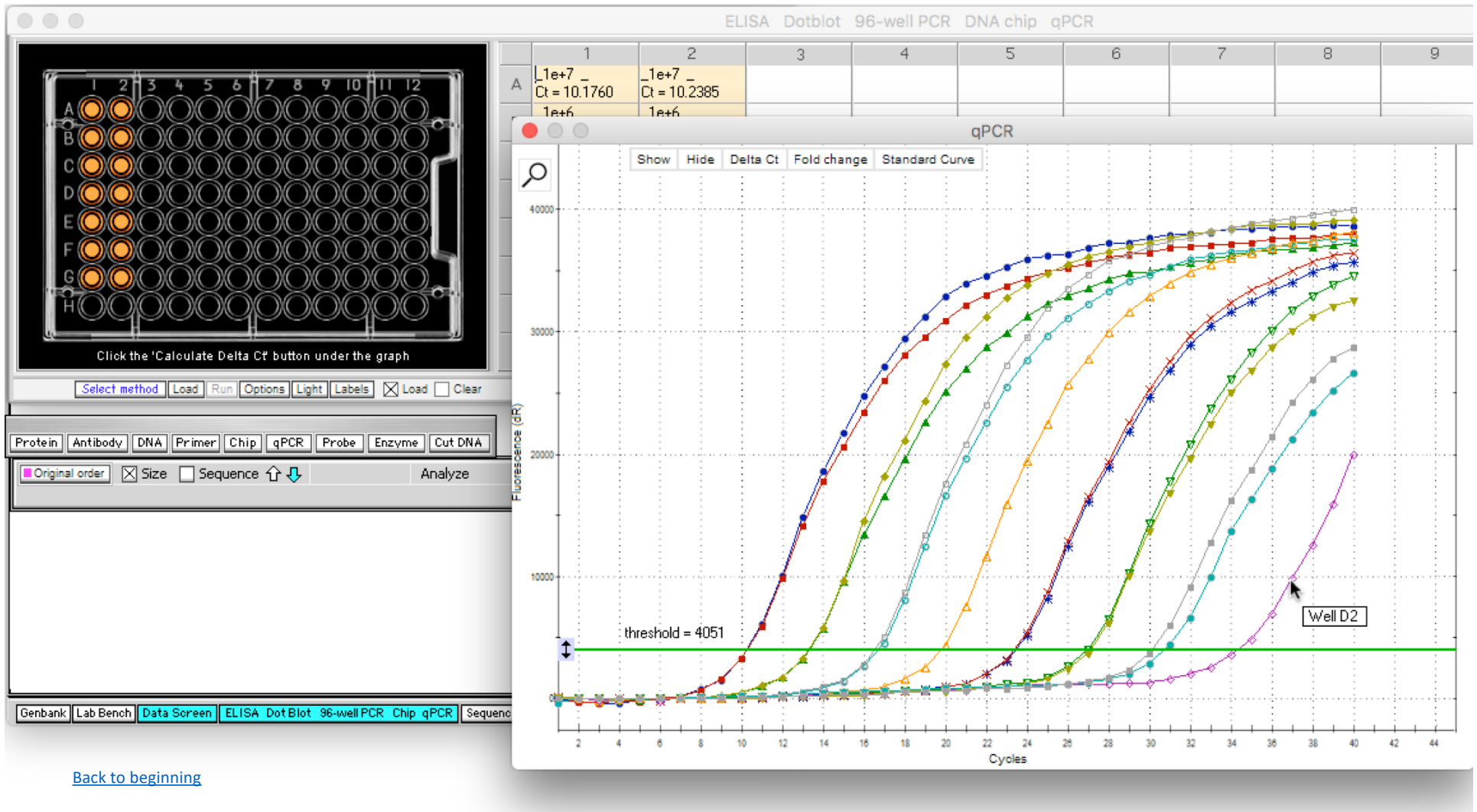


The standard curve plot appears, with a regression line for Ct plotted against initial quantity (copies). Note that an outlier is present in the data set, because a standard curve data points should fall close to the regression line. The outlier can be removed from the data set. First, hold the mouse cursor over the outlier; a box will pop up indicating that it is associated with **Well D2**, then click the **Amplification plot** button at upper left.



