Technical Note
Plus-Minus Scoring Analysis

General Introduction to Data Analysis

The aim of this Technical Note is to explain the principle of plus-minus scoring analysis and to guide you through the experimental set up and analysis of data. It begins with a general introduction to data analysis.

Results Editor

The starting point for data analysis is the Results Editor. Before an analysis method has been set, the Results Editor home screen will display the plate layout and thermal cycling program. Each stage where readings have been made will have its own tab showing a graph of raw data.

Selecting the Analysis Method

The Analysis Selection box on the Results Editor home screen allows the user to define the method of analysis to be applied to the readings gathered during the PCR run. Highlighting a stage name and pressing the Edit button will launch the Analysis Wizard Selection screen and allow an Analysis Method to be assigned for that stage.

The Analysis Wizards

Once an analysis method has been defined, a series of default settings will automatically analyse the data. The defaults can be viewed in the Analysis Wizards and edited if required. The intuitive Analysis Wizards explain in detail the mathematics behind the analysis settings and will lead you through each stage of the analysis setup. With experience, analysis can be quickly performed using the Analysis Properties displayed in the Parameters (PAR) box feature found on each data graph.
Analysis Method Options

The following analysis options are available in Quansoft:

**Baseline**
This simple analysis method allows for correction of differences in background fluorescence. It is also incorporated into many of the other analysis methods.

**Quantification**
Quantification analysis is used to determine the absolute or relative quantity of a target DNA template in a given test sample by measuring the cycle-to-cycle change in the fluorescent signal. The fluorescent signal increases proportionately to the amount of amplified DNA and quantification is performed either by comparison of the fluorescence of a PCR product of unknown concentration with that of several dilutions of an external standard, or by comparing the fluorescence of one product relative to another. To be able to make this comparison, the fluorophore is measured at a point in the amplification where the reaction efficiency can be considered optimal. This is generally around the cycle at which an increase in fluorescence is first detected.

**Dissociation curve**
Dissociation curve analysis can add to the information obtained from the PCR. Also known as melting curve analysis, it measures the temperature at which the DNA strands separate into single strands. This provides a measurement of the melting temperature or Tm, taken as the point at which 50% of the double stranded DNA (dsDNA) molecules are dissociated. Using the easy-to-program ‘ramp’ function, PrimeQ will perform a thermal ramping program that can be used to determine the Tm of the PCR product. This analysis provides the user with extra confidence in experiments using intercalating dye chemistry for identifying amplification of non-specific products or contamination.

**Plus-minus scoring**
This analysis exploits PrimeQ’s fluorescence technology to determine with ease and accuracy the presence or absence of a PCR product in any given sample. Input data can either be kinetic (where readings are taken throughout the amplification stage) or end-point (readings taken at the end of the run). The software scores the samples as positive or negative according to user-defined thresholds.

**Allelic discrimination**
Users of PrimeQ have the option of this powerful technique capable of detecting single nucleotide differences (SNPs). It can be used to discriminate between genotypes, mutations and polymorphisms within or between samples simply by comparing the fluorescence signal obtained using allele-specific, dye-labeled probes.

**Multi-read**
This is a simple end point analysis method which will report the average fluorescence of a selected number of readings. It is useful for assays other than PCR, for example fluorescence-based DNA assays, where just the fluorescence of a sample needs to be measured; in this way PrimeQ can be used as a simple fluorescence plate reader or fluorimeter.
Analysis method: Plus-Minus Scoring

This analysis method is used to record the presence or absence of a PCR product; it is therefore a qualitative rather than a quantitative analysis method. The input data for the analysis is usually the end-point fluorescence (a few readings taken at the end of the run – at least two are required). Since it is not strictly necessary to gather fluorescence data during the amplification cycles, the amplification can be performed in a non-real time thermal cycler as long as the reaction contains an appropriate fluorescence detection system for the target. The plate can then be read a few times in PrimeQ to generate the fluorescence data required for the analysis. Note that consumables appropriate for use in PrimeQ must be used.

Plus-minus assays can be performed with or without an internal positive control (IPC), although its use can prove helpful to ensure that a failed PCR is not mistaken for a negative result. A no template control (NTC) is used to set two confidence thresholds above which all unknowns will be scored as positive and below which they will be scored as negative. Samples between the two threshold values are scored as undetermined. With no user input, Quansoft will automatically analyse the raw data (or baseline corrected data) to measure the difference in fluorescence between the unknown samples and NTC.

Setting up and running the experiment

If the entire run is to be performed on PrimeQ, set up the thermal cycling profile in the usual way. You may or may not wish to collect fluorescence data at each cycle to view the progress of the amplification; however you will need to use fluorescence data from a few cycles at the end of the run for data analysis. This may be the last few cycles of the amplification stage, or a separate stage may be programmed for this purpose. Examples of programs are given in Figure 3 below.

![Figure 3: Example programs for plus-minus assays.](image)

- **(A)** A standard two-step program with reads for both the target and IPC throughout the amplification stage. Analysis would be performed on the data from the final few cycles.
- **(B)** As A, but with an additional stage at the end of the amplification stage with a few reads for the plus-minus analysis.
- **(C)** No readings are taken during amplification but an additional stage is included at the end with a few reads for the plus-minus analysis.

If multiplexing is possible, an IPC can be amplified simultaneously with the target. This will highlight any samples which fail to amplify due to inhibition of the reaction. The IPC can be a target known to be present in all the test samples or can be a target “spiked” into the master mix. It is good practice to also include both positive and negative controls as well as a NTC.

During the run, the real-time collection of data can be monitored on the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed. When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.
**Plus-Minus Scoring Analysis Wizard setup**

Once the PCR has completed, open the results file and from the Results Editor home screen set up the plus-minus scoring analysis as described below.

