



**Promega**

Technical Manual

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# **Plexor<sup>®</sup> HY System for the Bio-Rad iQ<sup>™</sup>5 Real-Time PCR Detection System**

INSTRUCTIONS FOR USE OF PRODUCTS DC1000 AND DC1001



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# Plexor® HY System for the Bio-Rad iQ™5 Real-Time PCR Detection System

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## 1. Description

The Plexor<sup>®</sup> HY System<sup>(a-f)</sup> is a novel real-time PCR system for quantitation of total human (autosomal) and human male (Y) DNA within a sample. This Technical Manual describes the Plexor<sup>®</sup> HY System and provides instructions for use with the Bio-Rad iQ<sup>™</sup>5 Real-Time PCR Detection System, including reaction setup, real-time PCR instrument programming and data analysis using the Plexor<sup>®</sup> Analysis Software.

The Plexor<sup>®</sup> technology takes advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis (1-3). One of the PCR primers contains a modified nucleotide (iso-dC) linked to a fluorescent label at the 5' end. The second PCR primer is unlabeled. The reaction mix includes deoxynucleotides and iso-dGTP modified with the quencher dabcyI. DabcyI-iso-dGTP is incorporated opposite the iso-dC residue in the primer. The incorporation of the dabcyI-iso-dGTP at this position results in quenching of the fluorescent dye on the complementary strand and a reduction in fluorescence, which allows quantitation during amplification (Figure 1).

With the Plexor<sup>®</sup> technology, accumulation of product is accompanied by a decrease in fluorescence as shown in Figure 2.

The Plexor<sup>®</sup> technology allows the use of a melt curve or dissociation curve to determine the melting temperature ( $T_m$ ) of the products following amplification (Figure 3). This is useful in assessing the specificity of the reaction.

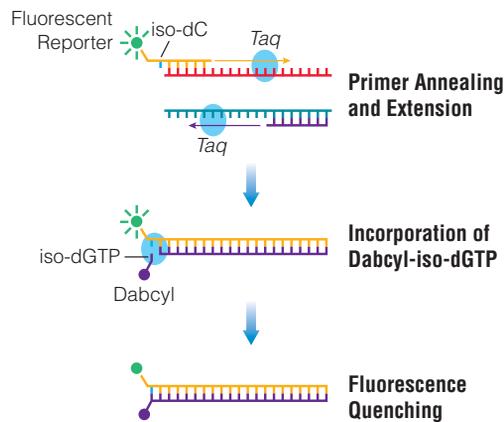
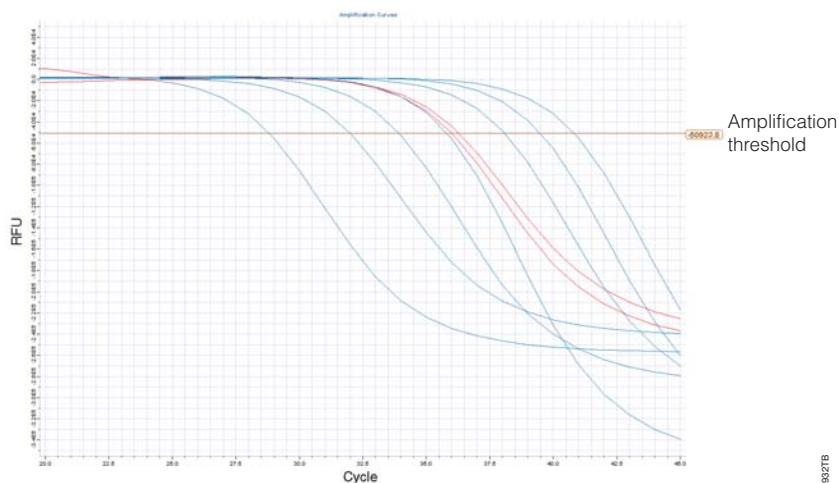
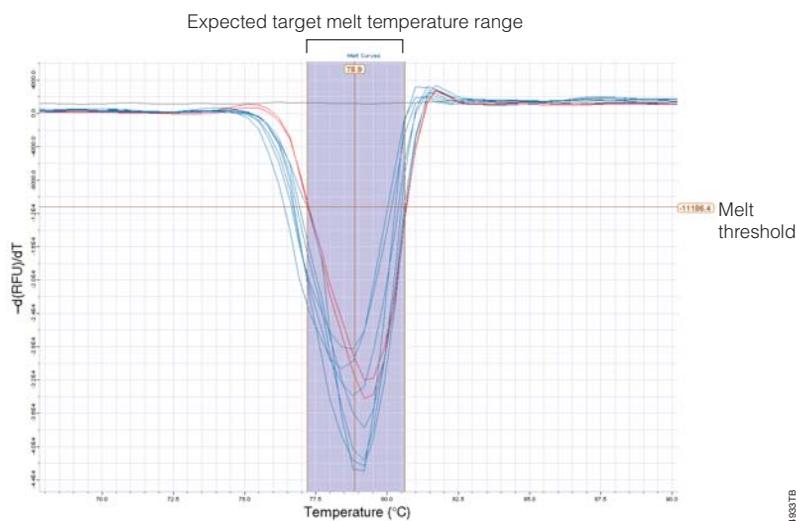


Figure 1. Schematic diagram illustrating the Plexor<sup>®</sup> real-time PCR process.



**Figure 2. Representative Plexor® amplification curves.** The amplification curves show the fluorescence (in relative fluorescence units, RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This line is used to determine the cycle threshold ( $C_t$ ) for the samples.



**Figure 3. Representative Plexor® melt curves.** Melting temperature is empirically determined by plotting the change in fluorescence with temperature ( $-dRFU/dT$ ) versus temperature and determining the temperature at which the greatest rate of change in fluorescence occurs.

The fluorescein dye of the Plexor® HY System is used to detect a human autosomal DNA target. The primers amplify a multicopy, 99bp target on chromosome 17. Data from this reaction are used to quantify the total amount of human DNA in a sample.

The CAL Fluor® Orange 560 dye of the Plexor® HY System is used to detect a Y-chromosomal DNA target. The primers amplify a multicopy, 133bp target on the Y chromosome. Data from this reaction are used to quantify the total amount of human male DNA in a sample.



## 1. Description (continued)

The CAL Fluor® Red 610 dye of the Plexor® HY System is used to detect the Internal PCR Control (IPC), which is added to every reaction. The primers amplify a novel DNA sequence added as a template to the reaction mix. The amplified product is 150 base pairs. Data from this target are used to monitor inhibition of amplification in samples.

The IC5 dye of the Plexor® HY System is used as a passive reference. IC5-labeled reagent is added to the reaction mix for every sample. Data from the three amplification channels (fluorescein, CAL Fluor® Orange 560 and CAL Fluor® Red 610) can be normalized to this signal.

The Plexor® HY System uses a hot-start chemistry. Reaction setup can be performed at room temperature and is amenable to automation.

## 2. Product Components and Storage Conditions

Product	Size	Cat.#
Plexor® HY System	200 reactions	DC1001

Not for Medical Diagnostic Use. Includes:

- 2 × 1ml Plexor® HY 2X Master Mix
- 200µl Plexor® HY 20X Primer/IPC Mix
- 150µl Plexor® HY Male Genomic DNA Standard, 50ng/µl
- 2 × 1.25ml Water, Amplification Grade
- 1 Protocol

Product	Size	Cat.#
Plexor® HY System	800 reactions	DC1000

Not for Medical Diagnostic Use. Includes:

- 8 × 1ml Plexor® HY 2X Master Mix
- 4 × 200µl Plexor® HY 20X Primer/IPC Mix
- 3 × 150µl Plexor® HY Male Genomic DNA Standard, 50ng/µl
- 5 × 1.25ml Water, Amplification Grade
- 1 Protocol

**Storage Conditions:** Store the Plexor® HY System at -20°C. Once thawed, store the Plexor® HY Male Genomic DNA Standard at 4°C; do not refreeze. For short-term storage, less than 1 week, the other components of the system can be stored at 4°C. Minimize the number of freeze-thaw cycles.

