Plexor™ Systems Instrument Setup and Data Analysis for the Roche LightCycler® 480 System

INSTRUCTIONS FOR USE OF PRODUCTS A4011, A4021, A4031, A4041, A4051 AND A4061.

Plexor™ Systems Instrument Setup and Data Analysis for the Roche LightCycler® 480 System

> **All technical literature is available on the Internet at: www.promega.com/tbs/**
> **Please visit the web site to verify that you are using the most**
> **current version of this Technical Manual.**

I.   Description

The Plexor™ qPCR and qRT-PCR Systems[a,b,c] are compatible with a variety of real-time PCR instruments. Data from these instruments can be analyzed with one dedicated software program, the Plexor™ Analysis Software. This manual includes instructions and thermal cycling conditions specific for use of the Plexor™ qPCR System, Plexor™ One-Step qRT-PCR System and Plexor™ Two-Step qRT-PCR System with the Roche LightCycler® 480 System. Instructions are included for instrument setup, data transfer from the instrument to the Plexor™ Analysis Software and data analysis.

II.   Plate Preparation and Amplification

Detailed instructions describing assay setup are provided in the *Plexor™ qPCR System Technical Manual* #TM262, *Plexor™ One-Step qRT-PCR System Technical Manual* #TM263 or *Plexor™ Two-Step qRT-PCR System Technical Manual* #TM264.

When using the Plexor™ qPCR System for the first time, we recommend programming the thermal cycling conditions and checking that the instrument is compatible with the dyes used and is configured for those dyes before assembling the reactions, so the reactions are not kept on ice for prolonged periods of time. Once you are familiar with the programming process, the instrument can be programmed after reaction assembly.

Materials to Be Supplied By the User
- LightCycler® 480 multiwell reaction plate
- centrifuge compatible with a 96-well plate
- optical adhesive covers and applicator

1. After the amplification reactions have been assembled, cover the reaction plate with an optical adhesive cover using the applicator. Centrifuge briefly to collect contents at the bottom of each well.

   Note: Keep the plate on ice during reaction setup and programming of the thermal cycling conditions.

2. Program the Roche LightCycler® 480 System. The proper thermal cycling conditions and instructions for programming the instrument are provided in Section III (generating color compensation files), Section IV (qPCR and two-step qRT-PCR assays), Section V (one-step qRT-PCR assays) and Section VI (genotyping assays).

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III. Generating Color Compensation Files

For multiplex assays, a color compensation file (cc object) must be created and applied to multicolor data to allow proper interpretation. To generate the color compensation file, labeled Plexor™ primers are used as dye calibrators in an initial color compensation cycling experiment. The color compensation cycling experiment contains separate color compensation reactions corresponding to each individual dye in your multiplex, as well as a blank reaction with no dye. Any subsequent runs performed with the same dyes and similar cycling conditions can be analyzed using this color compensation file.

Notes:

- The Roche LightCycler® 480 instrument should be programmed before preparing the reagents.

- The color compensation file can be generated after the experimental run and applied to the multiplex experiment before final analysis.

- A list of LightCycler®-compatible dyes is available at:
  www.promega.com/plexorresources/

III.A. Thermal Cycling Program for Color Compensation

The thermal cycling program is shown in Table 1. Primers designed using the Plexor™ Primer Design Software have an annealing temperature of approximately 60°C.

Table 1. Color Compensation Thermal Cycling Program.

Step	Temperature	Time	Number of Cycles
Initial Denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	40 cycles
Annealing and Extension:	60°C	35 seconds	
Denaturation:	95°C	5 seconds	1 cycle
Melt Temperature Curve:	50°C to 95°C, 2.5 acquisitions per °C		
Instrument Cool:	50°C	30 seconds	1 cycle

1. Open the LightCycler® 480 software.

2. To create a new dye set detection format, select "Tools" in the startup screen. The detection format specifies the channels in which fluorescence will be read.

Tools button
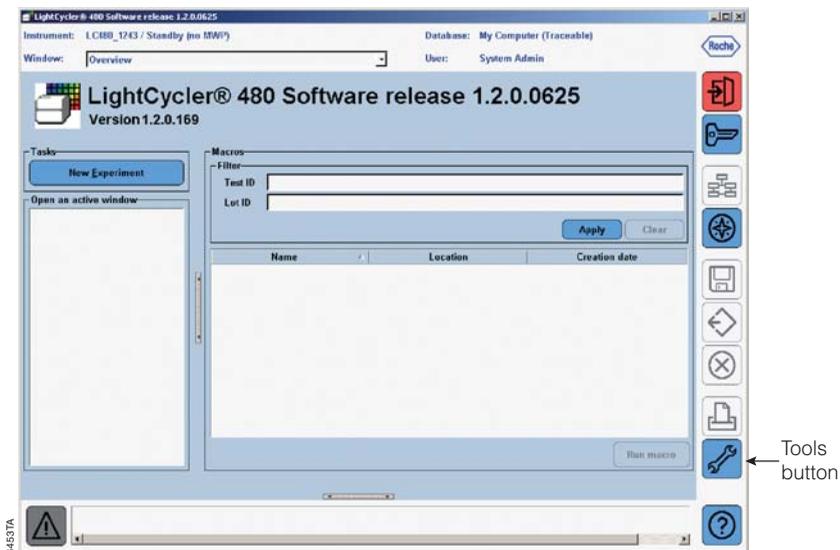
3. Select "Detection Formats", then choose "New", and select the appropriate instrument. A new detection format will be created.

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4. Select the appropriate excitation and emission filter combinations for the dyes in your multiplex using the "Filter Combinations Selection" window. Set the max integration time to 0.25 for all four color channels.
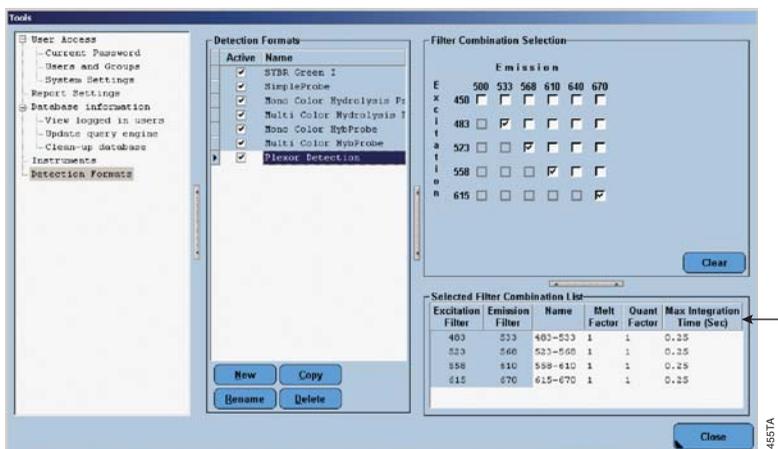
5. Select "Close" to save the new detection format into the database, and return to the startup screen.

6. To access this newly created detection format, the LightCycler® 480 software must be closed, then reloaded.

7. After closing and reloading the software, select "New Experiment" in the startup screen

8. Choose the appropriate detection format from the pull-down menu.
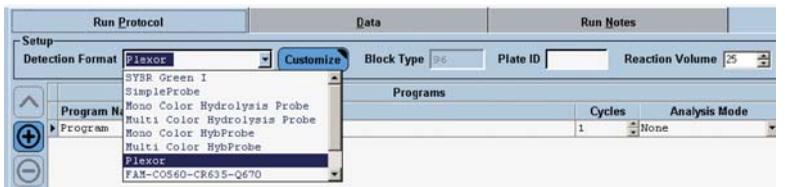
9. Enter the reaction volume.

10. The cycling program should mimic a typical PCR. This cycling will consist of four programs. Program the instrument by adding the program names in the top window. Select the "+" icon to add a program. In the "Program Name" section, name the first program of this experiment "Denature" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

11. With the "Denature" program name selected, change "Program Temperature Targets" to the values indicated below.

Add new program →

Add new step to selected program →

12. Next to "Program Name", select the "+" button to add a program. Name the second program of this experiment "PCR" or a similar title. Enter the number of amplification cycles. In the "Analysis Mode" pull-down menu, choose "Quantification".

13. With the "PCR" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 60°C target temperature selected, change the acquisition mode to "Single".

14. Next to "Program Name", select the "+" button to add a program. Name the third program of this experiment "Melt" or a similar title. In the "Analysis Mode" pull-down menu, choose "Color Compensation".

15. With the "Melt" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 95°C target temperature selected, change the acquisition mode to "Continuous" and change "Acquisitions (per °C)" to 2.5.