- In the Analysis Selection box, highlight the stage name for analysis to be applied and click **Edit**. The Analysis Wizard will launch.
- Select **Plus-Minus Scoring** from the drop-down menu in the Analysis Method selection box.
- Assign a dye usage for each of the reads (e.g. Reporter, IPC etc.) and click **Next**. The plus-minus scoring Analysis Wizard will launch.
- If a PRD was assigned in the dye usage menu, the next screen will offer the option of PRD correction. Click **Next**.

**Baseline Correction**

Baseline correction is only appropriate if fluorescence data has been collected throughout the amplification stage. In this case, follow the directions in the Operator’s Manual in section 4.5. If a separate stage has been programmed for collecting the fluorescence data for plus-minus scoring analysis, set baseline correction to **None** then click on **Next** to progress to the next screen.

**Plus-Minus Analysis Method**

The next screen allows you to choose which readings are to be used for results scoring. Select the appropriate data points then click **Next**.
Threshold Settings
Clicking **Next** leads through to the **Threshold Settings** screen. In plus-minus scoring, a no template control (NTC) is used to set confidence thresholds above which all unknowns are scored as positive and below which as negative. Samples between the two threshold values are scored as undetermined. This screen provides options for these threshold settings.

![Threshold Settings Screen](image)

**Figure 6: Threshold Settings.**

Set the threshold range using the drop down menus.

The confidence threshold default settings are:
Upper threshold: Average of NTC samples + 6 SD
Lower threshold: Average of NTC samples + 3 SD

Report Options and Summary
The Report Options screen allows you to select data to appear in a printable report. The selection can be changed from the Report tab in the Results Editor if necessary. The Summary screen provides a quick checklist of the parameters set up in the Analysis Wizard.

- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the set up.

Viewing the analysed results
Click on the results tab for the stage which has been set up for plus-minus scoring analysis. The analysed data will be displayed as a series of graphs similar to those shown in **Figure 7** below. Individual graphs can be closed or enlarged for easier viewing. Clicking an individual well or a selection of wells in the plate layout will highlight just the selected well(s) on the graphs. Clicking the **Show All Wells** button will re-select all wells.

![Results Editor Screen](image)

**Figure 7: The layout of data in the Results Editor screen for plus-minus scoring analysis.**

Dye to view options
Table of results
Baseline correction graph
Plate layout with individual well view of fluorescent data
Plus minus graph
The Plus-Minus Graph

The plus-minus graph shows a plot of the averaged fluorescence data of the range selected relative to the fluorescence of the NTC on a percentage scale. The upper and lower thresholds (red and blue respectively) are also displayed in their default positions. The position of the thresholds can be moved by dragging with the mouse or adjusting the value in the Analysis Properties accessed via the PAR button at the top of the graph.

Changing the position of either threshold will update the results and scores given in the Results Table.

The Results Table

Each sample is categorized in the results table as positive, negative or undetermined for each reporter (including the IPC) based on the following criteria:

(+): Positive: sample value is greater than the upper threshold.
(-): Negative: sample value is less than the lower threshold.
(?): Undetermined: sample value is between the lower and upper thresholds.

A score is also given if one of the dyes is designated an IPC (when dye usages are assigned). In this case, the following criteria are used to score the sample:

<table>
<thead>
<tr>
<th>IPC</th>
<th>Target</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Retest</td>
</tr>
<tr>
<td>+</td>
<td>?</td>
<td>Retest</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>Positive</td>
</tr>
</tbody>
</table>

In the example shown in Figure 9 below, a FAM™ labelled probe was used to detect the IPC and Yakima Yellow® to detect the target. Ideally the IPC should appear positive in each well unless there is a fault with the PCR e.g. inhibitor present.
**T08-004A: Plus-Minus Scoring Analysis**

### Viewing and Changing the Parameters

The analysis parameters as set up in the plus-minus scoring Wizard can be changed at any time and can be easily accessed via the PAR box on the plus-minus graph.

#### Figure 9: Results Table.

Results and scores for triplicate tests on three different samples. One of the replicates for Unknown 5 shows an undetermined result whilst the other two replicates tested negative.

<table>
<thead>
<tr>
<th>No</th>
<th>Well ID</th>
<th>Sample ID</th>
<th>FAM</th>
<th>Yakima Yellow</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>B10</td>
<td>Unknown 5</td>
<td>+</td>
<td>?</td>
<td>Retest</td>
</tr>
<tr>
<td>5</td>
<td>B11</td>
<td>Unknown 5</td>
<td>+</td>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>B12</td>
<td>Unknown 5</td>
<td>+</td>
<td>-</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>C1</td>
<td>Unknown 6</td>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>C2</td>
<td>Unknown 6</td>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>C3</td>
<td>Unknown 6</td>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>C4</td>
<td>Unknown 7</td>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>C5</td>
<td>Unknown 7</td>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>C6</td>
<td>Unknown 7</td>
<td>+</td>
<td>+</td>
<td>Positive</td>
</tr>
</tbody>
</table>

#### Figure 10: Analysis Properties.

Click the PAR button next to one of the graphs to bring up the analysis settings for plus-minus scoring. If any settings are changed, the data will be recalculated and the graphs and results table updated accordingly.

**PrimeQ Report**

If a printed report of the analysed data is required, open the Report tab of the Results Editor to view the plus-minus scoring analysis report. To change any of the report contents, click on the Report Options icon to open up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click Done to finish. The report can be printed or saved as a PDF file for future reference.

**Saving**

Saving will overwrite any previous analysis, therefore to preserve a particular analysis set up, click on File followed by Save As... to save as a different file name.

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