### Item Available Separately

Product	Cat.#
Plexor® Calibration Kit, Set A	DC1500

- 50µl Plexor® Concentrated Fluorescein Calibrator
- 50µl Plexor® Concentrated CAL Fluor® Orange 560 Calibrator
- 50µl Plexor® Concentrated CAL Fluor® Red 610 Calibrator
- 50µl Plexor® Concentrated IC5 Calibrator
- 11ml Plexor® 1X Calibration Buffer

### 3. General Considerations

The key to the Plexor® technology is the quenching of a fluorescent reporter due to the site-specific incorporation of dabcyI-iso-dGTP. As a result, the fluorescent signal from a Plexor® HY reaction decreases as PCR product accumulates. Real-time instrument software can record the quenching data, but most cannot determine amplification thresholds, threshold cycle ( $C_t$ ) or melting temperatures. Data analysis is accomplished by exporting the data to the Plexor® Analysis Software. The Plexor® Analysis Software is compatible with data from all supported instruments. The software is compatible with Windows® 98 and later operating systems.

Before using the Plexor® HY System, be sure you have the forensic release of the Plexor® Analysis Software. All instructions in this Technical Manual refer to the forensic release of the software. The Plexor® Analysis Software and instructions for software installation can be downloaded free of charge from the Promega Web site at: [www.promega.com/plexorhy/](http://www.promega.com/plexorhy/)

We recommend using designated work areas and pipettes for pre- and postamplification steps to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acid from one run to the next. Wear gloves, and change them often. Prevent contamination by using aerosol-resistant pipette tips. Always include a no-template control (NTC) reaction to detect contamination. We recommend performing NTC reactions in duplicate.

 Do not unseal reaction plates after amplification is complete. Unsealing the plates increases the risk of contaminating subsequent reactions with amplified product.

### 4. Calibration Protocol

Before using the Plexor® HY System, the instrument must be calibrated for fluorescein, CAL Fluor® Orange 560, CAL Fluor® Red 610 and IC5. The Plexor® Calibration Kit, Set A, includes aliquots of these four calibrators at a 100X concentration, along with a calibration buffer for use as a diluent to create a pure dye calibration plate.

Before performing the pure dye calibration, perform the mask and background calibrations as recommended by the instrument manufacturer. Follow the guidelines in the iQ™5 optical system software help file or instrument manual (see Notes 1 and 2).

#### Materials to Be Supplied By the User

- Plexor® Calibration Kit, Set A (Cat.# DC1500)
  - real-time PCR instrument and related consumables, such as the appropriate PCR plate and plate film or caps
1. Thaw the four Concentrated Calibrators (fluorescein, CAL Fluor® Orange 560, CAL Fluor® Red 610 and IC5) and Calibration Buffer.
  2. Vortex the Concentrated Calibrators and Calibration Buffer to mix.
  3. For each spectral calibrator, dilute 5µl of Concentrated Calibrator in 495µl of Calibration Buffer.
  4. Vortex the diluted spectral calibrators for 3-5 seconds to mix.
  5. For each diluted spectral calibrator, dispense 20µl to each well in a separate column of a 96-well optical plate (8 wells per dye).
  6. Apply the appropriate plate seal. Record the bar code number or mark the side of the plate skirt to designate the Plexor® HY spectral calibration plate.

4. **Calibration Protocol (continued)**
7. Centrifuge plate briefly. Protect plate from light.
8. Follow the protocol in the instrument guide to perform pure dye calibration with the Plexor® HY calibration plate. The protocol is summarized below with information specific for Plexor® HY dye calibration:
  - a. Open the iQ™5 optical system software. Select the Calibration Module, then the Pure Dye tab. Select “Create New” in the Plate Setup window (Figure 4, lower right panel).
  - b. Choose “Select/Add Fluorophores” to launch the Fluor Selector (Figure 5). Select/add the Plexor® HY fluors, specifying the following names and filter sets:
    - Fluorescein: “FAM”, Filter Position 2
    - CAL Fluor® Orange: “CO560”, Filter Position 3
    - CAL Fluor® Red “CR610”, Filter Position 5
    - IC5: “IC5”, Filter Position 6
 Select “OK”.
  - c. In the Plate Setup Editor, specify the wells containing each dye (Figure 6).
    - In the Plate Setup Editor, click the FAM button in the Fluorophore list to select the first fluorophore (FAM™).
    - Select “PureDye” above the plate map. Use the cursor to define sample wells with the selected fluorophore (click on the column header to fill in an entire column, or drag across wells).
    - Select the next fluorophore (e.g., CO560) from the Fluorophore list. Define wells as above. Repeat with the remaining two fluorophores (e.g., CR610 and IC5).
  - d. Specify “Sample Volume” (20), “Seal Type” and “Vessel Type”. Give the file an appropriate name (e.g., “PlexorHYPureDye”), then select “Save & Exit Plate Editing”.
  - e. Select “Run Pure Dye Calibration”.

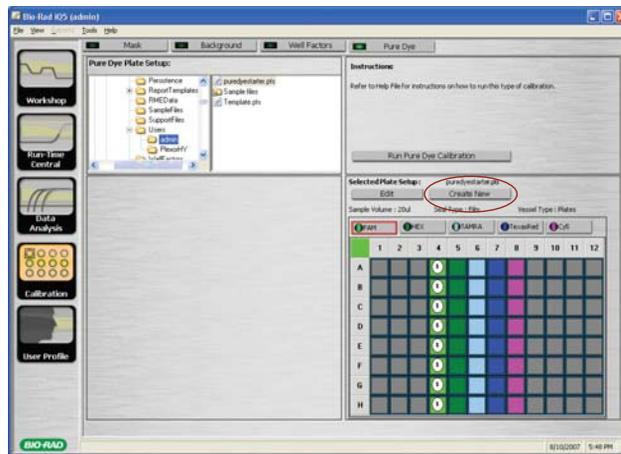


Figure 4. The iQ™5 Calibration Module, Pure Dye tab.

**Notes:**

1. It is not necessary to perform the external well factor calibration (see Note at the end of Section 6.C).
2. For mask alignment, the 1X fluorescein calibrator from the Plexor® Calibration Kit may be substituted for the Bio-Rad 10X external well factor solution to prepare the mask alignment plate. Filter position 2 would still be used.
3. After calibration, store the calibration plate, protected from light, at -20°C. Reuse the plate if reanalysis is necessary.

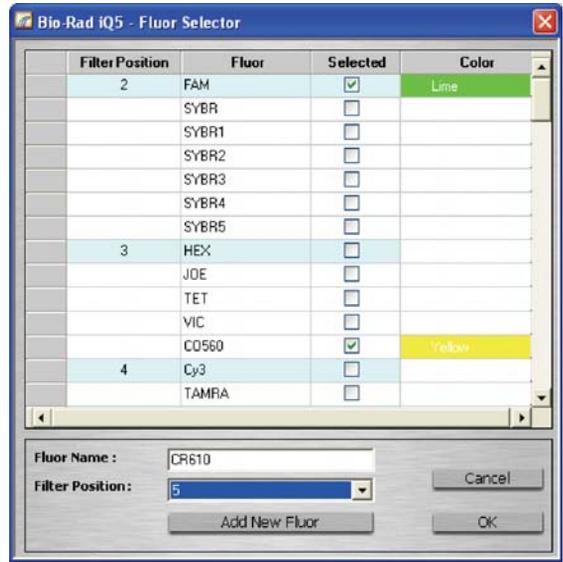


Figure 5. The iQ™5 Fluor Selector.

