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IX.G. Saving and Printing the Analysis File

1. The Plexor™ Analysis Software saves the analysis as an *.aan file. The current analysis can be saved at any time by selecting "Save Analysis File (.aan)" in the "File" menu.
2. Selected wells can be exported into a new analysis file. In the "File" menu, select "Export Selected Wells as New Analysis File (*.aan)".
3. The analysis screen can be printed or saved as a screenshot. In the "File" menu, select "Save a Screenshot (.png)" or "Print a Screenshot".
4. A Run Template and Analysis Template from an existing analysis can be exported and used in future analyses (Section XI.C).

X. Troubleshooting

Symptoms	Causes and Comments
Flat amplification curve in the amplification curves window (no apparent amplification)	Be sure that the reactions were assembled correctly. See the Technical Manual supplied with the Plexor™ Systems.
	Template was degraded or of insufficient quantity. Verify the integrity of the DNA or RNA template by electrophoresis. Repeat the DNA or RNA purification if necessary. Add RNasin® Ribonuclease Inhibitor to the reaction to inhibit a broad spectrum of RNases.
	Amplification inhibitor is present in the DNA or RNA template. Reduce the volume of template in the reaction. Repeat the DNA or RNA purification if necessary. Add the template in question to the positive control reaction. A significant increase in the C_t value or no amplification in the positive control reaction indicates the presence of inhibitors in the template.
	Thermal cycler was programmed incorrectly. Verify cycle times and temperatures (Section IV, V or VI).
	Data collection settings were incorrect. Data collection must occur during the extension step. The extension time must be sufficient for data collection. Verify the data collection settings.
	The wrong dye or detector was selected, or the dye was incompatible with the instrument. Be sure the selected detectors are appropriate for the fluorescent dyes used.
	The Plexor™ Master Mix may have lost activity. Be sure to store the Plexor™ qPCR and qRT-PCR Systems at –20°C to avoid loss of enzyme activity. Confirm the instrument settings and perform a positive control reaction to determine if there is a problem with the Plexor™ System reagents.
	The primer sequence is incorrect. Verify the primer sequence.
	Poor primer design. Redesign primers, targeting a different region of the gene of interest. We strongly recommend using the Plexor™ Primer Design Software, which is available at: **www.promega.com/plexorresources/**
	Primers may have been synthesized incorrectly. Resynthesize primers.

Symptoms	Causes and Comments
Flat amplification curve in the amplification curves window (no apparent amplification) (continued)	Primer was degraded. Use MOPS/EDTA Buffer to resuspend and dilute primers. Iso-dC-containing primers are sensitive to pH. Rehydrating or storing the primer in water or a buffer with a pH less than 7.0 will result in primer degradation. Do not use water to resuspend or dilute primers or make primer mixes.
	Primer concentration was incorrect. Verify the primer concentration by measuring the absorbance at 260nm.
	The scale of the Y axis was inappropriate. If the scale of the Y axis was too broad, the change in fluorescence may not be visible. Adjust the scale of the Y axis.
Increasing fluorescence over time	Excessive template was added to the reactions. Dilute the template and re-amplify.
	The baseline region was set in a region with significant fluorescence fluctuation. The baseline within the baseline region should be flat. Manually adjust the baseline region (Section XI.D).
	The baseline region was set too close to the signal change. Manually adjust the baseline region Section XI.D.
Two or more distinct melt curves in the melt curves window	For the Plexor™ qRT-PCR Systems, both RNA and DNA templates can be amplified. Treat the RNA template with DNase to eliminate contaminating genomic DNA.
	Poor primer specificity. Design new primers with higher specificity to the target. To verify primer specificity, perform a BLAST search with the primer sequence. The primer should not exhibit regions of identity with other sequences.
	Optimize the annealing temperature. Increase the annealing temperature by increments of 2°C to reduce the synthesis of primer-dimers or nonspecific amplification products.
	Pseudogenes or polymorphic genes may exist. Design new primers to avoid regions of identity between gene family members.
	Assemble the reactions on ice to minimize the synthesis of primer-dimers or nonspecific amplification product.
	Reduce the number of amplification cycles to minimize the synthesis of primer-dimers or nonspecific amplification product.
	Check for signal bleedthrough. Calibrate the instrument as instructed by the manufacturer for the dye set used.
	Decrease the primer concentration (e.g., 0.1μM).
	Primer pairs in a multiplex reaction can interact to form undesired amplification products. Perform a BLAST search to reveal regions of identity with undesirable target sequences. Label the primer with the lowest homology to other sequences. Alternatively, design new primers using the Plexor™ Primer Design Software, which is available at: **www.promega.com/plexorresources/**

