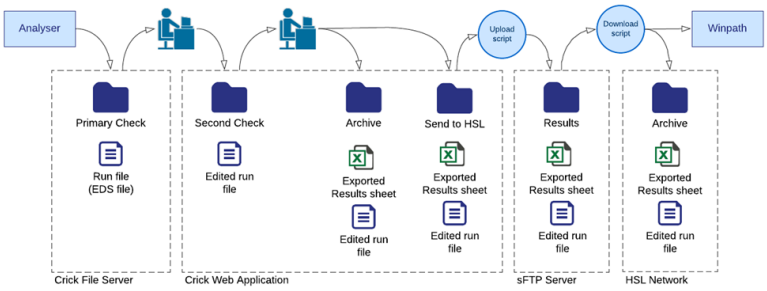
Supplementary Method 3: RT-LAMP Results Reporting

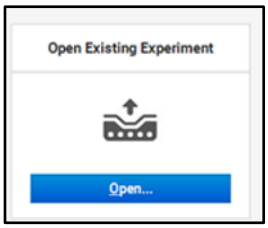
In the CCC pipeline, samples have been submitted to an accredited reporting laboratory with testing being completed in a research laboratory. The test results have been analysed by both a research scientist (First reporter) and an individual with BMS, CS or FRCPath clinical registration (Second reporter) and reported via the accredited reporting laboratory.



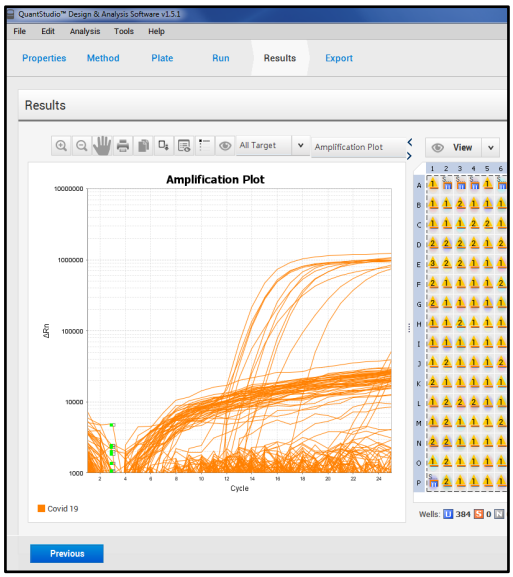
Equipment

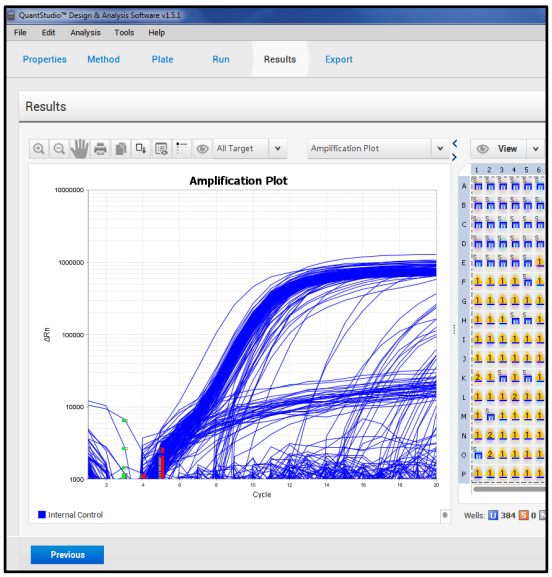
- Access to Windows PC with QuantStudio Software
- First Reporter Analysis
- The first checker would export the run result (EDS file) to a shared drive into a primary review folder. The file would have a unique name that identifies the run and could be linked to the sample auditing in the research laboratory internal LIMS.

First reporter/checker should:



- access the run in Quantstudio 1.4 →
- Ensure threshold is set to automatic.
- Check the run as a whole on the amplification plot.





- If the run shows a lack of typical PCR amplification across the plate (whole plate failure), reject the run and repeat set up.