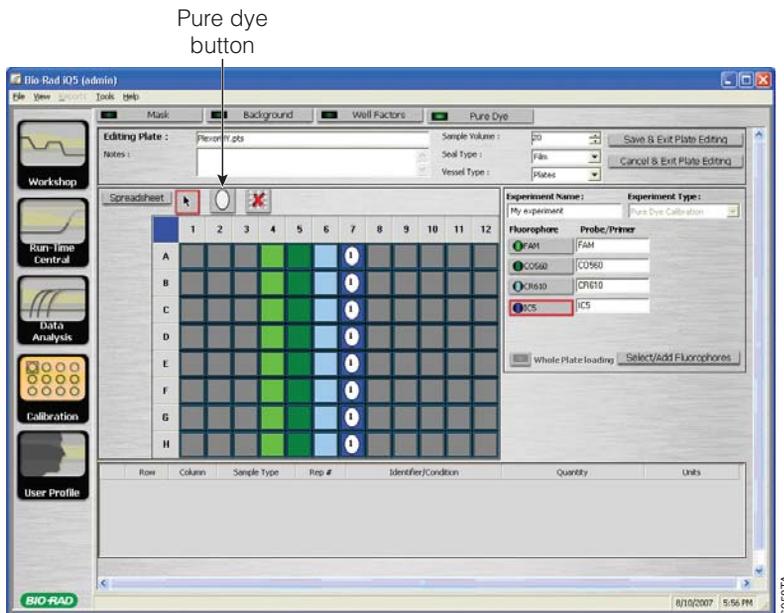


Figure 6. The Plate Setup Editor.

## 5. Reaction Plate Setup

When using the Plexor® HY System for the first time, we recommend programming the instrument (Section 6) before assembling the reactions. Once you are familiar with the programming process, the instrument can be programmed after reaction setup.

### Materials to Be Supplied By the User

(Solution compositions are provided in Section 10.J.)

- sterile, aerosol-resistant pipette tips
- pipettes dedicated to preamplification work
- real-time PCR instrument and related consumables, such as the appropriate PCR plate and plate covers or caps
- TE<sup>-4</sup> buffer
- Plexor® Analysis Software (forensic release)

### 5.A. Template Preparation

Change gloves often, especially after handling high-concentration DNA. These instructions use 2µl of template DNA per reaction. The sample volume can be increased as long as the final reaction volume stays constant. Sample volumes and final reaction volumes must be the same for both the DNA standards and the unknown samples, allowing the DNA standards to be considered as a concentration (in ng/µl) instead of input amount in ng (ng/µl × volume). The standard curve described below uses DNA standards in the range of 3.2pg/µl to 50ng/µl. These values can be modified if desired. See Section 10.B.

#### Sample DNA Templates (Unknowns)

Use 2µl of sample DNA per reaction. Performing duplicate analysis of each sample DNA and averaging the quantitation results can reduce variability. The sample DNA can be diluted in TE<sup>-4</sup> buffer, if desired.

#### DNA Standards

The Plexor® HY System is supplied with the Plexor® HY Male Genomic DNA Standard, 50ng/µl. This DNA is a mixture of human male DNA. Use of other DNA (i.e., DNA isolated from a cell line) as a standard is not recommended.

Serial dilutions of the DNA standard are amplified in the same plate as the unknown samples, and the results are used to generate a standard curve in the autosomal (fluorescein) and Y (CAL Fluor® Orange 560) channels to determine the concentrations of unknown samples. We recommend performing duplicate or triplicate amplification reactions with each dilution of the DNA standard. Use of a dedicated set of pipettes can increase run-to-run consistency. Use the same pipettes to dispense the DNA standards and unknown samples.

Multiple freeze-thaw cycles of the Plexor® HY Male Genomic DNA Standard, 50ng/µl, can increase variability in the standard curve. High-molecular-weight DNA may form aggregates or concentration gradients when frozen, which may result in sampling error. Store the DNA standard at 4°C overnight before the first use, and vortex prior to use. We recommend long-term storage at 4°C.

1. Thaw and vortex the Plexor® HY Male Genomic DNA Standard, 50ng/μl, at high speed for 5 seconds. After initial thawing, store at 4°C.
2. Prepare fresh serial dilutions of the Plexor® HY Genomic DNA Standard as indicated in Table 1. Vortex each dilution for 3–5 seconds before removing an aliquot for the next dilution. Be sure to change pipette tips between dilutions. Change gloves after handling high-concentration DNA.

**Table 1. Serial Dilution of the Plexor® HY Genomic DNA Standard.**

Concentration	Volume of DNA	Volume of TE <sup>-4</sup> Buffer
50ng/μl	Use undiluted DNA	0μl
10ng/μl	10μl of undiluted DNA	40μl
2ng/μl	10μl of 10ng/μl dilution	40μl
0.4ng/μl	10μl of 2ng/μl dilution	40μl
0.08ng/μl	10μl of 0.4ng/μl dilution	40μl
0.016ng/μl	10μl of 0.08ng/μl dilution	40μl
0.0032ng/μl	10μl of 0.016ng/μl dilution	40μl

### No-Template Control

Include a no-template control (NTC) reaction for each set of reactions. Add 2μl of TE<sup>-4</sup> buffer to these reactions in place of the template DNA. There should be no specific amplification product (e.g., <1.0pg/μl with a 2μl input volume) detected in the NTC reaction. Amplification of >1.0pg/μl of DNA in the NTC reaction indicates nonspecific amplification or the presence of contaminating DNA.

**Note:** The Plexor® HY System is extremely sensitive. The NTC reaction may show amplification products in the subpicogram range.

### 5.B. Reaction Setup

1. Thaw the Plexor® HY 2X Master Mix, Plexor® HY 20X Primer/IPC Mix and Water, Amplification Grade.
 

**Note:** Do not thaw the Plexor® HY 2X Master Mix and Plexor® HY 20X Primer/IPC Mix at temperatures above room temperature.
2. Briefly vortex the Plexor® HY 2X Master Mix and Plexor® HY 20X Primer/IPC Mix for 3–5 seconds to mix. Do not centrifuge after vortexing, as this may cause the primers to be concentrated at the bottom of the tube.
3. Determine the number of reactions to be set up. This should include negative control reactions. Add 1 or 2 reactions to this number to compensate for pipetting error. While this approach does require using a small amount of extra reagent, it ensures that you will have enough PCR master mix for all samples. It is critical that the same reaction mix is used for the entire run.
4. Prepare the reaction mix by combining the Water, Amplification Grade, Plexor® HY 2X Master Mix and Plexor® HY 20X Primer/IPC Mix as

## 5.B. Reaction Setup (continued)

indicated in Table 2. The reaction mix will be used to perform the NTC reaction and amplify samples and DNA standards. To amplify a larger input template volume, see Section 10.B.

**Table 2. Preparation of Reaction Mix for Quantitation Assays (assumes 2 $\mu$ l of template per reaction).**

Component	Volume (Per Reaction)
Plexor <sup>®</sup> HY 2X Master Mix	10 $\mu$ l
Water, Amplification Grade	7 $\mu$ l
Plexor <sup>®</sup> HY 20X Primer/IPC Mix	1 $\mu$ l
final volume	18 $\mu$ l

5. Vortex briefly to mix.
6. Add 18 $\mu$ l of the reaction mix prepared in Step 4 to each of the appropriate wells of an optical-grade PCR plate.
7. Add 2 $\mu$ l of TE<sup>-4</sup> buffer to the NTC reactions (Figure 7).
8. Add 2 $\mu$ l of DNA standard or unknown sample to the reaction mix in the appropriate wells (Figure 7). Duplicate amplification of standards is required. Duplicate analysis of unknown samples and subsequent averaging of the DNA concentration will reduce the impact of variability due to pipetting, sampling or amplification.
9. Apply the appropriate plate seal.
10. Centrifuge the plate briefly to collect the contents of the wells at the bottom. The plate is ready for thermal cycling. Protect the plate from extended light exposure or elevated temperatures before cycling. Handle the plate by the edges, and avoid touching the bottom of the plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50 <sup>ng</sup> / $\mu$ l	50 <sup>ng</sup> / $\mu$ l	UNK									
B	10 <sup>ng</sup> / $\mu$ l	10 <sup>ng</sup> / $\mu$ l	UNK									
C	2 <sup>ng</sup> / $\mu$ l	2 <sup>ng</sup> / $\mu$ l	UNK									
D	0.4 <sup>ng</sup> / $\mu$ l	0.4 <sup>ng</sup> / $\mu$ l	UNK									
E	0.08 <sup>ng</sup> / $\mu$ l	0.08 <sup>ng</sup> / $\mu$ l	UNK									
F	0.016 <sup>ng</sup> / $\mu$ l	0.016 <sup>ng</sup> / $\mu$ l	UNK									
G	0.0032 <sup>ng</sup> / $\mu$ l	0.0032 <sup>ng</sup> / $\mu$ l	UNK									
H	TE <sup>-4</sup>	TE <sup>-4</sup>	UNK									

5890MB

**Figure 7. Schematic diagram showing distribution of the diluted DNA standard and unknown samples (UNK) in a 96-well PCR plate.**

## 6. Bio-Rad iQ<sup>TM</sup>5 System Setup

### 6.A. Thermal Cycling Program

The thermal cycling program is shown in Table 3.