X. Troubleshooting (continued)

Symptoms	Causes and Comments
Broad melt curve or a shoulder on the melt curve	Pseudogenes and polymorphic genes may exist. Perform a BLAST search of the target sequence. When designing primers, choose target sequences that have the fewest regions of identity with pseudogenes and polymorphic genes.
	Check for signal bleedthrough. Calibrate the instrument as instructed by the manufacturer for the dye set used.
	Decrease the primer concentration (e.g., 0.1µM).
	Be sure the thermal cycler is programmed correctly (Section IV, V or VI).
No melt curve observed in the melt curve window	Poor amplification. See causes and comments for "Flat amplification curve in the amplification curves window (no apparent amplification)" above.
	Problems with data export or instrument analysis have occurred. Review the instructions for data export and instrument setup.
	Data collection settings were incorrect. Verify the thermal cycling program and data collection settings are correct (Section IV, V or VI).
	Incorrect files were imported. Be sure to import the proper files containing related amplification data and dissociation data.
	Instrument was programmed incorrectly. Verify the thermal cycling program is correct (Section IV, V or VI).
Variability in signal among replicate samples	Calibrate your pipettes to minimize variability in pipetting.
	Small volumes are difficult to pipet accurately. Do not pipet volumes <1µl; dilute the template, so larger volumes are pipetted.
	Some variation is normal. A difference of 1–2 cycles for the C_t values is within the normal variation associated with an exponential amplification reaction.
	There will be statistical variation in the amount of template in a reaction with targets present at low copy number. Poisson distribution predicts difficulty associated with reliable detection of very dilute samples with few target molecules.
	Mixing was inadequate. Vortex reagents to mix well prior to pipetting.
	Use capillaries recommended by the instrument manufacturer.
	Instrument was improperly calibrated. Calibrate instrument as instructed by the manufacturer.
	Thermal cycling conditions were suboptimal. Optimize the annealing temperature.
	Thermal cycling conditions were suboptimal. Redesign your primers, so the melting temperatures are 60°C. We strongly encourage using the Plexor™ Primer Design Software.
	Viscous samples (e.g., high-molecular-weight genomic DNA) are difficult to pipet accurately. Dilute the DNA template. Shear high-molecular-weight DNA by vortexing or pipetting.

Symptoms	Causes and Comments
Variability in signal among replicate samples (continued)	The baseline region was not set correctly. The baseline should be flat. The baseline region can be adjusted manually for each well to account for sample-to-sample variation (Section X.ID).
	Be sure the capillaries are properly sealed to avoid evaporation.
Fluorescence decrease observed in the no-template control	Nonspecific product can accumulate at higher cycle number in reactions with targets present at low copy numbers. Assemble the reactions on ice to reduce the accumulation of nonspecific amplification products.
	Decrease the cycle number to reduce the accumulation of nonspecific amplification products.
	Design new primers using the Plexor™ Primer Design Software.
	Reactions were contaminated with target DNA or RNA. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Take precautions to prevent contamination (see the *Plexor™ qPCR System Technical Manual* #TM262, the *Plexor™ One-Step qRT-PCR System Technical Manual* #TM263 or the *Plexor™ Two-Step qRT-PCR System Technical Manual* #TM264).
	An improperly calibrated instrument can lead to erratic fluorescence readings. Calibrate the instrument as instructed by the manufacturer.
Vertical fluorescence spikes or significant "noise" in the amplification curve	Consult the instrument manufacturer's user's manual for information about potential instrument problems that can cause spikes or noise.
	No amplification or poor amplification for the entire run. Poor amplification can lead to improper data scaling, making the fluorescence measurements appear erratic. See possible causes and comments for "Flat amplification curve in the amplification curves window (no apparent amplification)" above.
	Instrument was improperly calibrated. Calibrate instrument as instructed by the manufacturer.
Small signal change in amplification curve and melt curves	No amplification or poor amplification. See causes and comments for "Flat amplification curve in the amplification curves window (no apparent amplification)" above.
	Incorrect filter was selected. Verify the presence of the appropriate filter.
	Primer concentration was incorrect. Verify primer concentration by measuring the absorbance at 260nm.
	The scale of the Y axis of the amplification curve was affected by other reactions on the plate. A high fluorescent signal for one or more reactions can cause the scale of the Y axis of the amplification curve to be too high to see changes in some data. Adjust the scale of the Y axis to accommodate samples with smaller changes in fluorescence. See Section IX.D.