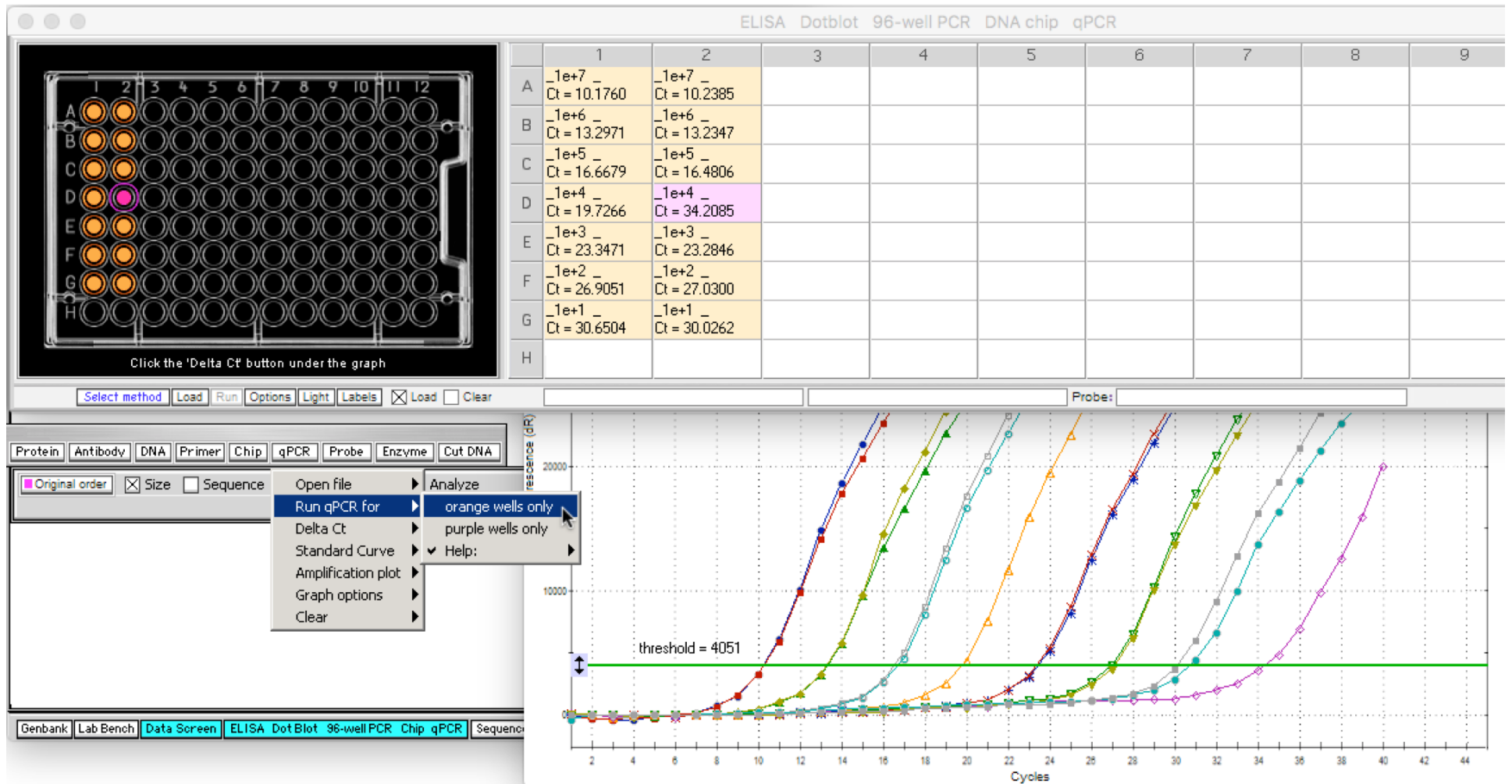
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Hold the mouse cursor over the purple curve on the far right, and a box pops up indicating that the curve is for Well D2, the outlier identified on the standard curve.