Table 3. Thermal Cycling Program.

Step	Temperature	Time	Number of Cycles
“Well Factor” collection from experimental plate	Automatically inserted into program (90 seconds at 90°C)		
Initial denaturation:	95°C	30 seconds	1 cycle
Denaturation:	95°C	5 seconds	38 cycles
Annealing and extension:	60°C	35 seconds	
Melt curve:	Use the default “Melt Curve” settings.		

1. Open the iQ<sup>TM</sup>5 optical system software. Select the Workshop Module, then Setup tab (Figure 8). Select “Create New” in the Protocol window to open the Protocol Editor.

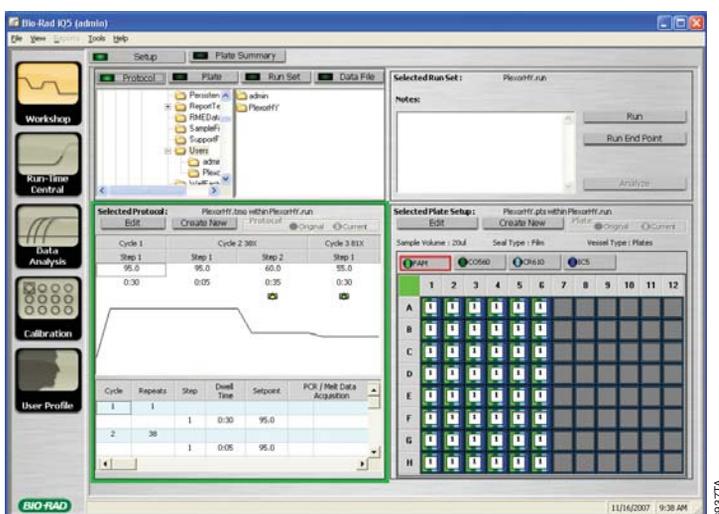


Figure 8. The iQ<sup>TM</sup>5 Workshop module, Setup tab, displaying the browser window at upper left, the selected Protocol window (active) at bottom left, the selected Plate Setup window at bottom right, and the selected Run Set window at upper right.

## 6.A. Thermal Cycling Program (continued)

2. Define the Plexor® HY profile by editing the default settings of the protocol template in the table at the bottom of the Protocol Editor window (Figure 9):
  - a. In Cycle 1, Step 1, change “Dwell Time” to 0:30 by clicking in the appropriate cell.
  - b. In Cycle 2, change “Repeats” to 38.
  - c. In Cycle 2, Step 1, change “Dwell Time” to 0:05.
  - d. In Cycle 2, Step 2, change “Dwell Time” to 0:35 and “Setpoint” to 60.0.
  - e. Add Cycle 3 by clicking on the “+” button in the Insert column in the Cycle 2 row (cycle rows have a blue background). This will insert a cycle containing 1 repeat of 1 step at the end.
  - f. Define Cycle 3 by choosing the Melt Curve option from the pulldown menu in the Data Acquisition column. Use the default Melt Curve profile (55°C to 95°C in 0.5°C steps).
3. Enter an appropriate file name (e.g., “Plexor HY”), and select “Save & Exit Protocol Editing” to save the protocol to an appropriate directory. The new protocol is now active in the Protocol window in the Setup tab.

**Note:** After programming the Protocol and Plate setup files, the user can link them in a Run Set file to simplify initiating Plexor® HY runs. Runs can be initiated by selecting the appropriate Run Set file, editing the Plate file, if necessary (defining occupied sample wells), and then initiating the run. See Note at the end of Section 6.B.

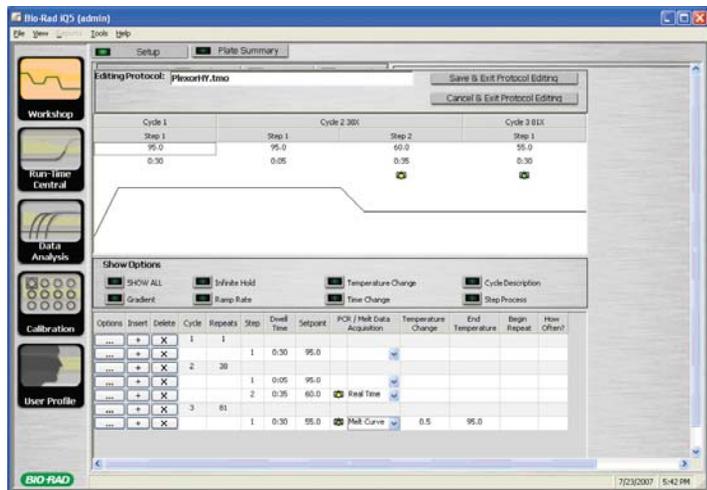


Figure 9. The iQ™5 Protocol Editor, displaying the correct Plexor® HY protocol.

## 6.B. Plate Setup

1. In the Workshop module Setup tab, select “Plate”, and then select “Create New” in the selected Plate Setup window (Figure 8, lower right panel). The Plate Setup Editor will launch (Figure 10).

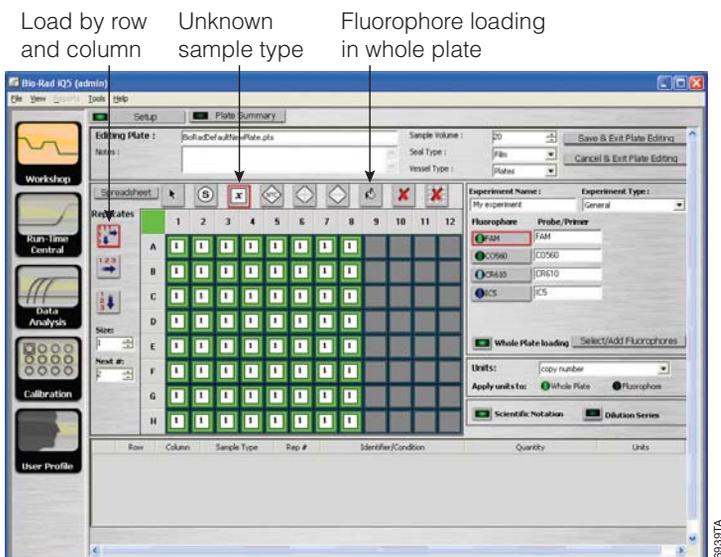


Figure 10. The iQ™5 Plate Setup Editor.

2. In the Plate Setup Editor, select “Select/Add Fluorophores” to launch the Fluor Selector. Select “FAM”, “CO560”, “CR610”, and “IC5”, and select “OK”.
3. Fill in the plate map:
  - a. In the Plate Setup Editor, select a button in the Fluorophore list to select the first fluorophore (e.g., FAM).
  - b. Select the Unknown sample type button above the plate map. Select the replicate loading type desired from the options on the left of the plate map (e.g., “By Row and Column”). Use the cursor to define sample wells with the selected fluorophore (click and drag across the plate).