X. Troubleshooting (continued)

Symptoms	Causes and Comments
Nonlinear standard curve, low R^2 values	An amplification inhibitor is present in the standard reference template. Determine whether the template contains inhibitors by adding the DNA template to the positive control reaction; a significant increase in the C_t value or no amplification of the positive control in the presence of the DNA template indicates the presence of inhibitors. Repeat purification of the standard reference template used to generate the standard curve.
	Calibrate your pipettes to minimize variability in pipetting.
	Small volumes are difficult to pipet accurately. Do not pipet volumes <1µl; dilute the template, so larger volumes are pipetted.
	Viscous samples (e.g., high-molecular-weight genomic DNA) are difficult to pipet accurately. Dilute the DNA template. Shear high-molecular-weight DNA by vortexing or pipetting.
	Adjust the baseline region. The baseline region can be manually adjusted for each reaction. See Section XI.D.
	Some variation is normal. Perform duplicate or triplicate reactions for the standard curve to minimize the effect of this variation.
	There will be statistical variation in the amount of template in a reaction with targets present at low copy number. Perform duplicate or triplicate reactions for the standard curve.
	An error was made during dilution of the standard reference template. Verify all calculations, and repeat dilution of the standard reference template. Do not pipet volumes <1µl.
	Incorrect concentration values were entered in the Plexor™ Analysis Software. Verify the concentrations for all samples used to generate the standard curve.
	Reactions were contaminated with target DNA or RNA. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Take precautions to prevent contamination (see the *Plexor™ qPCR System Technical Manual* #TM262, the *Plexor™ One-Step qRT-PCR System Technical Manual* #TM263 or the *Plexor™ Two-Step qRT-PCR System Technical Manual* #TM264).
	Carefully seal the capillaries to avoid evaporation.
	Aberrant fluorescence can be caused by writing on capillaries, contamination, fingerprints, etc. Do not write on the capillary. Use caution when handling capillaries. Wear gloves. Do not place capillaries on surfaces that might be contaminated with a fluorescent material. If you suspect contamination of the benchtop, thermal cycler block or any other area, clean it thoroughly.