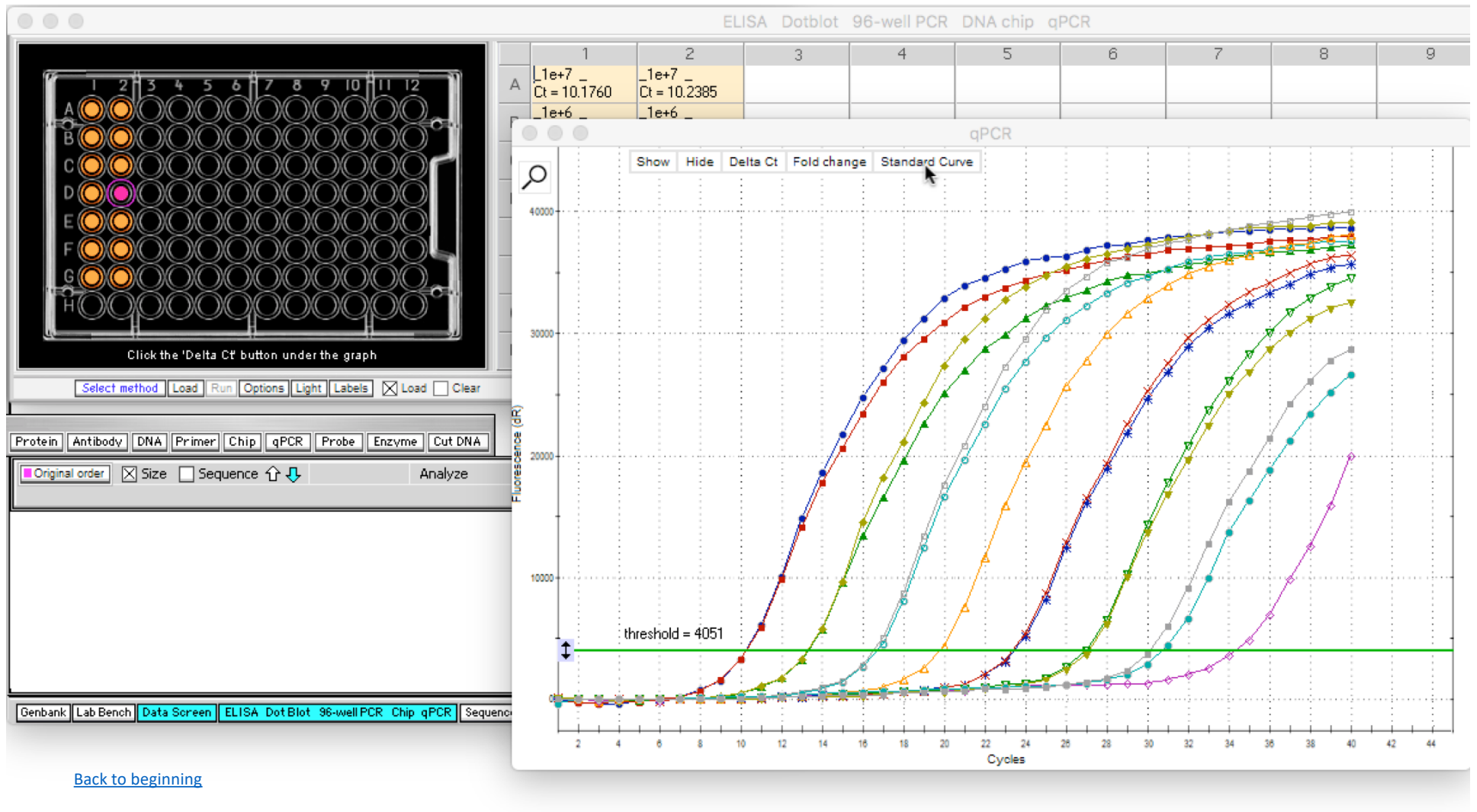
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Click well D2 to make it purple, then re-run the standard curve for **orange wells only**, to exclude the outlier well data.