Setup						
Detection Format	Flexor	Customize	Block Type 96	Plate ID	Reaction Volume 25	

Programs		
Program Name	**Cycles**	**Analysis Mode**
Denature	1	None
PCR	40	Quantification
▶ Melt	1	Color Compensation
cooling	1	None

Melt Temperature Targets								
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (cycles)**	
▶ 95	None	00:00:05	4.4					
50	None	00:00:30	2.2					
95	Continuous			2.5				

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16. Next to "Program Name", select the "+" button to add a program. Name the fourth program of this experiment "cooling" or a similar title. In the "Analysis Mode" pull-down menu, choose "None".

Setup						
Detection Format	Flexor	Customize	Block Type 96	Plate ID	Reaction Volume 25	

Programs		
Program Name	**Cycles**	**Analysis Mode**
Denature	1	None
PCR	40	Quantification
Melt	1	Color Compensation
▶ cooling	1	None

Cooling Temperature Targets								
Target (°C)	**Acquisition Mode**	**Hold (hh:mm:ss)**	**Ramp Rate (°C/s)**	**Acquisitions (per °C)**	**Sec Target (°C)**	**Step Size (°C)**	**Step Delay (cycles)**	
▶ 50	None	00:00:30	2.2		0	0	0	

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17. With the "cooling" program name selected, change "Program Temperature Targets" to the values indicated below.

III.B. Color Compensation Sample Editing

1. Sample information can be entered before, during or after a run.

2. Select "Subset Editor".

3. Click "New" to create a new subset. Highlight the wells in the plate that contain the color compensation reactions, then select "Apply".
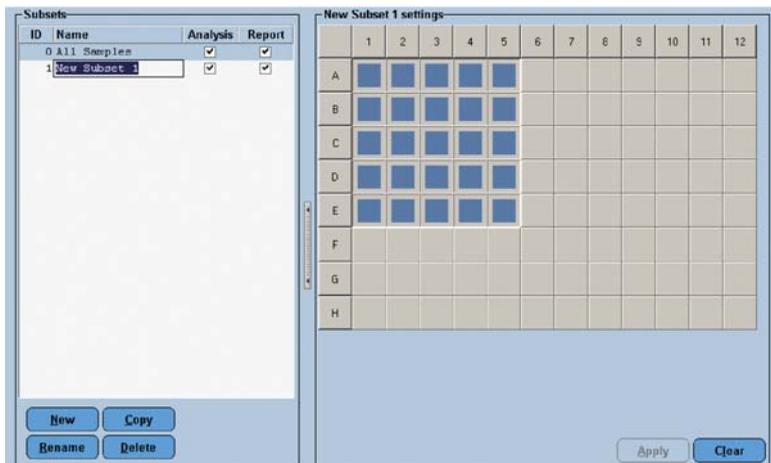
4. Select "Sample Editor". In the "Subset" pull-down menu, choose the subset containing your color compensation reactions.

5. Under the "General" tab, indicate which are replicate wells. Color compensation reactions should be run in replicates of 3–5. Name the samples if desired.

---
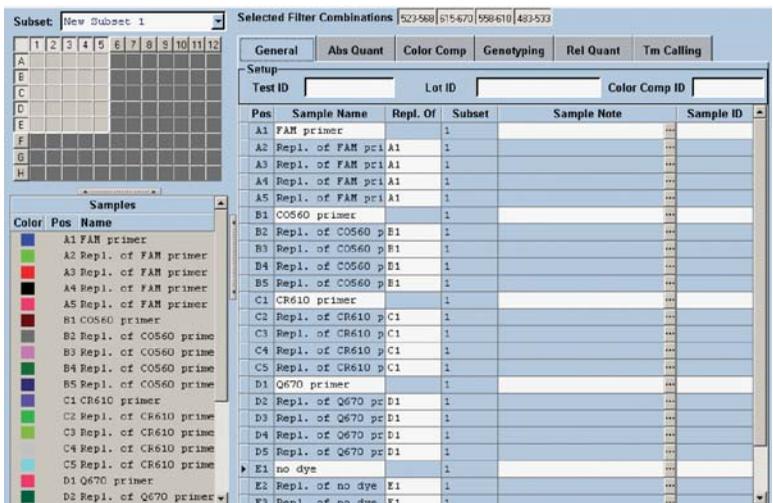
6. Under the "Color Comp" tab, indicate the dominant color channel for each color compensation sample, using the "Dominant Channel" pull-down menu. For "No Dye" samples, "Water" should be selected as the dominant channel.

7. Select "Experiment" to return to the experiment window.

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III.C. Color Compensation Reaction Setup

You will need a separate color compensation reaction dedicated to each individual dye in your multiplex, as well as a reaction with no dye (Table 2). Each of these color compensation reactions should be run in replicates of 3–5. The same Plexor™ primers that will be used in your experimental samples can be used to set up the color compensation reactions. The primers in the color compensation reactions should be used at similar concentrations to those in the experimental reactions. When using Plexor™ primers, it is not necessary to add template to the color compensation reactions.

Note: The volumes listed in Table 2 are for use with a 96-well reaction plate. When using a 384-well reaction plate, reaction components should be scaled down to a final volume that is compatible with that plate. Plexor™ reaction components have been used successfully in reaction volumes as low as 5µl. In a 384-well reaction plate, 10µl final volumes are commonly used.

Table 2. Color Compensation Reaction Setup

Reaction Components	Plexor™ Dye Reaction	No Dye Reaction (Blank)
2X Plexor™ Master Mix	12.5µl	12.5µl
Plexor™ primer	1µl	–
MOPS/EDTA Buffer	11.5µl	12.5µl
Total Volume	25µl	**25µl**

1. Setup the color compensation reactions on ice in a LightCycler® 480 multiwell reaction plate.

2. Mix each color compensation reaction thoroughly.

3. Cover the reaction plate with an optical adhesive cover using the applicator. Centrifuge briefly to collect contents at the bottom of each well.

4. Load the reaction plate into the LightCycler® 480, and immediately press "Start Run" to begin thermal cycling.

III.D. Creating a Color Compensation Analysis File

1. When the run is complete, select "Analysis".

2. In the "Analyses" pull-down menu, choose "Overview".

3. In the "Create new analysis" list, select "Color Compensation".
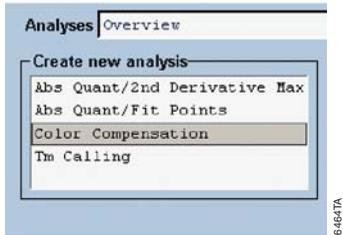
4. When the "Create new analysis" pop-up window appears, select the sample subset containing your color compensation reactions from the "Subset" pull-down menu. Select "OK".

"OK" button

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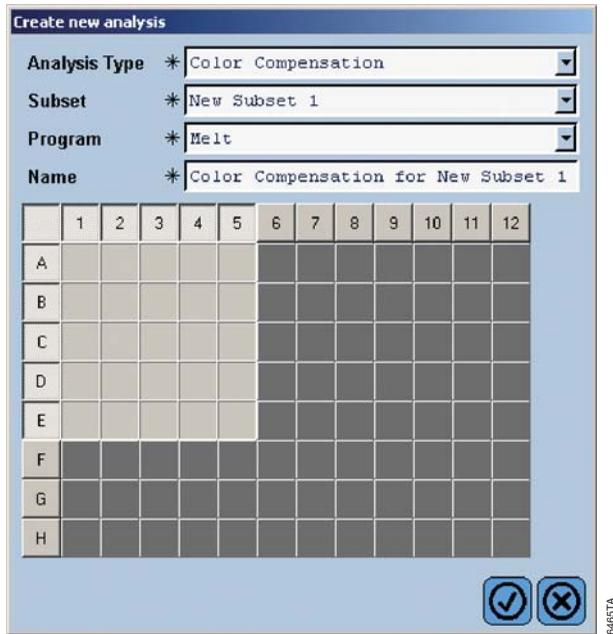
5. Select "Calculate" to perform the color compensation analysis. For each filter combination, the "Raw Data" chart shows the raw fluorescence data while the "Compensated Data" chart shows the fluorescence after the color compensation has been applied.
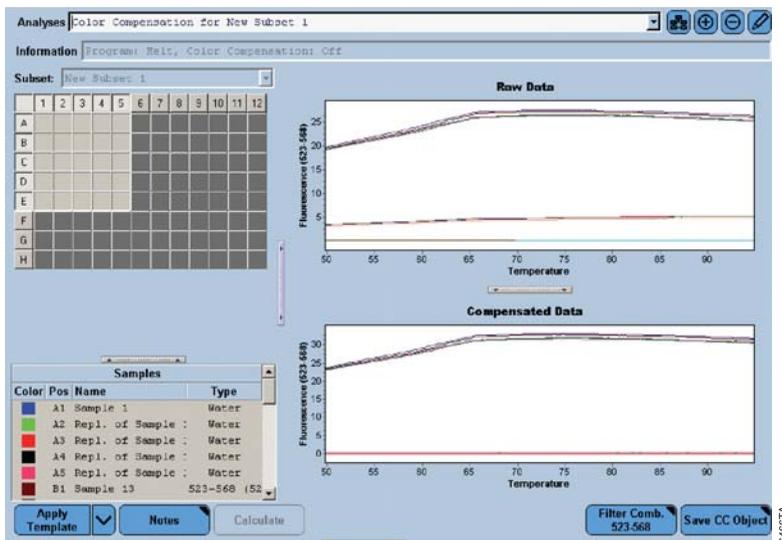
6. Select "Save CC Object" to save this color compensation analysis to the database. This color compensation file can now be applied to data in another experiment.