Symptoms	Causes and Comments
Slope less than 0.2 (inefficient amplification)	No amplification or poor amplification. See causes and comments for "Flat amplification curve in the amplification curves window (no apparent amplification)" above.
	Nonspecific amplification can become a problem in later amplification cycles with samples containing small amounts of target template. Decrease the number of amplification cycles.
	Poor primer design. Design new primers.
	Annealing temperature was too high. Design new primers with melting temperatures of 60°C. We strongly recommend using the Plexor™ Primer Design Software.
	Annealing temperature was too high. Optimize the annealing temperature.
Amplification in no-reverse transcription control for the Plexor™ qRT-PCR Systems	Contaminating DNA sequences related to the RNA template are present in the RNA preparation. Treat the RNA template with DNase to remove contaminating DNA.
	Design new primers to span introns to avoid amplification of contaminating genomic DNA.
	Nonspecific amplification occurring in reactions that contain a low number of copies of the template. Assemble reactions on ice.
	Decrease the number of amplification cycles to reduce accumulation of nonspecific amplification products.
	Design new primers to minimize the synthesis of nonspecific amplification products.
	Reactions were contaminated with target DNA or RNA. Clean pipettes and workstations with a mild bleach solution before and after use. Use new reagents and solutions. Use positive-displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettes for pre- and postamplification. Wear gloves and change them often.
No amplification in the positive control reaction	No amplification or poor amplification. See causes and comments for "Flat amplification curve in the amplification curves window (no apparent amplification)" above.
	Verify that the thermal cycling program and data collection settings were correct (Section IV, V or VI).
	Instrument setup problems can cause amplifications to fail. Consult the instrument manufacturer's user's manual for more information about potential instrument problems.
	The Plexor™ Master Mix may have lost activity. Be sure to store the Plexor™ qPCR and qRT-PCR Systems at –20°C to avoid loss of enzyme activity. Confirm the instrument settings, and perform a positive control reaction to determine if there is a problem with the Plexor™ System reagents.
	The RNA template used in the Plexor™ qRT-PCR System was contaminated with ribonuclease (RNase). Take precautions to prevent RNase contamination. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions.

X. Troubleshooting (continued)

Symptoms	Causes and Comments
No amplification in the positive control reaction (continued)	The RNA template used in the Plexor™ qRT-PCR Systems was degraded. RNA storage conditions are very important. Store RNA template at –70°C in single-use aliquots to minimize the number of freeze-thaw cycles. Once thawed, keep RNA on ice.
	Always use nuclease-free, commercially autoclaved reaction tubes, sterile aerosol-resistant tips and gloves to minimize RNase contamination.
	Reactions were assembled incorrectly. Repeat the experiment and assemble reactions as described in the *Plexor™ qPCR System Technical Manual* #TM262, the *Plexor™ One-Step qRT-PCR System Technical Manual* #TM263 or the *Plexor™ Two-Step qRT-PCR System Technical Manual* #TM264.
Unable to import data. An error like "Expecting NEWLINE, found''" or "Unexpected Token Error" is encountered	The data has been altered after export from the real-time PCR instrument software. Any alteration of this data is likely to change the formatting and can cause import errors. Do not open the exported files with other software programs.
Data display in the Plexor™ Analysis Software appears abnormal (the screen appears compressed, lines are replaced with dots, etc.)	Be sure that the display settings for the computer are set to 32-bit color, rather than 16-bit color, when using the Plexor™ Analysis Software.
Genotyping: Miscalled known heterozygous samples: Product formed with only one of the two genotyping primers	Poor primer design. Redesign primers. We strongly recommend using the Plexor™ Primer Design Software, which is available at: **www.promega.com/plexorresources/**
	The annealing temperature was too high or too low. Optimize the annealing temperature.
Genotyping: Miscalled known homozygous samples: Product formed (signal decrease) with both primers	Poor primer design. Redesign your primers. We strongly recommend using the Plexor™ Primer Design Software, which is available at: **www.promega.com/plexorresources/**
	The annealing temperature is too high or too low. Optimize the annealing temperature.
Genotyping: Miscalled known homozygous samples: Product formed only with the mismatched primer but not with the matching primer	The primer sequence was incorrect. Verify that the primer sequence is correct.
	Genotyping primer #1 and primer #2 were switched. Verify that the correct primer was used.
Genotyping: No call	Add more template.
	Redesign primers.
	See comments for "Flat Amplification Curve."

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XI. Appendix

XI.A. Plexor™ Analysis Software Operating System Compatibility

The Plexor™ Analysis Software is compatible with the following operating systems: Windows® 98, Windows NT® 4, Windows® ME, Windows® XP and Windows® 2000. Other operating systems are not supported. The Plexor™ Analysis Software is not compatible with Macintosh® computers.

Be sure that the display settings for the computer are set to 32-bit color, rather than 16-bit color, when using the Plexor™ Analysis Software.