    - **For Covid 19 plate runs:**
      Ensure positive control is 'positive' for **Covid 19** and the negative controls are 'negative' for **Covid 19** signal:
        - **Negative controls (no template control, NTC):** 1) Ct = undetermined or 2) Ct > 23 with Tm < 84 or > 86 in SYBR channel. There are two negative controls – one with viral inactivation L6 buffer that has been through the inactivation process (*96 well plate format*: F12, *384 well plate format*: N24) and one NTC (*96 well plate format*: G12, *384 well plate format*: O24).
        - **Positive control:** 1) standard curve in SYBR channel shows exponential amplification, 2) Ct < 25, and 3) the melt curve using the derivative reporter displays a single peak that has a melting temperature: 84 ≤ Tm ≤ 86 (*96 well plate format*: H12, *384 well plate format*: P24).

    - **For Internal Control plate runs:**
      Ensure positive control is 'positive' for **Internal Control** and the negative controls are 'negative' for **Internal Control** signal:
        - **Negative controls (no template control, NTC):** 1) Ct = undetermined or 2) Ct > 18 with Tm < 88 or > 91 in SYBR channel. There are two negative controls – one with viral inactivation L6 buffer that has been through the inactivation process (*96 well plate format*: F12, *384 well plate format*: N24) and one NTC (*96 well plate format*: G12, *384 well plate format*: O24).
        - **Positive control:** 1) standard curve in SYBR channel shows exponential amplification, 2) Ct < 20, and 3) the melt curve using the derivative reporter displays a single peak (a slight shoulder appears on the left, but should not appear as a second distinct peak) that has a melting temperature: 88 ≤ Tm ≤ 91 (*96 well plate format*: H12, *384 well plate format*: P24).

- Above requirements should be met on the individual plate otherwise the entire plate is invalid. If entire plate has failed and needs to be retested, go back to stored RNA and repeat RT-PCR.

- The first reporter should then click on properties tab and put their name in the 'user' free text box for sample audit purposes. The text box to the right of the properties tab can be used to include any additional information that needs to be communicated to the second reporter. The exported eds is then saved on an internal drive to be uploaded to the online portal for the second checker.

- The first reporter will also extract plate genealogy data from the research laboratory internal LIMS system allowing them to document the history of a plate so the stages and operators involved. This will support their sign off decision.

  Example of sample audit trail:

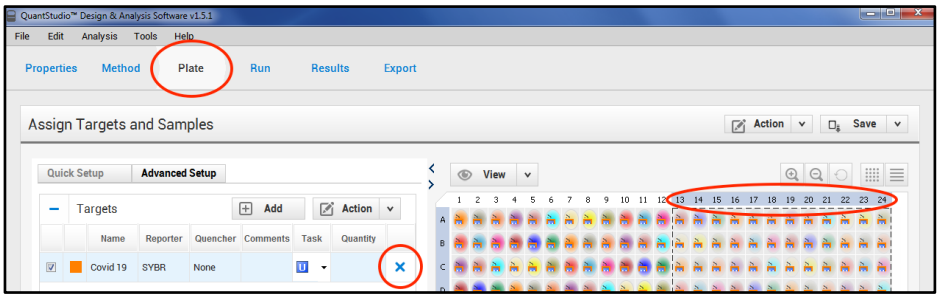
Sample information for plate CFH0PID8

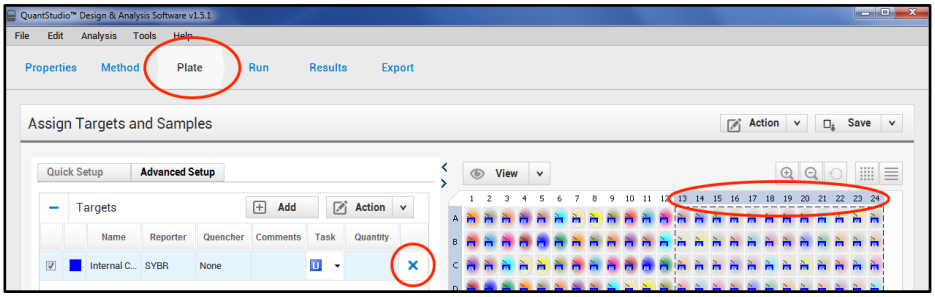
Sample barcode	Well location	RT-qPCR plate barcode	RT-qPCR operator	RT-qPCR date	RNA extraction plate barcode	RNA extraction operator	RNA extraction date	Sample consolidation plate barode	Sample consolidation operator	Sam cons date
21U325622	G:1	CFH0PID8	test	2020-03-29	SPL00005	Laura Cubitt	2020-03-29	LPL00505	LC	202(
50U080371	D:3	CFH0PID8	test	2020-03-29	SPL00005	Laura Cubitt	2020-03-29	LPL00505	LC	202(
21U325621	A:2	CFH0PID8	test	2020-	SPL00005	Laura	2020-03-	LPL00505	LC	202(