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IV. Instrument Setup and Thermal Cycling for qPCR and Two-Step qRT-PCR

These instructions describe instrument setup and thermal cycling conditions for DNA or cDNA quantitation using the Plexor™ qPCR or Plexor™ Two-Step qRT-PCR System. Thermal cycling programs described in this manual are optimized to work with primers designed using the Plexor™ Primer Design Software. The Plexor™ Primer Design Software can be accessed at: **www.promega.com/plexorresources/**

IV.A. Thermal Cycling Program

The thermal cycling program is shown in Table 3. Primers designed using the Plexor™ Primer Design Software have an annealing temperature of approximately 60°C.

Table 3. qPCR and Two-Step qRT-PCR Thermal Cycling Program.

Step	Temperature	Time	Number of Cycles
Initial Denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	40 cycles
Annealing and Extension:	60°C	35 seconds	
Denaturation:	95°C	5 seconds	1 cycle
Melt Temperature Curve:	50°C to 95°C, 2.5 acquisitions per °C		
Instrument Cool:	50°C	30 seconds	1 cycle

1. Open the LightCycler® 480 software.

2. Select "New Experiment" in the startup screen.

3. Select the appropriate detection format from the pull-down menu. The detection format specifies which fluorescent channels will be read (created in Section III.A).

4. Enter the reaction volume.

5. The cycling will consist of four programs. Program the instrument by adding the program names in the top window. Select the "+" icon to add a program. In the "Program Name" section, name the first program of this experiment "Denature" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

6. With the "Denature" program name selected, change "Program Temperature Targets" to the values indicated below.

7. Next to "Program Name", select the "+" button to add a program. Name the second program of this experiment "PCR" or a similar title. Enter the number of amplification cycles. In the "Analysis Mode" pull-down menu, select "Quantification".

8. With the "PCR" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 60°C target temperature selected, change the acquisition mode to "Single".

9. Next to "Program Name", select the "+" button to add a program. Name the third program of this experiment "Melt" or a similar title. In the "Analysis Mode" pull-down menu, select "Melting Curves".

10. With the "Melt" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 95°C target temperature selected, change the acquisition mode to "Continuous" and change "Acquisitions (per °C)" to 2.5.

11. Next to "Program Name", select the "+" button to add a program. Name the fourth program of this experiment "cooling" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

12. With the "cooling" program name selected, change "Program Temperature Targets" to the values indicated below.

IV.B. Sample Editing

1. Sample information can be entered in the LightCycler® 480 software before, during or after your experiment is run. Alternatively, sample names can be entered during the data analysis step using the Plexor™ Analysis Software.

2. To enter sample information using the LightCycler® 480 software, select "Sample Editor".

3. Under the "General" tab, indicate which are replicate wells. Name the samples if desired.

4. Select "Experiment" to return to the "Experiment" window.

5. Load the reaction plate into the LightCycler® 480, and immediately press "Start Run" to begin thermal cycling.

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V. Instrument Setup and Thermal Cycling for One-Step qRT-PCR

These instructions describe instrument setup and thermal cycling conditions for cDNA quantitation using the Plexor™ One-Step qRT-PCR System. The thermal cycling program includes the initial incubation for the reverse transcription. Thermal cycling programs described in this manual are optimized to work with primers designed using the Plexor™ Primer Design Software. The Plexor™ Primer Design Software can be accessed at: **www.promega.com/plexorresources/**

V.A. Thermal Cycling Program

The thermal cycling program is shown in Table 4. Primers designed using the Plexor™ Primer Design Software have an annealing temperature of approximately 60°C.

Table 4. One-Step qRT-PCR Thermal Cycling Program.

Step	Temperature	Time	Number of Cycles
Reverse Transcription[1]:	45°C	5 minutes	1 cycle
Initial Denaturation and Inactivation of the ImProm-II™ Reverse Transcriptase:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	40 cycles
Annealing and Extension:	60°C	35 seconds	
Denaturation:	95°C	5 seconds	1 cycle
Melt Temperature Curve:	50°C to 95°C, 2.5 acquisitions per °C		
Instrument Cool:	50°C	30 seconds	1 cycle

[1] The length of incubation for the reverse transcription reaction can be increased to up to 30 minutes. Longer incubation times can lead to increased sensitivity but also higher background.

1. Open the LightCycler® 480 software.
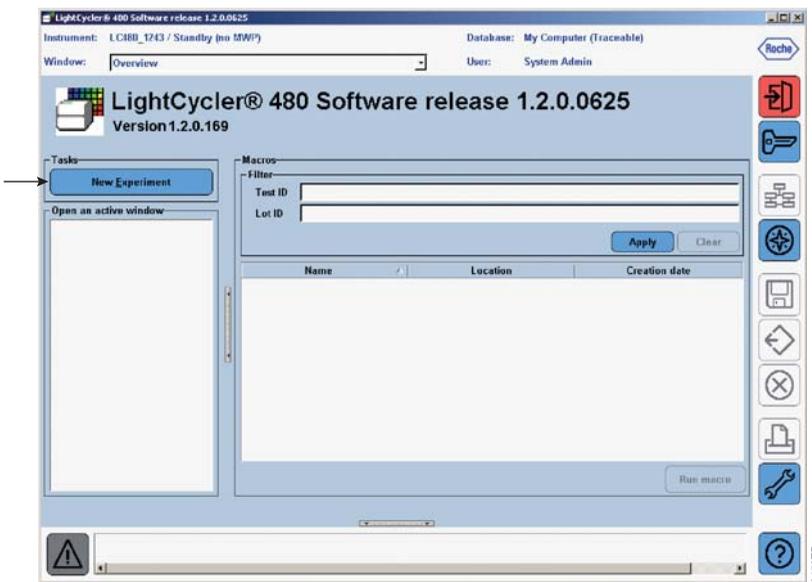
2. Select "New Experiment" in the startup screen.

3. Select the appropriate detection format from the pull-down menu. The detection format specifies which fluorescent channels will be read (created in Section III.A).
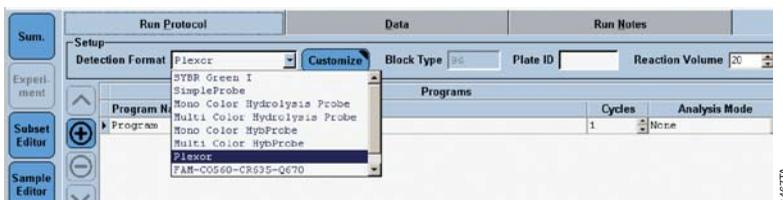
4. Enter the reaction volume.

5. The cycling will consist of four programs. Program the instrument by adding the program names in the top window. Select the "+" icon to add a program. In the "Program Name" section, name the first program of this experiment "Reverse Transcription and denaturation/inactivation" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

6. With the "Reverse Transcription and denaturation/inactivation" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window.

7. Next to "Program Name", select the "+" button to add a program. Name the second program of this experiment "PCR" or a similar title. Enter the number of amplification cycles. In the "Analysis Mode" pull-down menu, select "Quantification".

8. With the "PCR" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 60°C target temperature selected, change the acquisition mode to "Single".

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9. Next to "Program Name", select the "+" button to add a program. Name the third program of this experiment "Melt" or a similar title. In the "Analysis Mode" pull-down menu, select "Melting Curves".

10. With the "Melt" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 95°C target temperature selected, change the acquisition mode to "Continuous" and change "Acquisitions (per °C)" to 2.5.

Setup

Detection Format: Plexor | Customize | Block Type: 96 | Plate ID: | Reaction Volume: 25

Programs

Program Name	Cycles	Analysis Mode
Reverse Transcription and denaturation/inactivation	1	None
PCR	40	Quantification
Melt	1	Melting Curves
cooling	1	None

Melt Temperature Targets

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:05	4.4				
50	None	00:00:30	2.2				
95	Continuous			2.5			

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11. Next to "Program Name", select the "+" button to add a program. Name the fourth program of this experiment "cooling" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

12. With the "cooling" program name selected, change "Program Temperature Targets" to the values indicated below.

Setup

Detection Format: Plexor | Customize | Block Type: 96 | Plate ID: | Reaction Volume: 25

Programs

Program Name	Cycles	Analysis Mode
Reverse Transcription and denaturation/inactivation	1	None
PCR	40	Quantification
Melt	1	Melting Curves
cooling	1	None

cooling Temperature Targets

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
50	None	00:00:30	2.2		0	0	0

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V.B. Sample Editing

1. Sample information can be entered in the LightCycler® 480 software before, during or after your experiment is run. Alternatively, sample names can be entered during the data analysis step using the Plexor™ Analysis Software.