XI.B. Plexor™ Analysis Software Installation

The Plexor™ Analysis Software and installation instructions are available for download at: **www.promega.com/plexorresources/**. The software is also available free-of-charge on CD-ROM by request. Consult the Promega Web site to verify that you are installing the most recent version of the software. Following installation, the program can be accessed in the "Start" menu: Programs\Plexor\Analysis Desktop.

Instructions for Installing the Plexor™ Analysis Software from CD-ROM

1. Insert the CD-ROM into the CD-ROM drive.
2. Double-click the "Plexor.exe" installer icon on the CD-ROM and follow the on-screen instructions to install the software.

   Note: Installation of the software may take several minutes. There is a pause where the computer may appear to be inactive between the launch of the installer and the software installation.

XI.C. Advanced Options

At the "File Import" screen, Step 3 (Figure 18), there are two "Advanced Options" buttons: "Run Template" and "Analysis Template". These options allow plate configuration and assay parameter information to be saved for reuse during routine experiments.

Run Template: A run template is used to assign sample types, sample colors and concentrations of standards (Figure 33). If you routinely use the same setup for plates of standard samples and unknowns, a run template can be created, stored and applied to subsequent runs.

1. Select "Run Template".
2. Assign colors, sample types and concentrations to the standards in the "Plate Setup" tab.
3. Use the "Sample IDs" tab to label your samples. Simply select the sample you wish to name and start typing.
4. Select "Export" to save the plate configuration to a *.rtp file for later use.
5. Select "OK".

To import an existing *.rtp file that contains a saved plate configuration, select "Import" and browse to that file.

Figure 33. A Run Template.

Analysis Template and Definition of Analysis Functions: The analysis template is used to optimize the analysis settings for the experiment. If you routinely perform reactions with the same analysis conditions, an analysis template can be created, stored and applied to subsequent runs. These settings can be exported as a *.ntp file, then imported for subsequent experiments. A description of the functions for each setting follows.

1. Select “Analysis Template”.
2. Enter the desired values for the analysis defaults for each dye used (Figure 34).

   Note: Descriptions of the analysis details are provided below.
3. Select “Export” to save the default settings to a *.ntp file for later use.
4. Select “OK”.

To import an existing *.ntp file that contains the saved default settings, select “Import” and browse to that file.

Default Amplification Threshold (RFU) Baseline Noise Standard Deviations: The Plexor™ Analysis Software has a user-definable amplification threshold that determines the RFU value at which sample cycle thresholds are called.

This value is based on the variation (noise) in the baseline regions of all samples and is determined by taking the mean and standard deviation of all RFUs in baseline regions. The threshold is set a specified number of standard deviations below the mean. The default threshold is 10 standard deviations but can be changed in the Analysis Template or recalculated at any time by using “Set Amp Threshold from Selected Samples” option in the “Edit” menu (see Section IX.D).

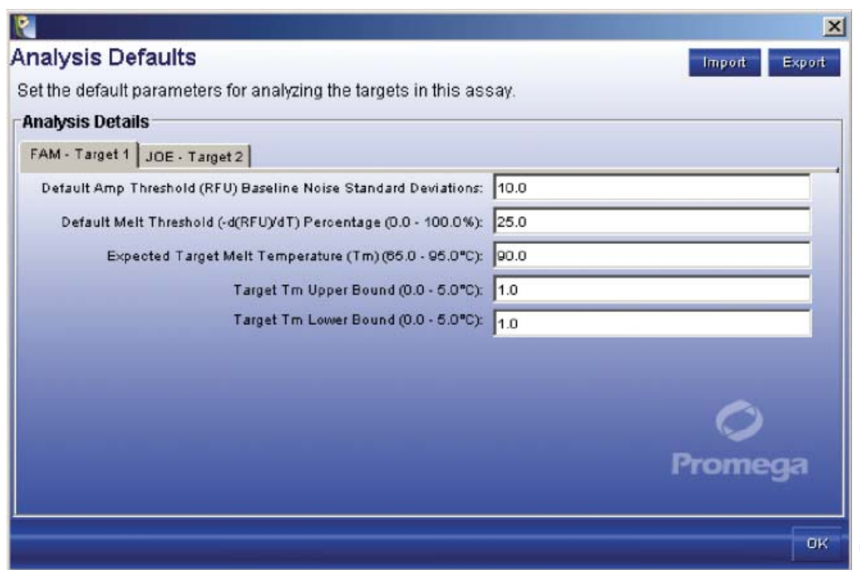


Figure 34. The Analysis Defaults tab.