⚠ Do not highlight wells that are not being used (i.e., wells that do not contain reaction mix). Including unused wells will significantly affect the scale of the Y axis when viewing the data.

  - c. Select the next fluorophore (e.g., CO560) from the Fluorophore list. Select “Fluorophore loading in whole plate mode” at the top (Paint Bucket icon). Click on the colored cell in the upper left corner of the plate map to designate that fluor in all mapped sample wells.
  - d. Repeat previous step with the remaining two fluorophores (i.e., CR610 and IC5).

## 6.B. Plate Setup (continued)

4. Specify "Sample Volume" (20 $\mu$ l), "Seal Type" and "Vessel Type".
5. Enter any notes about the plate setup in the Notes box. Specify a name for the plate setup file, then select "Save & Exit Plate Editing".
6. The "Plate Editor" will close, and the display will return to the Setup view in the Workshop module.

**Note:** We advise against designating sample types (e.g., standard, NTC, positive control) or naming samples during setup in the iQ<sup>TM</sup>5 optical system software, as this information will not be imported into the Plexor<sup>®</sup> Analysis Software with the data. Sample names can be entered in the Plexor<sup>®</sup> Analysis Software or copied and pasted into the Plexor<sup>®</sup> Analysis Software from a Microsoft<sup>®</sup> Excel spreadsheet. If the user wishes to record sample names in the iQ<sup>TM</sup>5 plate file, we suggest names be entered into a Microsoft<sup>®</sup> Excel spreadsheet and imported into the iQ<sup>TM</sup>5 optical system software during setup. To perform this function, refer to the guidelines in the iQ<sup>TM</sup>5 optical system software help file or instrument manual. The same Excel file can then be used to import sample information into the Plexor<sup>®</sup> Analysis Software.

**Note:** After programming the Protocol and Plate setup files, the user can link them in a Run Set file to simplify initiating Plexor<sup>®</sup> HY runs. Runs can be initiated by selecting the appropriate Run Set file, editing the Plate file, if necessary (defining occupied sample wells), and then initiating the run.

- a. Ensure that the appropriate Protocol and Plate setup files are selected in the Setup tab in the Workshop module.
- b. From the "File" pulldown menu, select "New", then "Run Set".
- c. When prompted, give the Run Set file an appropriate name and specify the desired file directory.
- d. Select "Save".

## 6.C. Initiating a Run

1. Ensure that the appropriate Protocol and Plate Setup files are selected in the Setup tab in the Workshop module. This can be most easily done by selecting the Run Set tab and using the browser to select the appropriate Run Set file.

**Note:** Make sure that the appropriate sample wells have been mapped in the active Plate Setup file. Including empty wells will significantly affect data presentation in Plexor<sup>®</sup> Analysis Software. Use the Edit button in the Plate window to launch the Plate Setup Editor, and change the mapped wells if necessary.

2. Select "Run" in the Run Set window in the Setup tab of the Workshop module (Figure 8).

- The Initiate Run window from the Run-Time Central module will open (Figure 11). Select the “Collect Well Factor from Experimental Plate” radio button (see Note at the end of this section).

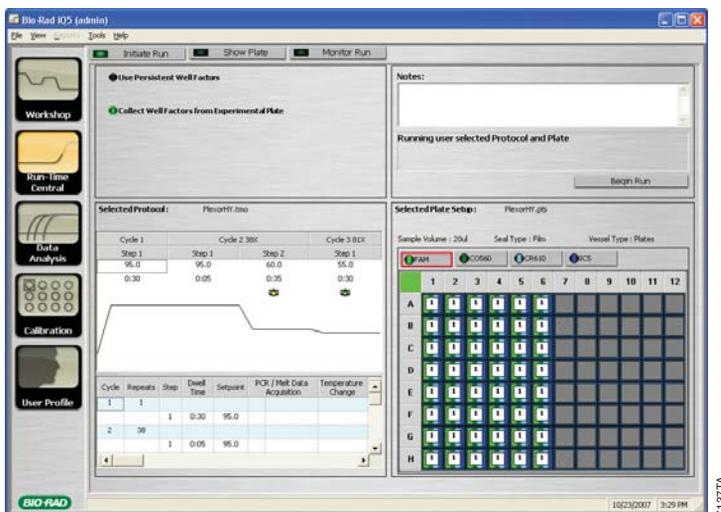


Figure 11. The iQ™5 Initiate Run window in Run-Time Central module.

- Select “Begin Run”.
- A “Save” dialog box will open; specify a name and directory to save the optical data (.opd) file. Select “Save”.
- Upon initiating the run, the iQ™5 optical system software will open the Monitor Run window.

**Note:** The iQ™5 system uses a “well factor” collection process to normalize signal intensity across a plate. Before starting a run, the iQ™5 system software will require the user to designate the source of this data, either the experimental plate or an external well factor plate (a plate analyzed separately, during an optional calibration step). The experimental plate method is the simplest and is recommended for Plexor® HY. When this method is specified, the internal well factor data are collected during an initial 90-second process (at denaturing temperatures), after which the programmed thermal profile is initiated.

## 7. Data Export from the iQ™5 Optical System Software and Import into the Plexor® Analysis Software

After initial processing in the iQ™5 optical system software (Section 7.A), data may be transferred from the iQ™5 optical system software to the Plexor® Analysis Software in two ways. The recommended method is to copy and paste data from the iQ™5 optical system software into the Plexor® Analysis Software (Section 7.B). Alternatively, data may be exported from the iQ™5 optical system software into Microsoft® Excel. The Excel files are saved, then imported into the Plexor® Analysis Software (Section 7.C).

## 7.A. Initial Data Processing in the iQ™5 Optical System Software

1. Upon completing the run, the iQ™5 optical system software will open a Run Status window (Figure 12). Choose “Yes” to display the completed run in the Data Analysis module. An automated analysis will be conducted, and the data will be presented in the PCR Quant tab of the Data Analysis module (Figure 13).
2. The default analysis presents data as “PCR Base Line Subtracted Curve Fit”. Select “Background Subtracted” from the Analysis Mode pulldown menu.
3. Select “Results”. Data for the first target dye (FAM™) will be presented in the spreadsheet in the lower window.
4. Select the Amplification Data/RFU tab.
5. Amplification data from these spreadsheets will be copied or exported for Plexor® analysis.
6. Data for other dyes in the assay can be viewed by selecting the corresponding button at the bottom of the screen, then selecting the Amplification Data/RFU tab.

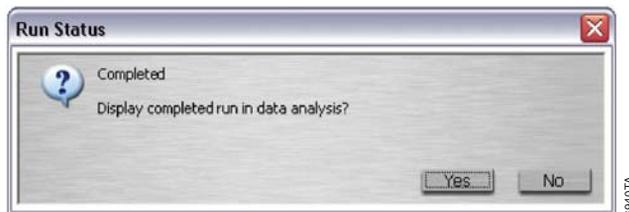


Figure 12. The iQ™5 Run Status window.

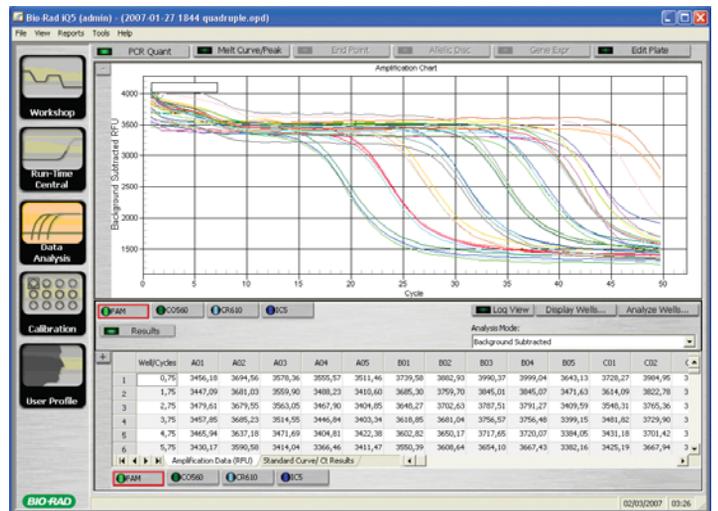


Figure 13. The PCR Quant tab of the iQ™5 Data Analysis module. Results view is selected; the Amplification Data (RFU) spreadsheet is displayed.