- First reporters would then place the eds file of any runs that passed the first check in a location where they would then be uploaded to the online portal and become available for external clinically registered second reporters to access as shown in the screenshot below

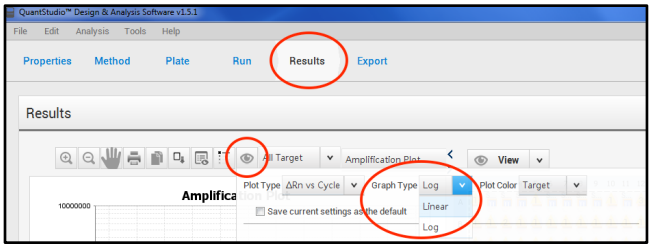
Second Reporter Analysis

Once the second reporter has downloaded the eds file from the online portal they would enter the password to allow for customisation of the eds file. The eds file will open to the results page in log format. If the plate to be analysed is only half full, to remove any of the wells that are not to be analysed go to the plate screen and select these wells and deselect the targets.

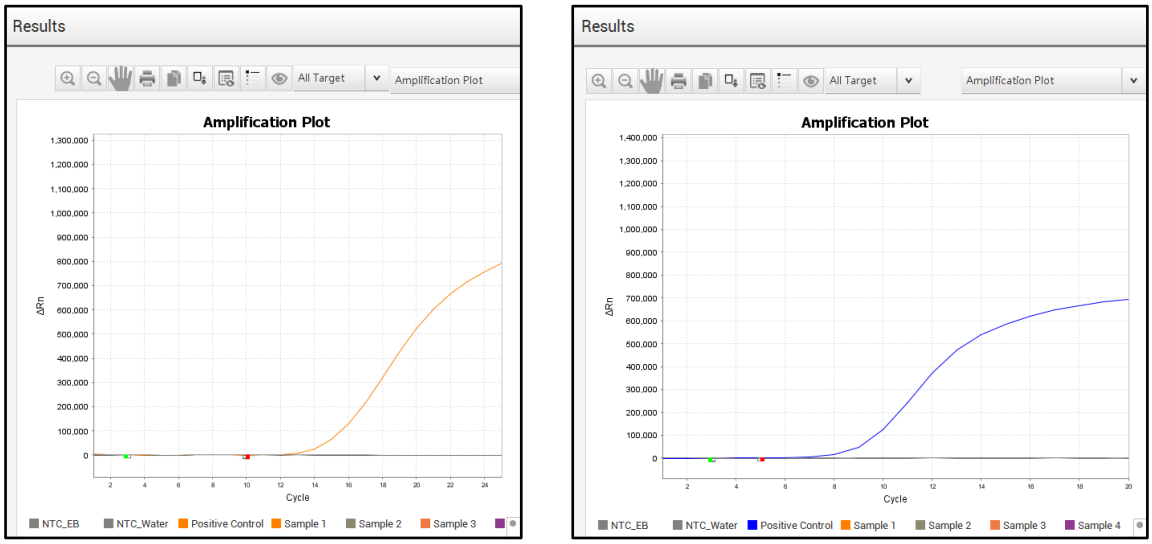




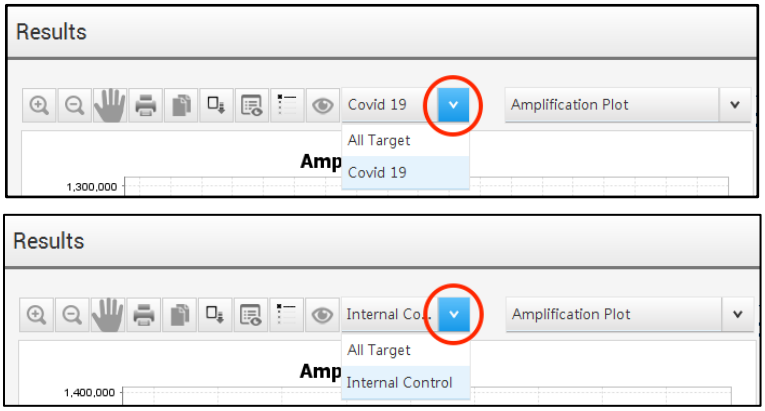
Curve visualisation: To visualise S curves back on the results page change to a linear graph by clicking the eye symbol and change this under graph type. Then press ANALYSE button, top right.