Default Melt Threshold –d(RFU)/dT Percentage: The melt curve allows you to distinguish amplification products with different sequences and lengths. In the absence of nonspecific amplification products, the melt curve will have one peak. Each sample has a melt curve, from which a T_m can be determined. A T_m value is reported for all melt curves that cross the melt threshold. The melt threshold represents the –d(RFU)/dT value that is required before a T_m value is reported for a sample. A sample's T_m value is calculated as the temperature at which the melt curve has the lowest (i.e., the most negative) –d(RFU)/dT value.

The default melt threshold –d(RFU)/dT percentage is preset at 25.0% and can be set between 0.0 and 100.0%. This value is used by the software to calculate the melt threshold value. The T_m threshold value is defined as a percent of the –d(RFU)/dT value for the sample with the lowest –d(RFU)/dT value in the data set.

The melt threshold value is recalculated when a standard curve is generated. The melt threshold value can be manually adjusted by clicking and dragging the horizontal melt threshold line.

Expected Target Melt Temperature: The expected target melt temperature is the melt temperature of the correct PCR product. The expected target melt temperature must be between 65°C and 95°C. The default expected target melt temperature is 90°C.

Target T_m Upper Bound: The target T_m upper bound is the number of degrees Celsius above the expected target melt temperature at which a sample T_m is considered to be suspect. The default target T_m upper bound is +1°C.

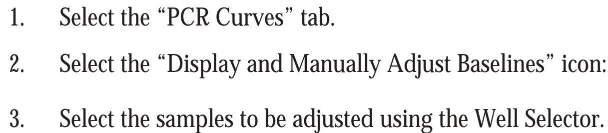
Target T_m Lower Bound: The target T_m lower bound is the number of degrees Celsius below the expected target melt temperature at which a sample T_m is considered to be suspect. The default target T_m lower bound is –1°C.

XI.D. Manual Baseline Adjustments

The proper baseline region is important for optimal analysis of Plexor™ System data. Baseline regions are automatically determined during import of data into the Plexor™ Analysis Software.

The baseline region is set in a flat region of the amplification curve before the beginning of the downward inflection that indicates product accumulation. In some instances, manual adjustment may provide optimal representation of the data. This may include samples with excessive noise, bleedthrough or early C_t values or situations where the real-time instrument shows early signal fluctuation.

1. Select the "PCR Curves" tab.
2. Select the "Display and Manually Adjust Baselines" icon:



3. Select the samples to be adjusted using the Well Selector.

   Note: The baseline region can be adjusted for individual samples or groups of samples by selecting or dragging the lower and upper limits. The shading in the baseline region will be gray if the selected samples do not share a common baseline region (Figure 35). For multiplex assays, the baseline is set independently for each dye.

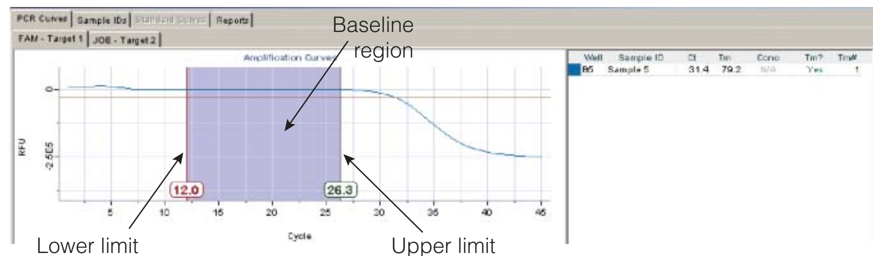
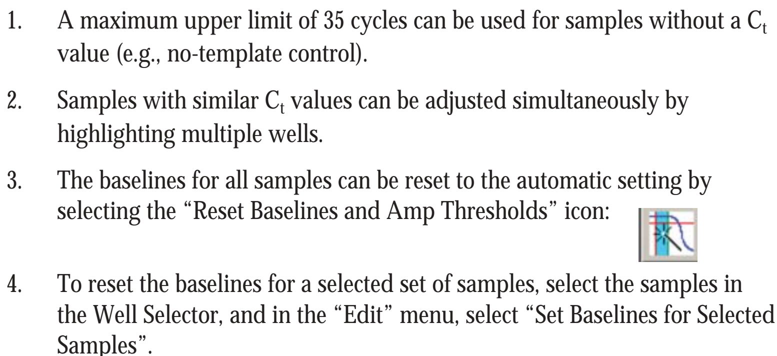