2. To enter sample information using the LightCycler® 480 software, select "Sample Editor".

3. Under the "General" tab, indicate which are replicate wells. Name the samples if desired.

4. Click "Experiment" to return to the "Experiment" window.

5. Load the reaction plate into the LightCycler® 480, and immediately press "Start Run" to begin thermal cycling.

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VI. Instrument Setup and Thermal Cycling for Genotyping (SNP) Assays

These instructions describe instrument setup and thermal cycling conditions for genotyping assays using the Plexor™ qPCR System. This cycling program is specific for genotyping primers designed using the Plexor™ Primer Design Software. Thermal cycling programs described in this manual are optimized to work with primers designed using the Plexor™ Primer Design Software. The Plexor™ Primer Design Software can be accessed at: **www.promega.com/plexorresources/**

VI.A. Thermal Cycling Program

The thermal cycling program is shown in Table 5. The cycling program will include one cycle with an annealing temperature of 50°C, followed by 40 cycles with an annealing temperature of 60°C. The first round of PCR is performed at the lower annealing temperature; additional rounds of PCR are performed at the higher annealing temperature to increase the specificity of amplification.

Table 5. Thermal Cycling Profile for Genotyping Assays.

Step	Temperature	Time	Number of Cycles
Initial Denaturation:	95°C	2 minutes	1 cycle
Annealing and Extension:	50°C	35 seconds	
Denaturation:	95°C	5 seconds	40 cycles
Annealing and Extension:	60°C	35 seconds	
Denaturation:	95°C	5 seconds	1 cycle
Melt Temperature Curve:	50°C to 95°C, 2.5 acquisitions per °C		
Instrument Cool:	50°C	30 seconds	1 cycle

1. Open the LightCycler® 480 software.

2. Select "New Experiment" in the startup screen.

3. Select the appropriate detection format from the pull-down menu. The detection format specifies which fluorescent channels will be read (created in Section III.A).

4. Enter the reaction volume.

5. The cycling will consist of four programs. Program the instrument by adding the program names in the top window. Select the "+" icon to add a program. In the "Program Name" section, name the first program of this experiment "Plexor first step genotyping" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

6. With the "Plexor first step genotyping" program name selected, change "Program Temperature Targets" to the values indicated below.

7. Next to "Program Name", select the "+" button to add a program. Name the second program of this experiment "PCR" or a similar title. Enter the number of amplification cycles. In the "Analysis Mode" pull-down menu, select "Quantification".

8. With the "PCR" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 60°C target temperature selected, change the acquisition mode to "Single".

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9. Next to "Program Name", select the "+" button to add a program. Name the third program of this experiment "Melt" or a similar title. In the "Analysis Mode" pull-down menu, select "Melting Curves".

10. With the "Melt" program name selected, change "Program Temperature Targets" to the values indicated below. To add new steps, select the "+" button in the lower window. With the 95°C target temperature selected, change the acquisition mode to "Continuous" and change "Acquisitions (per °C)" to 2.5.

11. Next to "Program Name", select the "+" button to add a program. Name the fourth program of this experiment "cooling" or a similar title. In the "Analysis Mode" pull-down menu, select "None".

12. With the "cooling" program name selected, change "Program Temperature Targets" to the values indicated below.

VI.B. Sample Editing

1. Sample information can be entered in the LightCycler® 480 software before, during or after your experiment is run. Alternatively, sample names can be entered during the data analysis step using the Plexor™ Analysis Software.

2. To enter sample information using the LightCycler® 480 software, select "Sample Editor".

3. Under the "General" tab, indicate which are replicate wells. Name the samples if desired.

4. Click "Experiment" to return to the "Experiment" window.

5. Load the reaction plate into the LightCycler® 480, and immediately press "Start Run" to begin thermal cycling.

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VII. Data Export from the Roche LightCycler® Data Analysis Software

Before the data can be analyzed using the Plexor™ Analysis Software, the data must be exported from the LightCycler® 480 software. Two *.txt files must be exported for **each** color channel used: one with the amplification data and one with the melt/dissociation data. Be sure to use a descriptive name when naming these files so that it is clear which file contains the amplification data and which file contains the dissociation data while still indicating that the files are related.

Preliminary analysis must be performed using the LightCycler® software.

1. Open the experiment to be analyzed.

2. Click on "Analysis" on the toolbar.

3. To analyze the amplification data, select "Overview" in the "Analyses" pull-down menu. From the "Create new analysis" list, select "Abs Quant/Fit Points".
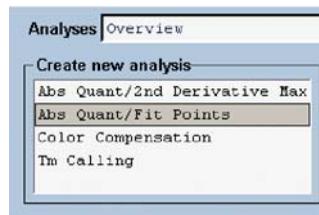
4. In the "Create new analysis" pop-up window, confirm that "PCR" is selected in the "Program" pull-down menu. Selecting "All Samples" in the "Subset" pull-down menu will result in analysis of the entire plate. Alternatively, if the experiment contains previously defined subsets of samples, a specific subset can be chosen for analysis. Select "OK" to proceed.

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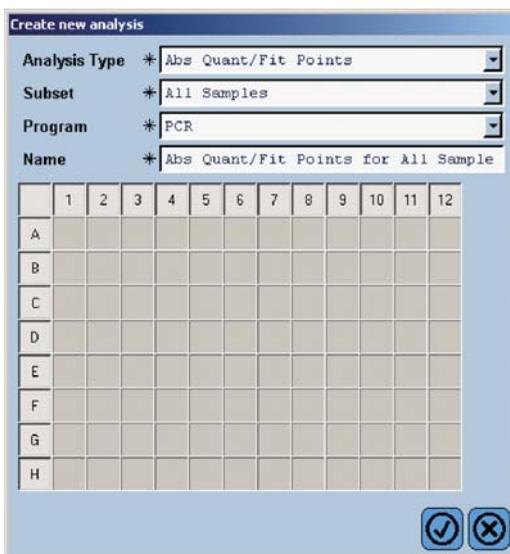
5. Amplification curves are displayed in the chart. For multiplex data (i.e., more than one dye), a color compensation file must be applied. Select the "Color Comp" button, and choose "In Database". From the "Available Color Compensations" list, select the appropriate color compensation file and select "OK". In the "Color Compensation Channels" box, choose the channels to compensate and select "OK".

6. The "Filter Comb" button indicates the color channel being displayed. To view each color channel, select "Filter Comb", and choose the channel you wish to display. The fluorescence data must be exported separately for **each** color channel.
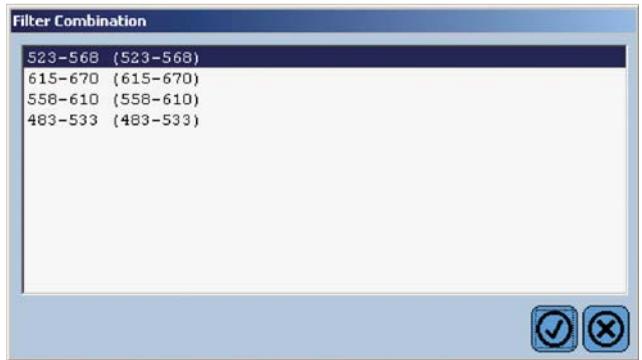
7. To export the amplification data, right-click on the "Amplification Curves" chart and select "Export" from the menu.
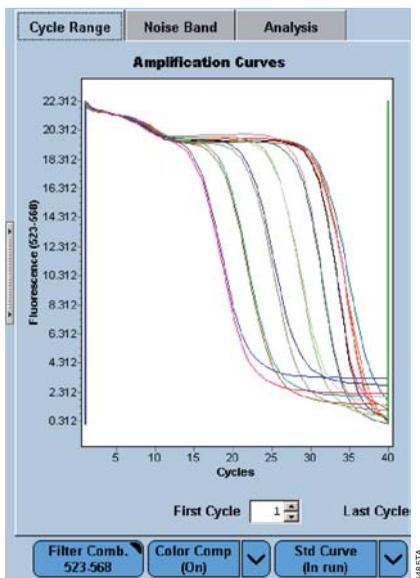
8. In the "Export" chart window, select the "Data" tab. Choose "Text" as the format. Include "Point Index", "Point Labels" and "Header". The file should be tab-delimited.