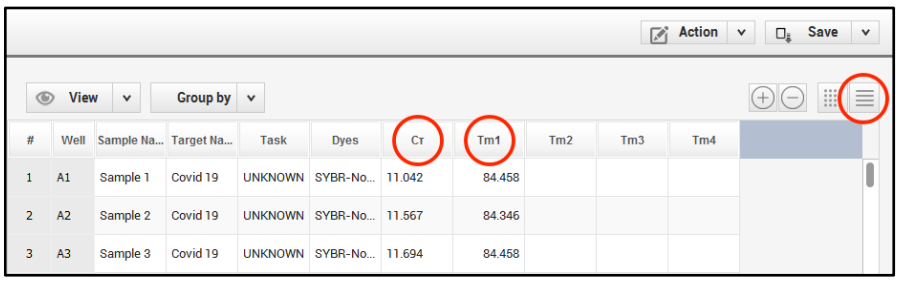
The controls from a **Covid 19** RT-LAMP run (left) and **Internal Control** RT-LAMP run (right) are shown below. The positive control for the Covid 19 graph is shown with an orange line and the positive control for the Internal Control graph is shown with a blue line. The negative controls for both plate runs are visualised with a gray line below for reference. To visualise the curves for individual samples click on their well on the plate layout.

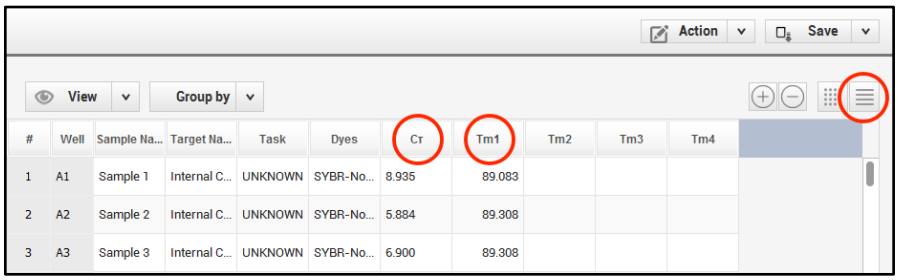


Targets: To visualise just one target at a time, either **Covid 19** or **Internal Control**, this can be done under the plot settings next to the eye symbol (red circle).