7. To view melt data, select “Melt Curve/Peak” in the Data Analysis module (Figure 14). Melt data for the first target dye (FAM™) will be presented in the spreadsheet in the lower window.
8. Select the  $-d(\text{RFU})/dT$  tab.
9. Melt data from these spreadsheets will be copied or exported for Plexor® analysis.
10. Data for other dyes in the assay can be viewed by selecting the corresponding button at the bottom of the screen, then selecting the  $-d(\text{RFU})/dT$  tab.

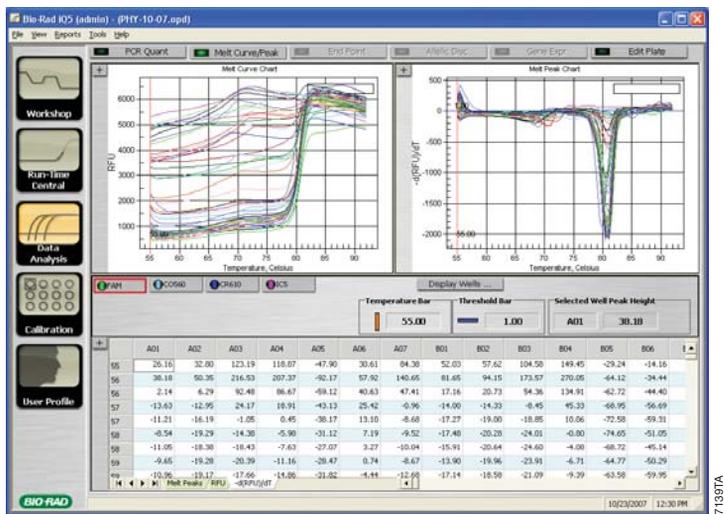


Figure 14. The Melt Curve/Peak tab of the iQ™5 Data Analysis module.

### 7.B. Pasting Data Directly into the Plexor® Analysis Software

This method requires that the iQ™5 optical system software and Plexor® Analysis Software both be open on the instrument workstation.

**Note:** Two sets of data (one for the amplification data and one for the melt data) must be copied from the iQ™5 optical system software and pasted into the Plexor® Analysis Software for each of the four dyes in the assay.

1. Launch the Plexor® Analysis Software; go to the Start menu and select “Programs”, then “Plexor”, then 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: 

**Note:** Make sure “Set Passive Reference on Import” in the “File” pulldown menu is selected.

3. **Optional:** Enter an assay name in the Assay Setup screen, Step 1 (Figure 15). This screen is used to enter general information about the type and format of the data that will be used for each assay.

## 7.B. Pasting Data Directly into the Plexor® Analysis Software (continued)



Figure 15. The Assay Setup screen when pasting data directly into the Plexor® Analysis Software.

4. Select “BioRad iQ™5 (via paste)” as the instrument type.
5. Select “Add Target” four times.
6. For each dye, enter a target name and select the dye name from the pulldown menu (e.g., “Auto” and FAM, “Y” and CO560, “IPC” and CR610, and “passive reference” and IC5). Indicate there is amplification data and dissociation (melt) data to be analyzed for the FAM™, CO560 and CR610 dyes.
7. Indicate that the IC5 data are reference data by checking “Pass. Ref.”. (The Amplification and Melt boxes for this target will automatically become deselected.)
8. Select “Next”.
9. **Optional:** Enter information specific to your run in the Run Info screen, Step 2 (Figure 16). Details such as the date, notes, title, name of the researcher, etc., can also be entered in the provided windows.

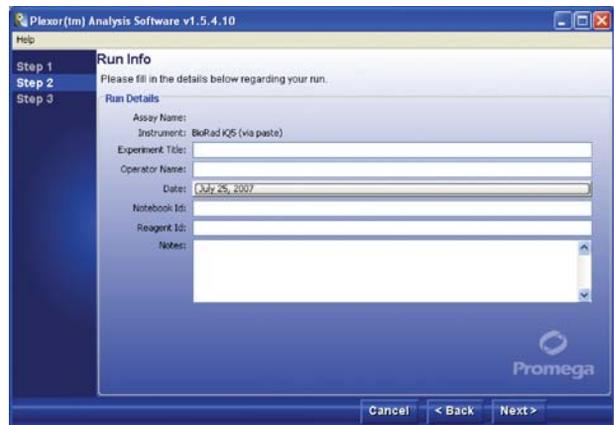
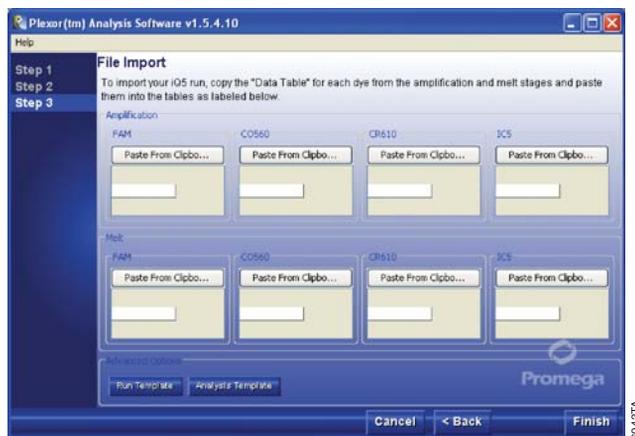


Figure 16. The Run Info screen when pasting data directly into the Plexor® Analysis Software.

10. Select “Next”. Use the File Import screen, Step 3 (Figure 17), to paste data copied from the iQ<sup>TM</sup>5 as described in Steps, 11–18, below.

**Note:** “Advanced Options” can be used to create templates for routine plate setups and analysis conditions. See Section 10.G for details concerning these advanced options and an explanation of the default analysis settings.



**Figure 17.** The File Import screen when pasting data directly into the Plexor<sup>®</sup> Analysis Software.

11. Return to the PCR Quant tab of the Data Analysis module in the iQ<sup>TM</sup>5 optical system software (Figure 13). Select the Amplification Data/RFU tab in the results spreadsheet for the first dye.
12. Select all data by clicking in the upper left corner of the spreadsheet. Use the “Control C” shortcut to copy the data to the computer clipboard.
13. In the Plexor<sup>®</sup> Analysis Software File Import screen, select “Paste From Clipboard” for the appropriate dye in the Amplification section to transfer the data.
14. Repeat Steps 11–13 to copy amplification data for each dye.
15. Return to the Data Analysis module in the iQ<sup>TM</sup>5 optical system software, and select the Melt Data/Curve tab (Figure 14). Select the –d(RFU)/dT tab in the results spreadsheet for the first dye.
16. Select all data by clicking in the upper left corner of the spreadsheet. Use the “Control C” shortcut to copy the data to the computer clipboard.
17. In the Plexor<sup>®</sup> Analysis Software File Import screen, select “Paste From Clipboard” for the appropriate dye in the Melt section to transfer the data.
18. Repeat Steps 15–17 to copy melt data for each dye.
19. When all data have been transferred, select “Finish” in the Plexor<sup>®</sup> Analysis Software File Import Screen to complete import and open the Analysis Desktop.

## 7.C. Data Export and Import via Microsoft® Excel

**Note:** Two files (one for amplification data and one for melt data) must be exported for each dye used in the run.

1. Navigate to the PCR Quant tab of the Data Analysis module in the iQ™5 optical system software. Select the Amplification Data/RFU tab in the results spreadsheet for the first dye (Figure 18).