9. Assign a descriptive name to the file in the "Filename" box, so that it is clear the file contains the amplification data for that specific color channel. Choose the "..." box to select a location to save the file, then select "Export".
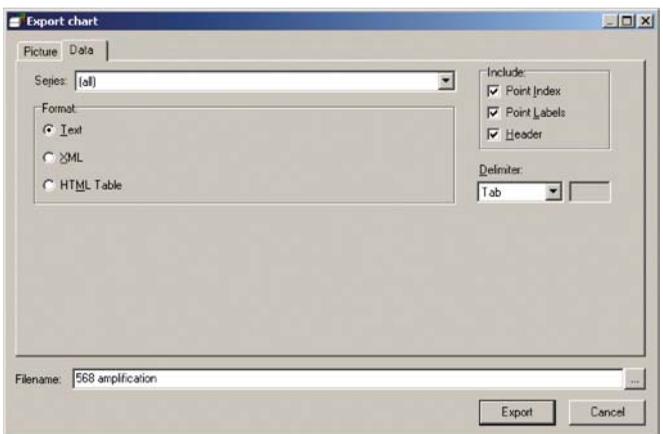
10. For multiplex reactions, each dye channel must be exported separately by repeating Steps 6–9. In Step 6, use the "Filter Comb" button to change the color channel being displayed to export data from each of the color channels.

11. To analyze the melt data, select "Overview" in the "Analyses" pull-down menu.

12. From the "Create new analysis" list, select "Tm Calling".

13. In the "Create new analysis" pop-up window, confirm that "Melt" is selected in the "Program" pull-down menu. Selecting "All Samples" in the "Subset" pull-down menu will result in analysis of the entire plate. Alternatively, if the experiment contains previously defined subsets of samples, a specific subset can be chosen for analysis. Select "OK" to proceed.

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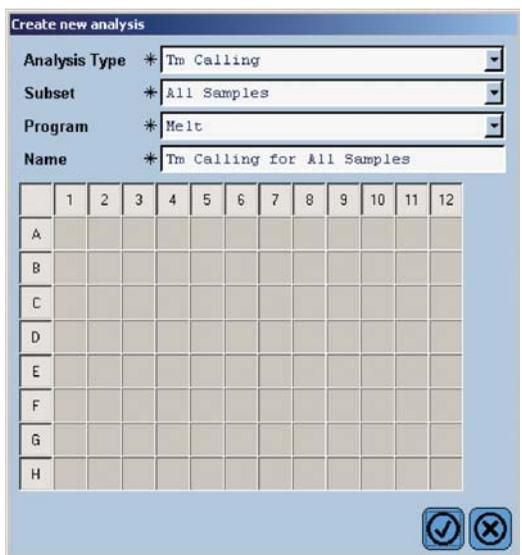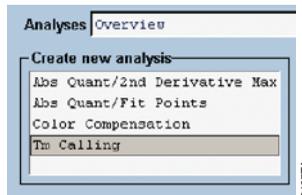
14. Melting curves are displayed in the upper chart. For multiplex data (i.e., more than one dye), a color compensation file must be applied. Select the "Color Comp" button, and choose "In Database". From the "Available Color Compensations" list, choose the appropriate color compensation file, and select "OK". In the "Color Compensation Channels" box, choose the channels to compensate, and select "OK".

15. The "Filter Comb" button indicates the color channel being displayed. To view each color channel, select "Filter Comb", and choose the channel you wish to display. The fluorescence data must be exported separately for **each** color channel.

16. To export the melt data, right-click on the "Melting Curves" chart, and select "Export" from the menu.
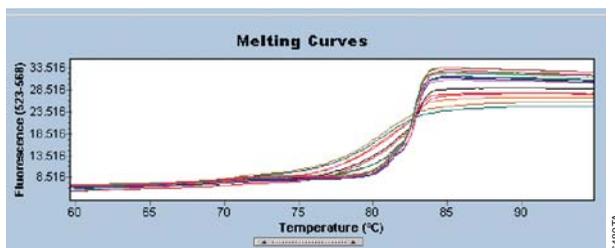
17. In the "Export" chart window, select the "Data" tab. Select "Text" as the format. Include "Point Index", "Point Labels" and "Header". The file should be tab-delimited.

18. Assign a descriptive name to the file in the "Filename" box, so that it is clear the file contains the melt data for that specific color channel. Choose the "…" box to select a location to save the file, then select "Export".

19. For multiplex reactions, each dye channel must be exported separately by repeating Steps 15–18. In Step 15, use the "Filter Comb" button to change the color channel being displayed in order to export data from each of the color channels.

20. If analysis will be done on a separate computer, save the files on removable media or an accessible network location. These exported files are now ready for use with the Plexor™ Analysis Software.

    Note: The Plexor™ Analysis Software will use the greatest change in signal throughout the imported data set to determine a signal threshold. This may affect sensitivity of assays with lower signal if multiple types of assays (in the same color) are being run simultaneously. This can be avoided by exporting the samples for each assay separately.

VIII. Data Import into the Plexor™ Analysis Software

The Plexor™ Analysis Software (Cat.# A4071) is available for download at: **www.promega.com/plexorresources/.** It is also available free-of-charge on CD-ROM by request. Software installation instructions are given in Section XI.B.

When exporting data for use with the Plexor™ Analysis Software, be sure to assign descriptive names to the files so that related amplification curve and melt curve files (e.g., files generated using the same data) can be easily identified during data import.

1. To launch the Plexor™ Analysis Software, go to the "Start" menu and select "Programs", then "Plexor"; select "Analysis Desktop".

    Note: A shortcut can be placed on the desktop by right-clicking on "Analysis Desktop", selecting "Copy", then right-clicking on the Windows® desktop and selecting "Paste Shortcut".

2. In the "File" menu, select "Import New Run" or select the icon:

3. **Optional:** Enter an assay name in the "Assay Setup" screen (Figure 1). This screen is used to enter general information about the type and format of the data that will be used for each assay.

4. Select "Roche LightCycler 480" as the "Instrument". Select either "96 Well Block" or "384 Well Block", as is appropriate for your sample run.
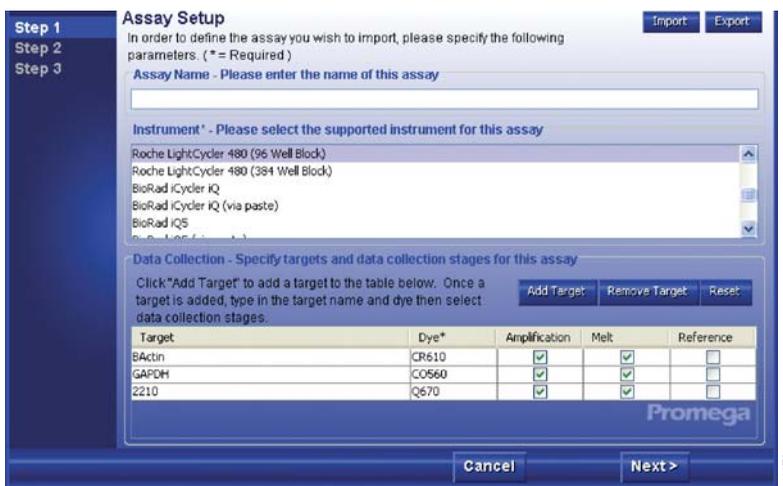
Figure 1. The Assay Setup screen.

5. Select "Add Target" for each fluorescent dye used in your assay. For each dye, assign a target name, enter the dye name and indicate that there is amplification data and dissociation (melt) data to be analyzed for that dye. The name of the dye must be the same as that in the original exported data file .

    Note: For frequently run assays, a template with the target information and dyes can be saved (Section XI.C).

6. Select "Next".

---

7. Enter information specific to your experiment in the "Run Info" screen (Figure 2). Details (date, notes, title, name of the person performing the experiment, etc.) can also be entered in the provided windows.

Figure 2. The Run Info screen.

8. Select "Next".

9. Use the "File Import" screen (Figure 3) to specify the data files exported from the instrument in Section VII. Use "Browse" to locate the appropriate exported amplification and dissociation data files.

When analyzing data with the Plexor™ Analysis Software, be sure to choose the amplification curve file and melt curve file generated using the same data. The file names assigned when exporting data must be descriptive so that the appropriate files can be easily identified and imported into the Plexor™ Analysis Software.