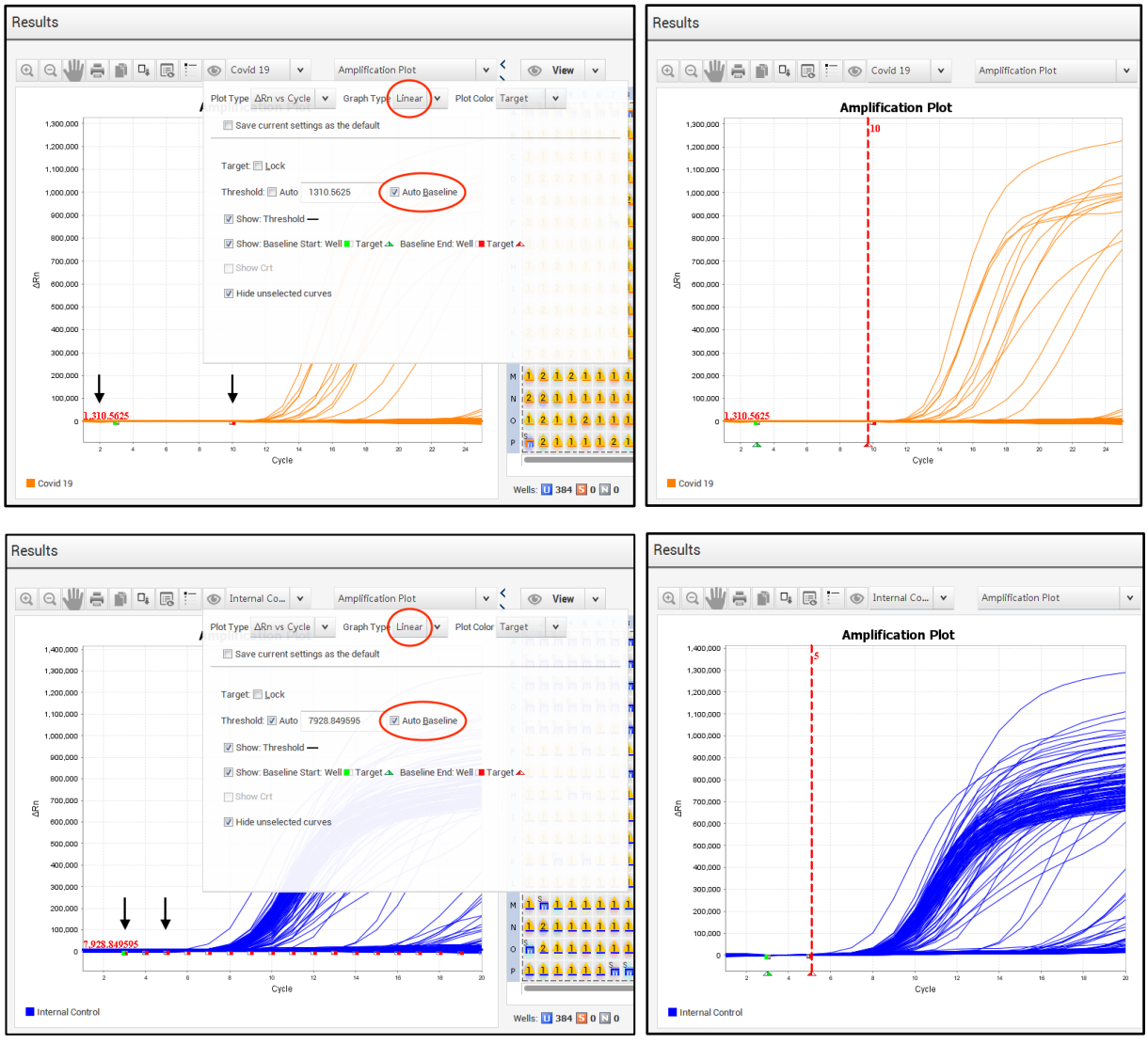


Ct and Tm (melting temperature) visualisation: For an easier view of the Ct and Tm produced by each curve use the list screen rather than the 96 or 384 well screen on the right hand side (button and Ct/Tm1 columns circled).