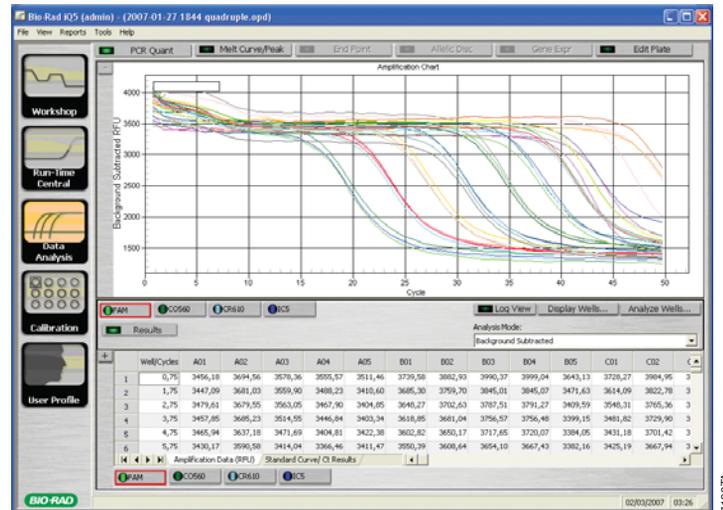


Figure 18. The PCR Quant tab of the iQ™5 Data Analysis module. Results view is selected; the Amplification Data (RFU) spreadsheet is displayed.

2. Right click in the data spreadsheet, and select “Export to Excel”. Specify a name for the file, and select “Save”. An Excel workbook will be produced automatically with the Amplification Data/RFU spreadsheet active.  
**Note:** The saved \*.xls file will not be used – data in the open Excel spreadsheet will resaved in a different format.
3. Save the Amplification Data/RFU spreadsheet as a tab-delimited text file (\*.txt) by using the Save As function from the Excel File pulldown menu. Specify a file name that describes the dye and indicates that it is an amplification data set (e.g., “PlexorHY FAM amp.txt”).
4. Return to the PCR Quant tab of the Data Analysis module in the iQ™5 software. Select the next fluorophore, select the Amplification Data/RFU tab in the results spreadsheet section, and repeat Steps 2 and 3 for each dye in the experiment.
5. Return to the Data Analysis module in the iQ™5 software, and select the Melt Data/Curve tab. Select the  $-d(\text{RFU})/dT$  tab in the results spreadsheet section for the first dye (Figure 19).

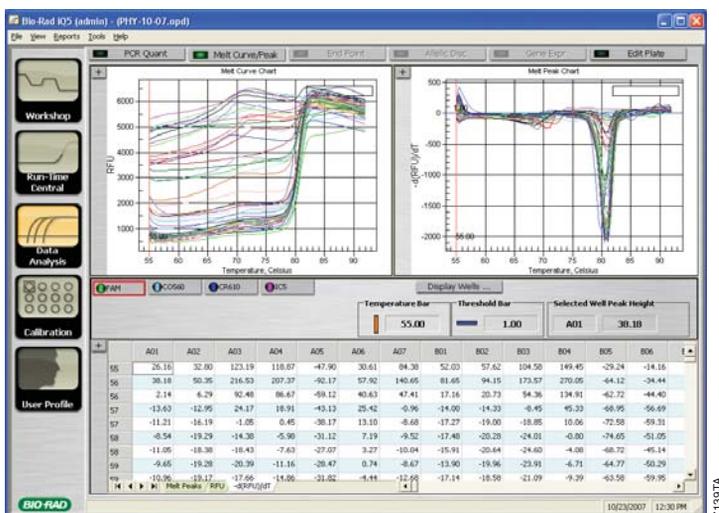


Figure 19. The Melt Curve/Peak tab of the iQ™5 Data Analysis module.

6. Right click in the data spreadsheet, and select “Export to Excel”. Specify a name for the file, and select “Save”. An Excel workbook will be produced automatically with the  $-d(\text{RFU})/dT$  spreadsheet active.  
**Note:** The saved \*.xls file will not be used – data in the open Excel spreadsheet will be resaved in a different format.
7. Save the  $-d(\text{RFU})/dT$  spreadsheet as a tab-delimited text file (\*.txt) by using the Save As function from the Excel File pulldown menu. Specify a file name that describes the dye and indicates that it is an amplification data set (e.g., “PlexorHY FAM melt.txt”).
8. Return to the Melt Data/Curve tab of the Data Analysis module in the iQ™5 software. Select the next fluorophore, select the  $-d(\text{RFU})/dT$  tab in the results spreadsheet section, and repeat Steps 6 and 7 for each dye in the experiment.
9. Launch the Plexor® Analysis Software; go to the Start menu and select “Programs”, then “Plexor”, then 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”.  
**Note:** Make sure the Set Passive Reference on Import option in the File pulldown menu is selected.
10. In the File menu, select “Import New Run” or select the icon: 
11. **Optional:** Enter an assay name in the Assay Setup screen, Step 1 (Figure 20). This screen is used to enter general information about the type and format of the data that will be used for each assay.
12. Select “BioRad iQ™5” as the instrument type.
13. Select “Add Target” four times.

## 7.C. Data Export and Import via Microsoft® Excel (continued)

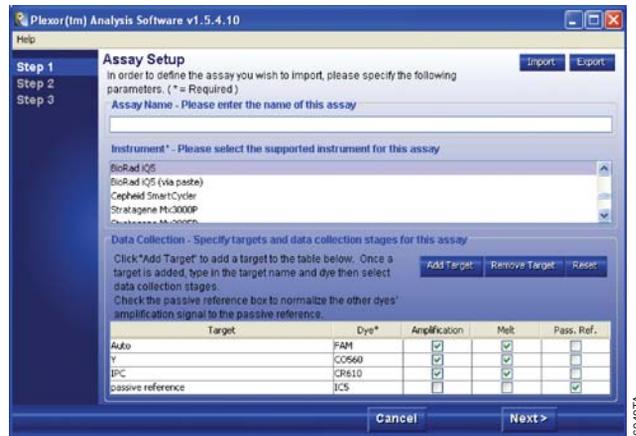


Figure 20. The Assay Setup screen when exporting and importing via Microsoft® Excel.

14. For each dye, enter a target name and select the dye name from the pulldown menu (e.g., “Auto” and FAM, “Y” and CO560, “IPC” and CR610, and “passive reference” and IC5). Indicate there is amplification data and dissociation (melt) data to be analyzed for the FAM™, CO560 and CR610 dyes.

**Note:** The dye names specified in import must match those in the data files generated in Section 4.

15. Indicate that the IC5 data are reference data by checking the Pass. Ref. box. (The Amplification and Melt boxes for this target will automatically become deselected.)
16. Select “Next”.
17. **Optional:** Enter information specific to your run in the Run Info screen, Step 2 (Figure 21). Details such as the date, notes, title, name of the researcher, etc., can also be entered in the provided windows.

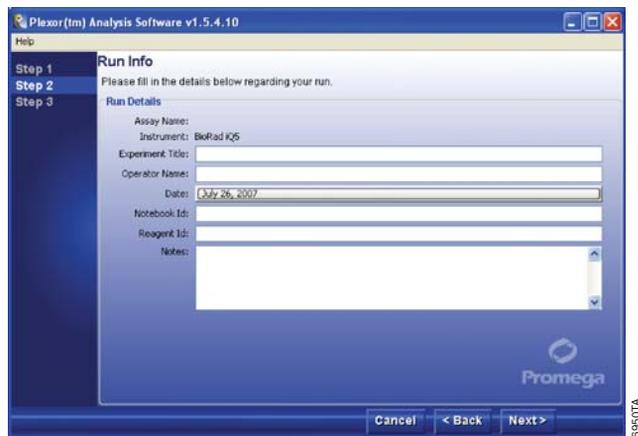


Figure 21. The Run Info screen when exporting and importing via Microsoft® Excel.

18. Use Step 3 of the File Import screen (Figure 22) to specify the data files exported from the instrument (Steps 1–10). Use “Browse” to locate the appropriate exported amplification and melt (dissociation) data files.

ⓘ When analyzing data with the Plexor® Analysis Software, be sure to choose the amplification and melt curve files 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 10.G for details concerning these advanced options and an explanation of the default analysis settings.