Note: "Advanced Options" can be used to create templates for routine plate setups and analysis conditions. See Section XI.C for details concerning these advanced options and an explanation of the default analysis settings.
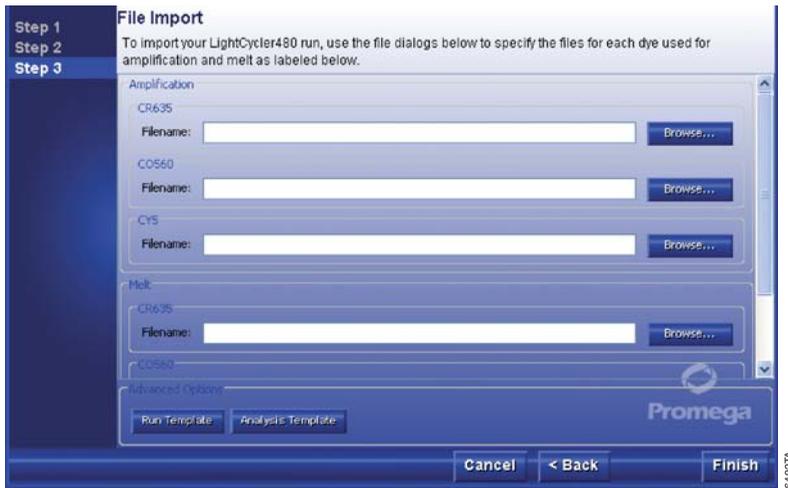
Figure 3. The File Import Screen.

10. Select "Finish" to complete the data import and to open "Analysis Desktop".

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IX. Data Analysis with the Plexor™ Analysis Software

After data import is complete (Section VIII), the "PCR Curves" tab of the "Analysis Desktop" is displayed (Figure 4).
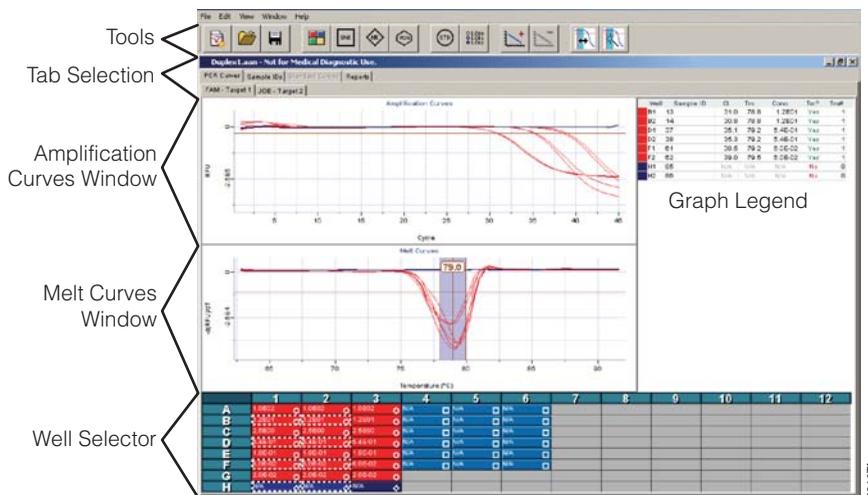
Figure 4. The PCR Curves tab of the analysis desktop. The amplification curves window, melt curves window and well selector are indicated.

IX.A. Sample Definition

1. Use the "Well Selector", which is shown in Figure 4, to select and define each well or group of wells. Choose one of the icons shown in Figure 5 to define the samples. See Notes 1–5.

2. To assign sample names, select the "Sample IDs" tab (Figure 6). To enter sample names manually, select the well, and enter the desired sample name. Repeat to enter sample names for other wells.

   To copy names from a MicroSoft® Excel spreadsheet, highlight the sample names in the spreadsheet and select "copy". In the "Edit" menu, select "Paste Sample IDs from Template" or use the control T shortcut. The layout of the sample names in the spreadsheet must be the same as the layout of the samples within the PCR plate.

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	Unknown
	No-template control
	Standard sample. The concentration is entered in a pop-up window following designation of the well as a standard.
	Selecting the wells and choosing the "Create Dilution Series" icon can automatically create a titration curve across several wells.
	Positive control
	Color assignment

Figure 5. The icons used to define samples in the Plexor™ Analysis Software.
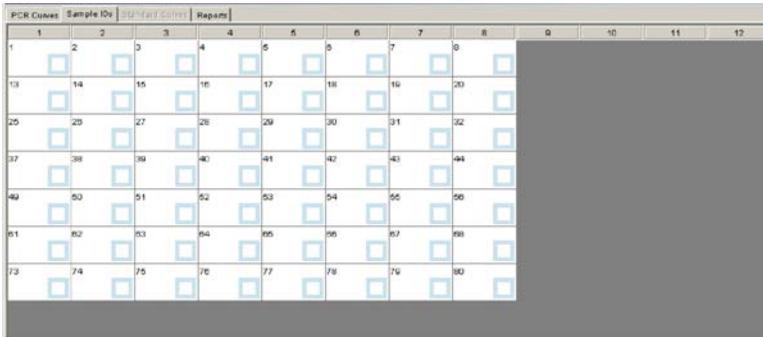
Figure 6. The Sample IDs tab.

Notes:

1. Sample definitions, color selection and concentration of standards will be entered for all dyes. To define samples separately in each dye, uncheck "Propagate Selection Across Dyes" in the "Edit" menu.

2. A sample or set of samples can be permanently deleted from a Plexor™ analysis. Go to the "PCR Curves" tab, and select the samples. Use the delete key or select "Remove Selected Wells" in the "Edit" menu.

3. All samples defined as standard reference templates must be assigned a concentration. 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.). Standard reference templates with the same concentration may be assigned simultaneously by highlighting multiple wells. The software does not accept commas in the concentration assignments.

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4. A row or column of contiguous wells within a dilution series of standard reference templates may be simultaneously assigned as standards using the "Assign Dilution Series" function by highlighting multiple wells (Figure 7). You must enter the initial concentration of the series, the dilution factor and whether the series is increasing or decreasing.

5. Colors can be assigned to samples to provide distinction to the displayed data. Select the samples, then select "color assignment" to apply a color to the selected sample(s).

   Assigning or changing a color does not alter the information associated with a sample. For example, the concentrations of standard samples will be retained if the display color associated with those samples is changed.
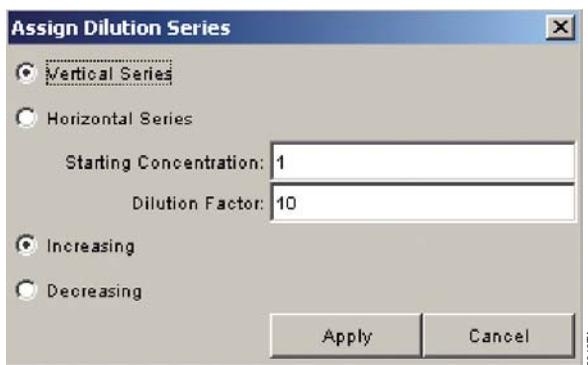
Figure 7. The Assign Dilution Series screen.

IX.B. Adjusting the Expected Target Melt Temperature

Set the expected target melt temperature and the expected target melt temperature range. Failure to set the range for the expected target melt temperature correctly will cause the results to be incorrectly reported in the graph legend and the "Reports" tab (Section IX.F). For multiplex assays, the expected melt temperature range must be adjusted for each dye.

1. Select the "PCR Curves" tab. The default setting for the expected target melt temperature is 90.0, and the default target T_m range is +/–1°C.

2. Select a well containing a standard reference template or genotyping control sample. The T_m for each selected sample will be displayed in a table to the right of the graph (Figure 8). The expected target melt temperature and associated target melt temperature range for all samples in this dye channel should be set based on the T_m of this standard or control sample. See Note 1.

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Graph Legend showing
Sample T$_m$ and
Target T$_m$ indicators

Melt Threshold
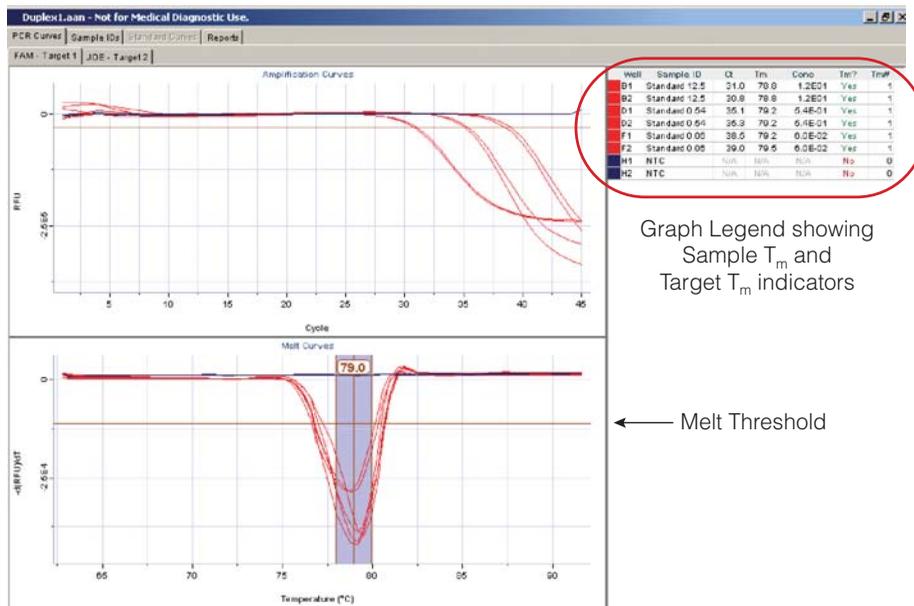
Figure 8. The expected target melt temperature is displayed in a table to the right of the graph.