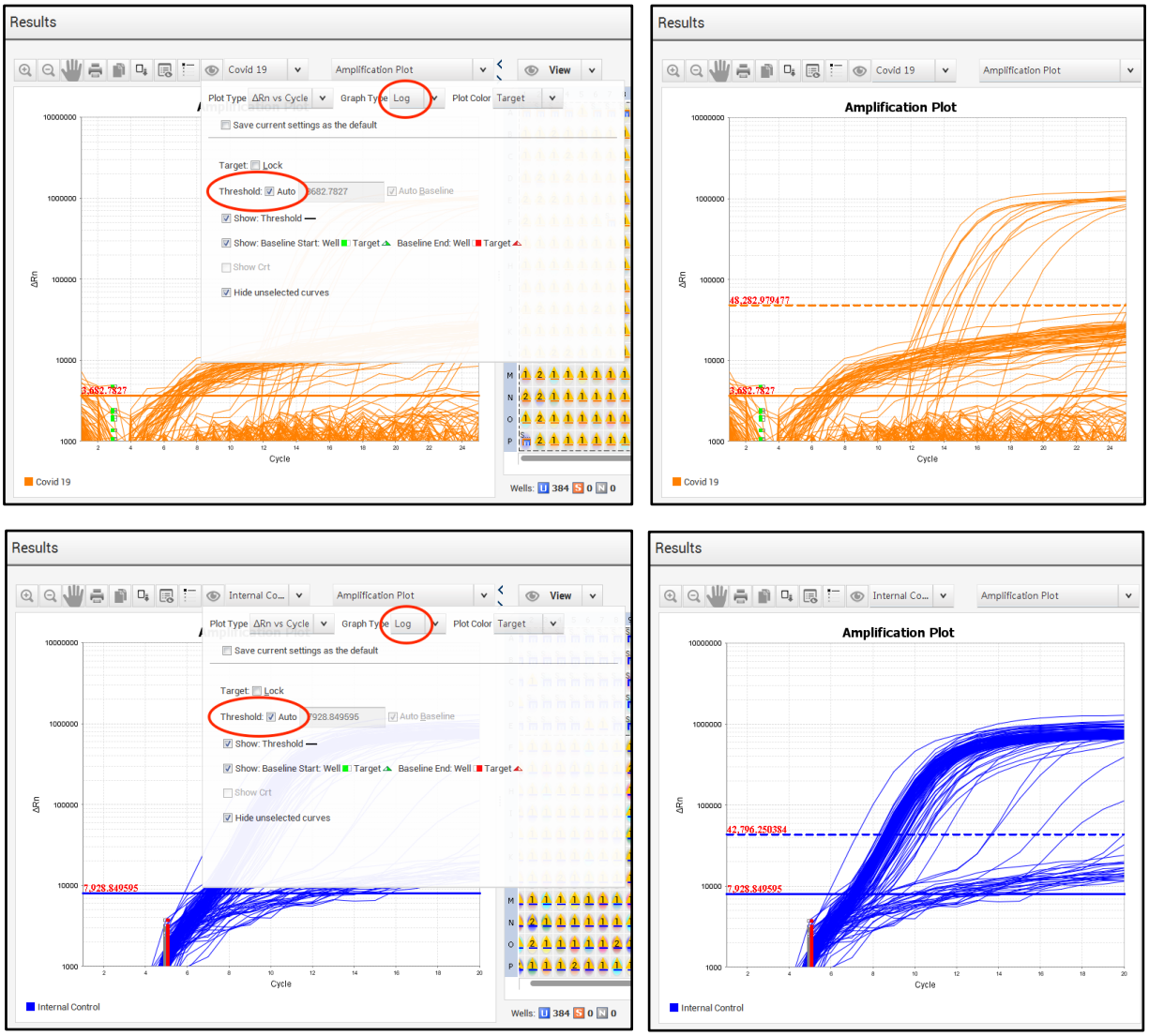


Manual baseline and threshold: The baseline should be set between **3 to 10 cycles** for **Covid 19** RT-LAMP assay runs and **3 to 6 cycles** for **Internal Control** RT-LAMP assay runs, however it may be manually adjusted to reflect the amplification on the plate. Click on the eye symbol and untick the auto baseline box (circled, left panel) then drag the baseline end to just before the first true amplification on the plate (dotted line, right panel). Click the blue Analyse button for the Ct values to be reanalysed once this has been moved. This is best done in the linear graph. A **Covid 19** run is displayed with orange lines and an **Internal Control** run is depicted with blue lines.