Figure 35. An amplification window showing the baseline region and baseline upper and lower limits.

4. Adjust the upper limit of the baseline region for each sample, so the upper limit is approximately 5 cycles before the decrease in fluorescence and in an area where the baseline is flat. The C_t values for selected samples are displayed in the table to the right of the graph. The C_t value may change when the limits are changed. See Notes 1 and 2.
5. If necessary, adjust the lower limit to a region that creates the flattest baseline given the selected upper limit.
6. **Optional:** The amplification threshold is based on noise within the baseline region for all of the samples. When manual baseline adjustments are complete, consider recalculating the amplification threshold for all samples. Select all samples, and in the "Edit" menu, select "Set Amp Threshold from Selected Samples" (Section IX.D). See Notes 3 and 4.

Notes:

1. A maximum upper limit of 35 cycles can be used for samples without a C_t value (e.g., no-template control).
2. Samples with similar C_t values can be adjusted simultaneously by highlighting multiple wells.
3. The baselines for all samples can be reset to the automatic setting by selecting the “Reset Baselines and Amp Thresholds” icon: 
4. To reset the baselines for a selected set of samples, select the samples in the Well Selector, and in the “Edit” menu, select “Set Baselines for Selected Samples”.

XI.E. Icon Definitions

Assign Color (shortcut = “q”)

The “Assign Color” function allows you to select a color in which a sample is displayed. This color selection is associated with those samples in the amplification and melt curves, Well Selector and any reports. Select one or more capillaries using the Well Selector, then select this button to choose the desired color for the selected samples. These colors are not transferred to printed copies or exported reports. Sample color does not change the analysis of a sample in any way.

Assign Unknown (shortcut = “w”)

The “Assign Unknown” function allows you to assign the sample type “unknown” to all selected samples. Select one or more capillaries using the Well Selector, then select this button to assign the sample type “unknown”. Unknown samples are displayed as open squares in the Well Selector. They are labeled “unknown” in reports. When included in a standard curve, the concentrations of unknown samples will be calculated and reported.

Assign NTC (shortcut = “e”)

The “Assign NTC” function allows you to assign the sample type “no-template control" to all selected samples. Select one or more capillaries using the Well Selector, then select this button to assign the sample type “no-template control”. No-template control reactions are displayed as diamonds in the Well Selector. They are labeled as “no-template control” in reports. When included in a standard curve, the concentrations of sample in the no-template control will be calculated and reported.

Assign Positive Control (shortcut = “t”)

The “Assign Positive Control” function allows you to assign the sample type “positive control” to all selected samples. Select one or more capillaries using the Well Selector, then select this button to assign the sample type “positive control”. Positive control samples are displayed as hexagons in the Well Selector. They are labeled “positive control” in reports. When included in a standard curve, the concentrations of positive control samples will be calculated and reported.

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XI.E. Icon Definitions (continued)

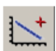
Assign Standard (shortcut = “r”)

The “Assign Standard” function allows you to assign the sample type “standard” to all selected samples. Select one or more capillaries using the Well Selector, then select this button to assign the sample type “standard”. Only samples that have been assigned a type of “standard” will be used to generate the best-fit line in standard curves. All standard samples must be assigned a concentration by the user when they are defined as a standard. Concentrations may be entered in standard format (0.01, 0.1, 1, 10, 100, 1000, etc.) or scientific format (1e-2, 1e-1, 1e0, 1e1, 1e2, 1e3, etc.). The software does not accept commas in the concentration assignments. Standard samples are displayed as circles in the Well Selector and standard curve graphs. They are labeled “standard” in reports.