3. In the melt curves window, move the mouse so the arrow is over the expected target melt temperature line, and drag it to the desired temperature. Alternatively, double-click on the line, and enter the desired temperature. See Notes 2–5.

4. **Optional:** The melt threshold may be reset to change the sensitivity in detecting the amplification product. See Note 6. The default melt threshold is set at 25% of the signal change for the sample within the set that has the greatest change in signal. In some instances, the sample(s) used to set this threshold may not be typical of the data set.

   To adjust the melt threshold line based on a selected set of samples, highlight the desired samples in the well selector. In the "Edit" menu, select "Set Melt Threshold From Selected Samples". Enter the desired percentage of signal change.

   To manually adjust the melt threshold line, place the cursor over the threshold line, and drag the line to the desired location. Alternatively, double-click on the threshold line, and enter the desired threshold value.

Notes:

1. The absence of peaks for the standard reference template or genotyping control sample indicates amplification problems. See Section X for more information about possible causes.

2. The target melt temperature range can be adjusted manually. Move the mouse so that the arrow is over the upper or lower limit, and drag the limit to the desired temperature. Alternatively, upper and lower limits can be adjusted by double-clicking on the appropriate lines and entering an exact value in the pop-up window that appears.

3. The melt threshold is the level of signal that must be reached for the Plexor™ Analysis Software to "call" the melt results. Target T_m indicators are included in the table to the right of the amplification and melt curve windows.

4. A "Yes" or "No" in the "Tm?" column indicates whether a sample T_m is within the expected target melt temperature range. A "No Call" in this column indicates the melt curve displays the correct expected target melt temperature, but there is insufficient amplification product to cause the amplification curve to cross the melt threshold.

5. The "Tm#" is the number of peaks that cross the melt threshold line. More than one peak indicates heterogeneous amplification products. This may be due to nonspecific amplification, secondary structure or a polymorphic target. See Section X for more information about possible causes.

6. Changes made to the melt threshold line will apply to the entire data set within the same dye channel, including those samples that were not selected.

IX.C. Adjusting the Y Axes of the Amplification and Thermal Melt Curves (Optional)

The scales of the Y axes for the amplification curve and melt curve in an experiment are determined by the sample that yields the most amplification product (i.e., the sample with the greatest decrease in signal). These scales are set for the entire data set.

The scale of the Y axis can be set manually by double-clicking on the Y axis of the graph and entering the new value in the pop-up window. This change will alter the scale for the entire data set.

IX.D. Adjusting the Baseline Region and Amplification Threshold Line (Optional)

The Plexor™ Analysis Software automatically sets the baseline region for each sample. The baseline is set in a flat region of the amplification curve before product accumulation. Manual adjustment of baseline is possible. See Section XI.D. for information on display and adjustment of baseline regions.

The amplification threshold is used to determine the C_t value for the samples (Figure 9). The default amplification threshold is based on the variation (noise) in the baseline regions of all samples. It is determined by taking the mean and standard deviation of all RFU values in the baseline regions and setting the threshold to 10 standard deviations below the mean.

Optional: If desired, the amplification threshold may be reset to change the sensitivity in detecting the amplification product.

1. To adjust the amplification threshold based on a selected set of samples, highlight the desired samples in the well selector. In the "Edit" menu, select "Set Amp Threshold From Selected Samples". Enter the number of standard deviations of the background within the baseline region to use. The default is 10 standard deviations.

2. To manually adjust the threshold, place the cursor over the threshold, and drag the line to the desired location. Alternatively, double-click on the threshold, and enter the desired value.

   Note: Changes made to the amplification threshold will apply to the entire data set within the same dye channel, including those samples that were not selected.

   The baseline and threshold reset button will reset the amplification threshold to the default using all samples.
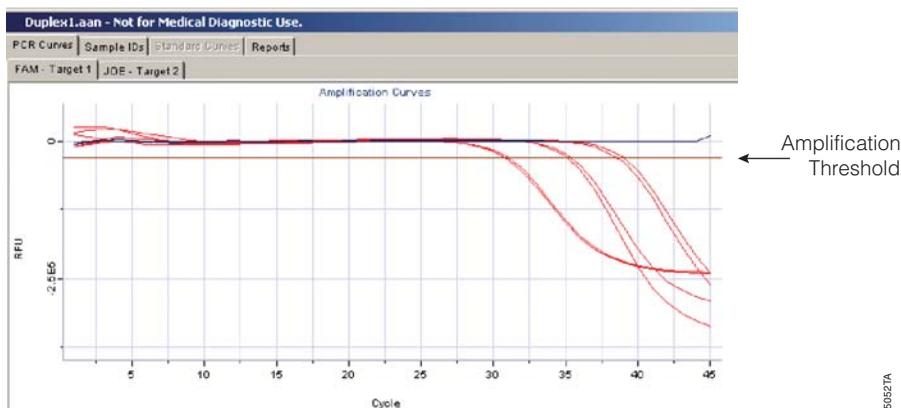
Figure 9. The amplification and melt thresholds.

IX.E. Generating a Standard Curve (Optional)

Amplification results from a dilution series of the standard reference template are used to generate a standard curve. This standard curve can be used to determine the concentration of unknown samples. A standard reference template with any unit of concentration or amount can be used to generate the standard curve. In general, copy number or mass is used, but other units that are appropriate for your experiment, such as plaque forming units or dilution factors from a known stock, can be used. Samples for generating the standard curve must be designated as standards (Section IX.A). For multiplex assays, standard curves must be generated for each dye label.

1. Select the desired standard samples and the samples you want to quantify. Select "Add Standard Curve" to generate a standard curve.

   Add Standard Curve

2. Select the "Standard Curves" tab to view the standard curve (Figure 10). The default display shows the log concentration on the Y axis and the cycle threshold on the X axis.

Alternatively the standard curve can be displayed with the cycle threshold on the Y axis and the log of the concentration on the X axis (Figure 11). To do so, select the "Standard Curves" tab to view the standard curve. In the "Edit" menu, select "Flip Std. Curve Axes". See Notes 1 and 2.

3. View the concentrations for all samples, including the unknown samples, in the table next to the standard curve graph (Figures 10 and 11). The calculated concentrations can also be viewed in the sample details report (Section IX.F).

4. Repeat Step 1 with any other desired set of standards and samples.

Notes:

1. A second standard curve can be created using a different set of samples. Repeat Step 1 using the new set of standard samples. Multiple standard curves may be created within the same data set if none of the samples and standards are shared.

   If you attempt to generate an additional standard curve using samples that are used in the current standard curve, the alert box "Confirm Standard Curve Replace" will appear. The currently assigned standard curve will be overwritten with the new standard curve if "OK" is selected.

2. An existing standard curve can be changed or additional unknowns added. To do so, delete the existing standard curve. Go to the "Standard Curves" tab, then select "Remove Standard Curve". The "Remove Standard Curve" button is only active in the "Standard Curves" tab.