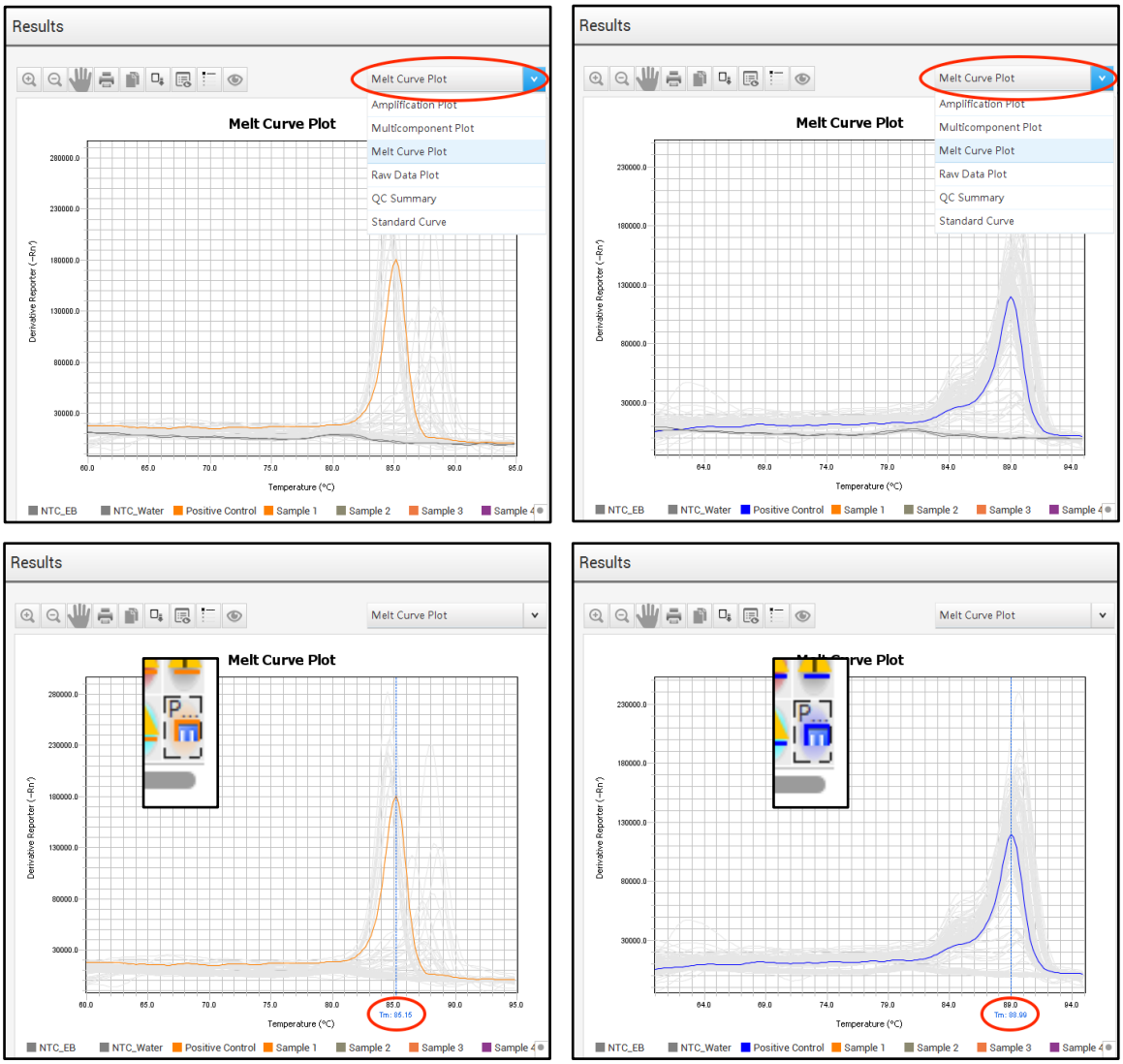


Once the baseline has been adjusted the result analysis can be done using manual thresholding to remove any noise or abnormal curves that could be interfering with the appropriate Ct being given for each sample. In some cases the automatic thresholding will have called all results appropriately and the threshold can be manually set to the same level.

Click the eye symbol and untick the auto threshold box (circled, left panels). The threshold line will then become draggable by mouse (dotted line, right panels) and can be raised to a level above any “noise” on the plate. The **Covid 19** threshold is usually set around 50,000. The **Internal Control** threshold is usually set around 50,000. Click the blue [Analyse] button for the Ct values to be reanalysed once this has been moved. This is best done in the log scale graph.



Melt curves: The melt curve can be displayed by clicking on the arrow next to amplification plot (circled, top plots) and selecting melt curve. Select the positive control well on the plate layout (insert, bottom plots) and the Tm will be displayed on the graph (circled, bottom plots)



Result Anaylsis

The second reporter should again check that the controls meet these criteria:

- **For Covid 19 plate runs:**
  Ensure positive control is 'positive' for **Covid 19** and the negative controls are 'negative' for **Covid 19** signal:
  - **Negative controls (no template control, NTC):** 1) Ct = undetermined or 2) Ct > 23 with Tm < 84 or > 86 in SYBR channel. There are two negative controls – one with viral inactivation L6 buffer that has been through the inactivation process (*96 well plate format*: F12, *384 well plate format*: N24) and one NTC (*96 well plate format*: G12, *384 well plate format*: O24).
  - **Positive control:** 1) standard curve in SYBR channel shows exponential amplification, 2) Ct < 25, and 3) the melt curve using the derivative reporter displays a single peak that has a melting temperature: 84 ≤ Tm ≤ 86 (*96 well plate format*: H12, *384 well plate format*: P24).