Create Dilution Series (shortcut = “f”)

The “Create Dilution Series” function creates a full dilution series within a row or column of capillaries. Select the capillaries that contain a dilution series of the standard, then select “Create Dilution Series”. You must enter the initial concentration of the series, the dilution factor and whether the series is increasing or decreasing (Figure 14). Concentrations may be entered in standard format (0.01, 0.1, 1, 10, 100, 1000, etc.) or scientific format (1e-2, 1e-1, 1e0, 1e1, 1e2, 1e3, etc.). The software does not accept commas in the concentration assignments.

All selected capillaries will be assigned the sample type “standard” with the appropriate concentration. This function can only be performed with standards within the same row or column. Using this function produces the same result as selecting each capillary in the series individually and assigning it the sample type “standard” with the appropriate concentration. Only samples that have been assigned the sample type “standard” will be used to generate the best-fit line in standard curves. Standard samples are displayed as circles. A row or column of capillaries within a dilution series of standards may be assigned as standards simultaneously by highlighting multiple capillaries and using the “Create Dilution Series” function.


Add Standard Curve (shortcut = “d”)

The “Add Standard Curve” function fits the experimentally measured C_t values and user-entered concentration values for standard samples to a straight line using the least mean squares method. It will calculate the concentrations of unknown samples, positive control reactions and no-template control reactions from their measured C_t values using the equation for the best-fit line. Any sample with a concentration of “N/A” on the report or elsewhere did not cross the cycle threshold, so the concentration of that sample cannot be calculated.

Select all of the samples you wish to use as standard samples, as well as all other samples for which you wish to calculate concentrations. Choose “Add Standard Curve” from the “Edit” menu. Type “d” or select the “Add Standard Curve” icon on the toolbar.

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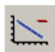
You may create as many standard curves as you wish for a single set of data, but no sample can be used to generate more than one standard curve. It is not possible to add samples to an existing standard curve, but a new curve can easily be constructed with a new selection. This action will remove the existing standard curve and generate the new standard curve using the samples you have selected. You may generate the original standard curve at any time.

 Remove Standard Curve (shortcut = "c")

The "Remove Standard Curve" function removes the standard curve on the tab that is currently selected. This function is only available in the "Standard Curves" tab.

Display and Manually Adjust Baselines

The "Display and Manually Adjust Baselines" function allows you to set the baseline range for a sample or set of samples. Select one or more wells using the Well Selector, then select this button. See Section IX.D.

Reset Baselines and Amp Thresholds

The "Reset Baselines and Amp Thresholds" function allows you to reset the baseline range and amplification threshold for all samples. See Section XI.C.

XI.F. Amplification Efficiency Calculations

The Plexor™ Analysis Software automatically calculates the equation for the best fit line and determines the R^2 value of the standard curve. The R^2 value is a measure of the fit of the data points to a straight line. An R^2 value of 1.0 is a perfect fit. R^2 values should be close to 1.0.

The software also calculates the slope of the standard curve. The slope is an indication of the efficiency of the PCR. At 100% efficiency, the amount of amplification product doubles with every cycle, so C_t values differ by 1 for each twofold dilution of the template. At 100% efficiency, the amount of product increases tenfold every 3.32 cycles ($2^{3.32} = 10$), so C_t values differ by 3.32 for each tenfold dilution. A reaction with 100% efficiency will have a slope of –3.32 when the amplification curve is displayed with the C_t values on the Y axis and log concentration on the X axis. When the amplification curve is displayed as C_t versus log concentration, the efficiency may be calculated as $[(10^{-1/slope}) - 1] \times 100\%$ (1).

XI.G. Reference

1. Bustin, S.A. (2004) *A–Z of quantitative PCR.* International University Line, La Jolla

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