   Remove Standard Curve.
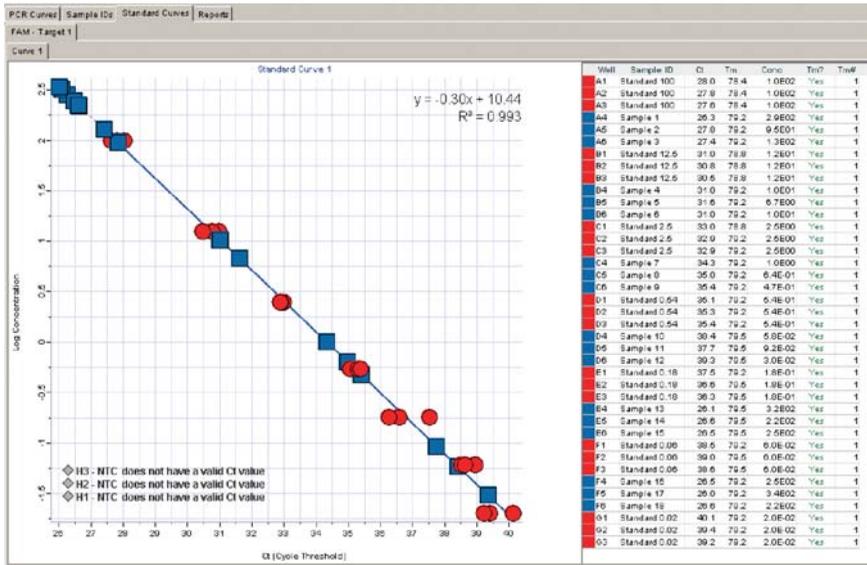
Figure 10. The Standard Curves tab. A standard curve with the log concentration on the Y axis and the cycle threshold on the X axis.
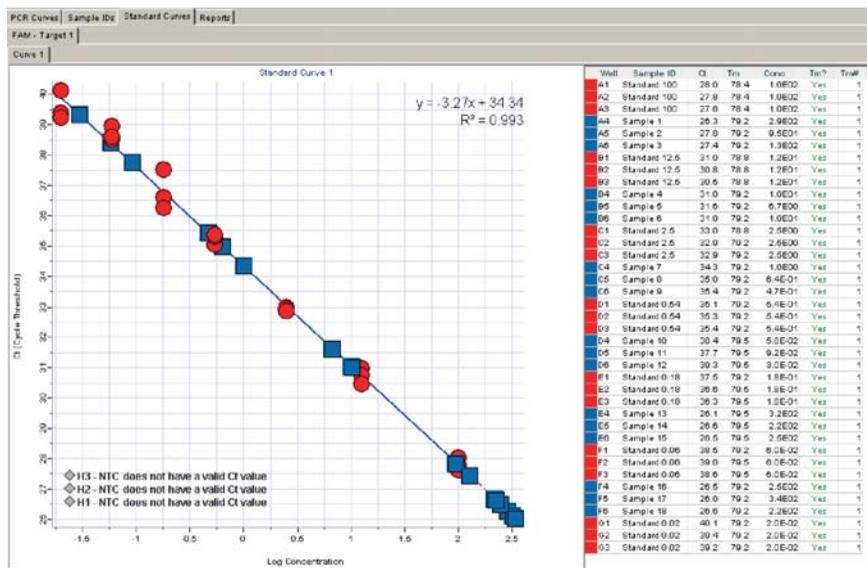
Figure 11. The Standard Curve tab. A standard curve with the cycle threshold on the Y axis and the log of the concentration on the X axis.

Note: Samples that do not cross the amplification threshold, such as the no-template controls, are listed in the graph as not having a valid C_t value.

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IX.F. Reports

The Plexor™ Analysis Software includes five report options: "Sample Details", "Thresholds", "Baseline Regions", "Run Info" and "Import Files", which are included as subtabs in the "Reports" tab. To view these report options, select the "Reports" tab. Information is presented in a tabular format that can be copied, saved or printed using the provided icons. The saved data can be opened using Microsoft® Excel.

For multiplex assays, the Plexor™ Analysis Software reports include information for all of the dye labels.

Sample Details: The sample details report includes well location, sample ID, dye channel, cycle threshold, thermal melt temperature, concentration (if applicable), whether the sample has the expected T_m and the number of melt curves that cross the melt threshold line (Figure 12).

A sample with a C_t value of "N/A" has an amplification curve that did not cross the amplification threshold.

Figure 12. The Sample Details tab.

Thresholds: The thresholds report includes the numerical values for the thresholds in the current analysis (Figure 13). This information can be used to develop an analysis template for assays where the same or similar thresholds will be used on a routine basis. See Section X.C for more information about creating an analysis template.
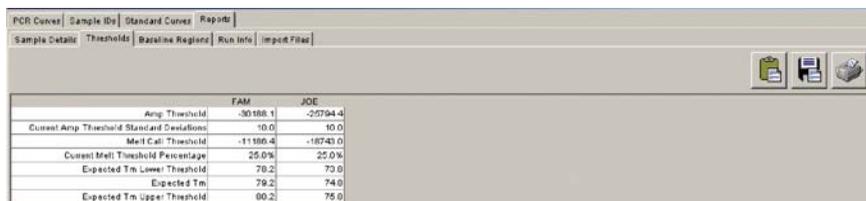
Figure 13. The Thresholds tab.

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Baseline Regions: The baseline regions report includes the numerical values for the cycle number used in each sample (Figure 14).

Run Info: The run info report includes the information from the data import (Figure 15).

Import Files: The Import Files report includes information on the data import files (Figure 16).

Figure 14. The Baseline Regions tab.
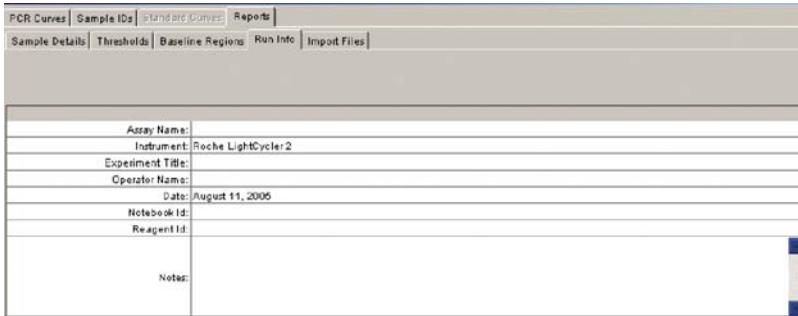
Figure 15. The Run Info tab.
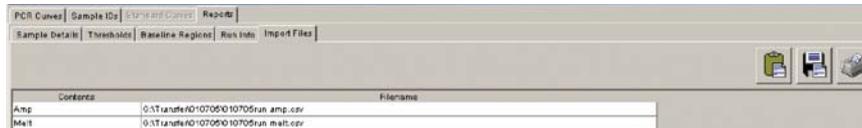
Figure 16. The Import Files tab.

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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 was 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 was 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/**

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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.
	Primers may have been synthesized incorrectly. Resynthesize primers.
	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 is 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 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/**

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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 in Section III or per manufacturer instructions.
	Decrease the primer concentration (e.g., 0.1µM).
	Be sure the thermal cycler was 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 reaction plates 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.

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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 XI.D).
	Be sure the reaction plates 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 in Section III or per manufacturer instructions.
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 in Section III or per manufacturer instructions.
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.C.

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Symptoms	Causes and Comments
Nonlinear standard curve, low R^2 values	An amplification inhibitor was 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 reaction plate to avoid evaporation.
	Aberrant fluorescence can be caused by contamination, fingerprints, etc. Do not write on the surface of the optical adhesive plate covers. Use caution when handling optical adhesive plate covers. Wear gloves.

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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 qRT-PCR Systems	Contaminating DNA sequences related to the RNA template were present in the RNA preparation. Treat the RNA Plexor™ 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 are 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.

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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 was 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 3), 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 17). 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.

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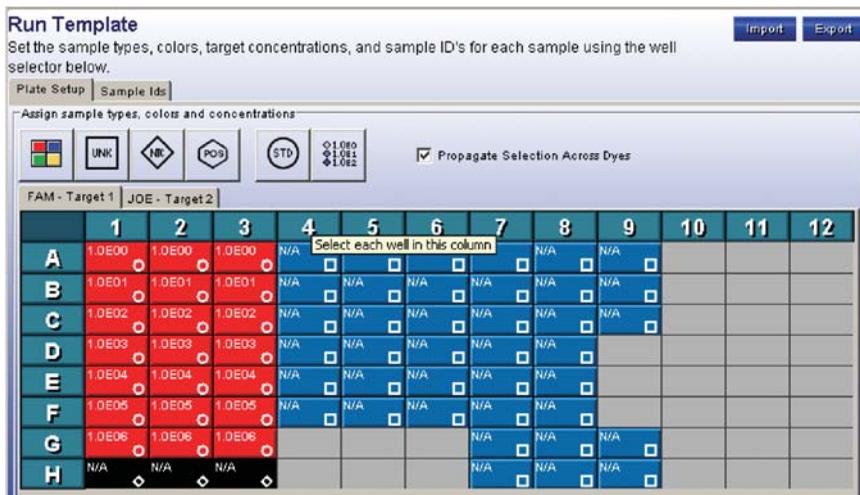
Figure 17. A Run Template.

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 18).

    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 18. 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.

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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 19). For multiplex assays, the baseline is set independently for each dye.
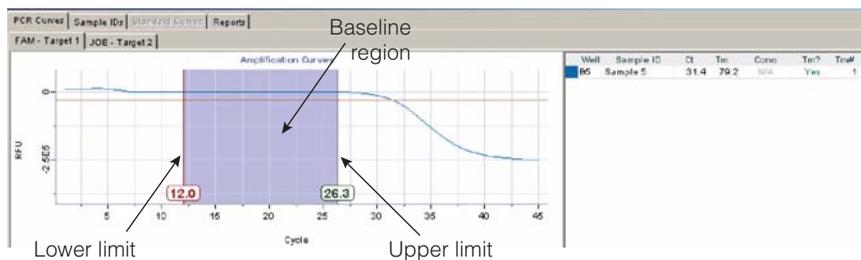
Figure 19. An amplification window showing the baseline region and baseline upper and lower limits.

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.

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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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(STD) 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.

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 XI.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.D.

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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