- **For Internal Control plate runs:**
  Ensure positive control is 'positive' for **Internal Control** and the negative controls are 'negative' for **Internal Control** signal:
  - **Negative controls (no template control, NTC):** 1) Ct = undetermined or 2) Ct > 18 with Tm < 88 or > 91 in SYBR channel. There are two negative controls – one with viral inactivation L6 buffer that has been through the inactivation process (*96 well plate format*: F12, *384 well plate format*: N24) and one NTC (*96 well plate format*: G12, *384 well plate format*: O24).
  - **Positive control:** 1) standard curve in SYBR channel shows exponential amplification, 2) Ct < 20, and 3) the melt curve using the derivative reporter displays a single peak (a slight shoulder appears on the left, but should not appear as a second distinct peak) that has a melting temperature: 88 ≤ Tm ≤ 91 (*96 well plate format*: H12, *384 well plate format*: P24).

Once the above criteria are met and the sample audit trail visualised by the second reporter, they would save the modified eds file and export the data from QuantStudio into an xls file. They would then upload the eds file and xls files to the online portal which releases results to the clinically accredited laboratory's LIMS. The second reporter would then be returned to the Referrals list on the online portal where any other reports awaiting validation would be available.

The automated outcome will be calculated by the logic in the online system that will take into account the Ct, Tm1 (1º melting temperature), and MTP (multiple Tm peaks) values. Any samples that have have an internal control failure will be failed. Once the results for the **Covid 19** and **Internal Control** runs have been uploaded the following outcomes will be possible:

Result Outcome	Covid 19			Internal Control		
	CT	**Tm1**	**MTP**	**CT**	**Tm1**	**MTP**
Positive	CT < 25	84 ≤ Tm ≤ 86	N	CT < 20	88 ≤ Tm ≤ 91	N
Negative	CT = UD	Any Tm	Y/N	CT < 20	88 ≤ Tm ≤ 91	N
	Any CT	< 84 or > 86	N			
	Any CT	Any Tm	Y			
Fail / Retest	CT = UD	Any Tm	Y/N	CT = UD	Any Tm	Y/N
				Any CT	< 88 or > 91	Y/N
				Any CT	Any Tm	Y
	Any CT	< 84 or > 86	Y/N	CT = UD	Any Tm	Y/N
				Any CT	< 88 or > 91	Y/N
				Any CT	Any Tm	Y
	Any CT	Any Tm	Y	CT = UD	Any Tm	Y/N
				Any CT	< 88 or > 91	Y/N
				Any CT	Any Tm	Y

Result File Structure

The result file is the Excel output from the QuantStudio. It has 5 tabs:

Sheet #	Sheet Name	Description
1	Sample Setup	Information about the specimens contained in each well, and the run setup.
2	Amplification Data	Raw data of the well curve X/Y coordinates.
3	Results	The main sheet containing the well numbers, sample code and result values (CT, Tm1, and MTP). This will be used for import to Winpath.
4	Melt Curve Raw Data	Raw data of the melt curve X/Y coordinates
5	Melt Curve Result	Raw data of the Tm and melting peak height

The results tab has a table starting on row 49 with the results. The key columns for import are:

Column position	Column header	Description
D	Sample Name	The lab number associated with the sample.
E	Target Name	The assay processed. This needs to be mapped to the result code in Winpath as part of the interface.
I	CT	The quantitative result. This will either be a number or 'Undetermined'. This will interface to an internal line in Winpath. Winpath rules will then generate the final result.
AA	MTP	Y or N value assigned based on the presence or absence of multiple Tm peaks
AC	Tm1	The primary melting temperature peak value
Y	Comment	A free-text comment that can be applied in QuantStudio to a single well. Used for manual overrides. This will be "INVALID" for sample failure.

Other notes:

- The files should have a unique name
- If a run is exported with failed run controls by mistake the interface should prevent it being transmitted to the reporting laboratory LIMS.
- The reporting laboratory LIMS rules will look for technical failures and prevent reporting if an invalid result is identified.