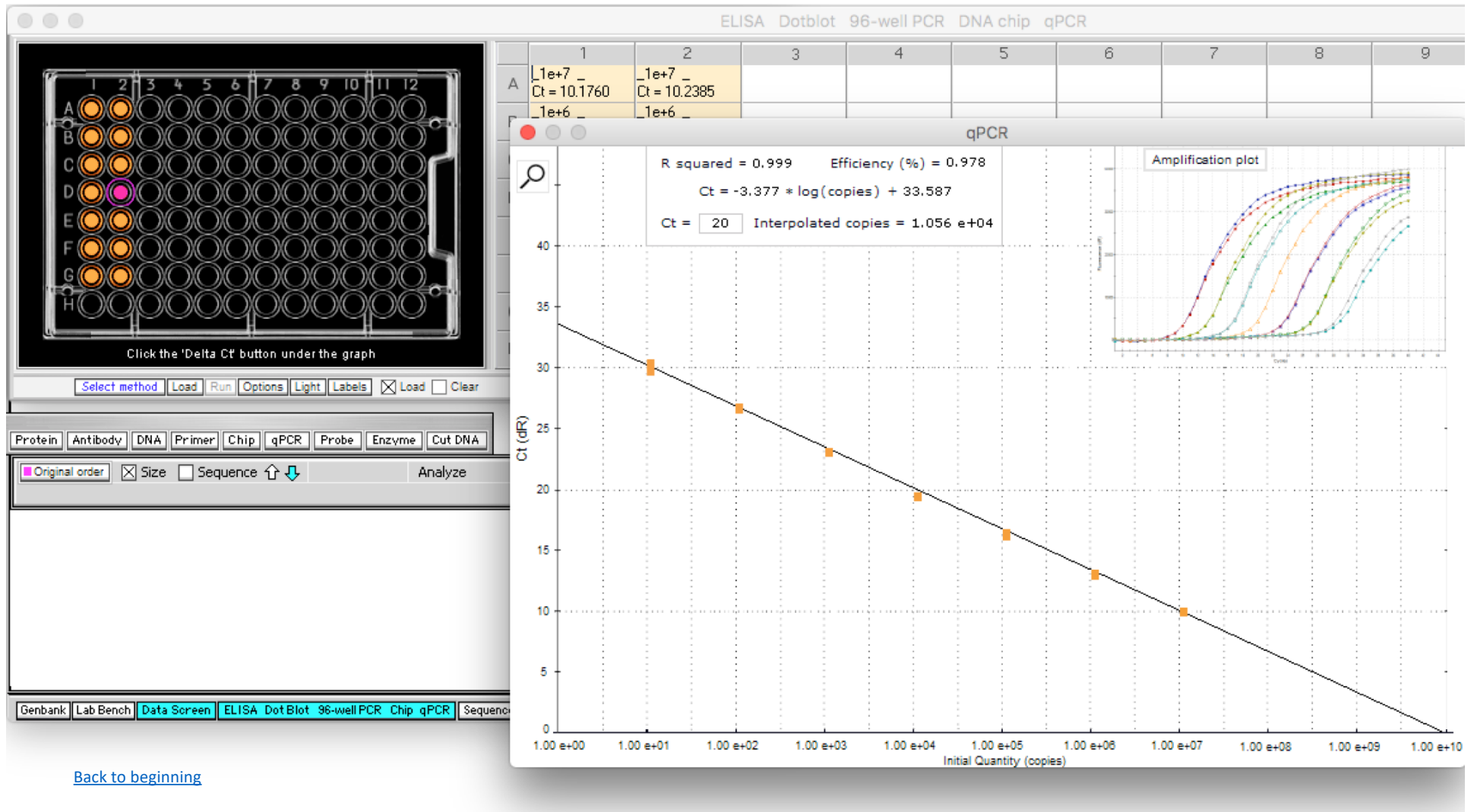
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Next, click the **Standard Curve** button to recalculate the standard curve with the outlier deleted.



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Data points now fall very close to the regression line, as verified by an R squared value of 0.999 (it was 0.774 with the outlier included). Normally you should be very careful about removing outliers from a data set, but for standard curve data we know that the points should fall on the regression line, if the experiment is done correctly. So for standard curve data, outliers definitely mean that something is wrong with the experimental setup.



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The equation for the regression line is shown in the box on the graph, so that the line can be used to estimate the number of copies present for experimental Ct values. For example, if 20 is entered in the box (the default value), then the number of copies interpolated from the line is 1.056 e+04, or 1056 copies (1.056*10⁴). You can also hold the mouse cursor over any point on the line to generate an interpolated copy number. Note that the number in the pop-up box will be slightly different (1.060 e+04 in this case), depending on where the mouse cursor is positioned on the line.