19. Select “Finish” to complete the data import and open the Analysis Desktop.

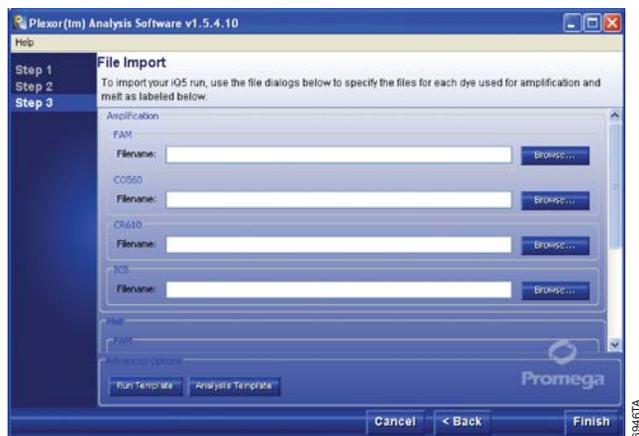
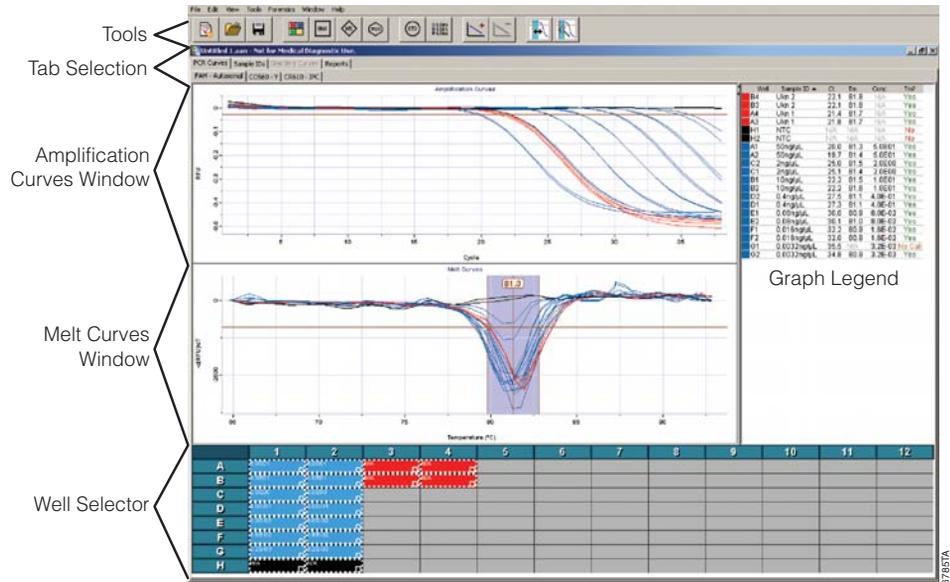


Figure 22. The File Import Screen when exporting and importing via Microsoft® Excel.

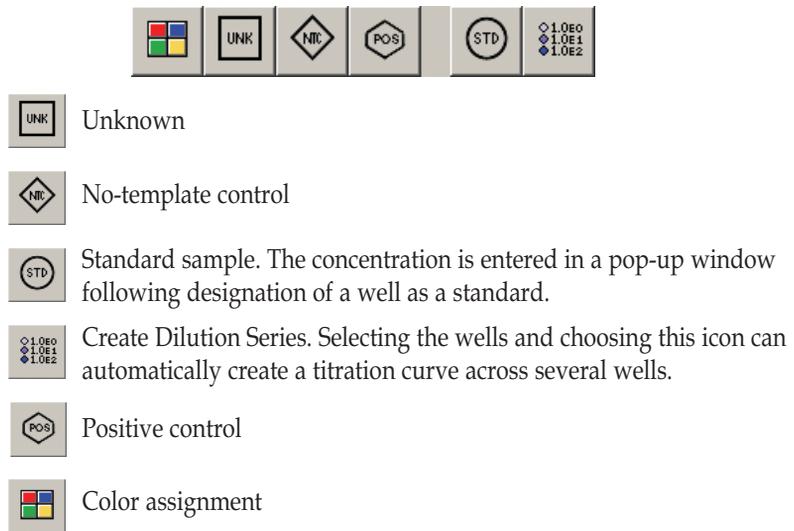
## 8. Data Analysis with the Plexor® Analysis Software

After data import is complete (Section 7), the PCR Curves tab of the Analysis Desktop is displayed (Figure 23).

**Note:** The columns within any report or table, such as the graph legends shown in Figures 23 and 27, can be reordered by placing the cursor over the column header, then clicking on that column and dragging it to the desired location. To add or delete columns from a table or report, right-click on a column, select “Change Columns Shown” and a list of all possible columns for display will appear. Check or uncheck the appropriate boxes to add or delete columns, respectively, from the display.



**Figure 23.** The PCR Curves tab of the Analysis Desktop. The amplification curves window, melt curves window and well selector are indicated.



**Figure 24.** The icons used to define samples in the Plexor® Analysis Software.

## 8.A. Sample Definition

Sample definition, color selection and DNA concentration of standards will be entered for all dyes.

Use the well selector, which is shown in Figure 23, to select and define each well or group of wells. Choose one of the icons shown in Figure 24 to define the samples.

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 DNA concentrations of standards will be retained if the display color associated with those samples is changed.

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.

These instructions are for DNA standard setup as described in Section 5.A, using concentrations in ng/μl. The volume of template used for the DNA standard reactions must be the same as that for unknowns.

1. Define the DNA standards.
  - a. Use the well selector to highlight wells that contain DNA standards.
  - b. Select the Create Dilution Series icon: The icon is a small square button with a light blue background. It contains three rows of text: the first row has a circle icon followed by '1.0E0', the second row has a diamond icon followed by '1.0E1', and the third row has a diamond icon followed by '1.0E2'.
  - c. Confirm that the series selected is “Vertical Series” and the series is “Decreasing”. Enter 50 for the starting concentration and 5 for the dilution factor. See Figure 25.

### Notes:

1. Results with the 0.0032ng/μl dilution of the DNA standard may exhibit increased variability compared to those of other standard dilutions. Definition of the 0.0032ng/μl dilution as a standard is optional. See Section 8.C. The 0.0032ng/μl dilution should be included on each plate. Amplification of the 0.0032ng/μl samples will confirm the sensitivity of the assay in each run.
2. The software does not accept commas or unit definitions in the concentration assignments.
3. The unit definition for DNA quantity can be changed throughout the data analysis to describe concentrations in pg/μl rather than ng/μl. To report concentrations in pg/μl, change the input values as follows:
  - a. In Step 1.c above, confirm that the series selected is “Vertical Series” and the series is “Decreasing”. Enter 50000 for the starting concentration and 5 for the dilution factor.
  - b. In Section 8.D, Steps 6–8, select the check boxes for picograms and enter values as pg/rxn and pg/μl.
  - c. Results in the report sections will now be in pg/μl.

## 8.A. Sample Definition (continued)

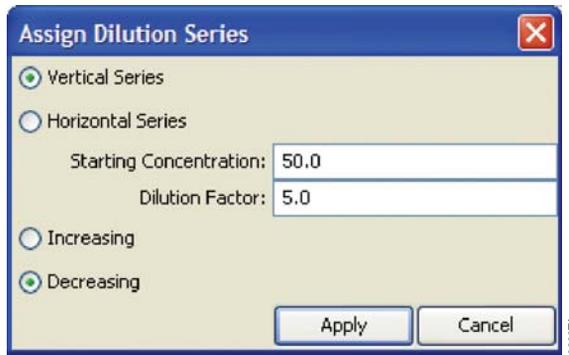


Figure 25. The Assign Dilution Series window.

2. Define the no-template control reactions.
  - a. Use the well selector to highlight the wells that contain NTC reactions.
  - b. Select the NTC icon: 
3. Assign sample names to the unknowns.
  - a. Select the Sample IDs tab (Figure 26). Select the well, and enter the desired sample name. Repeat to enter sample names for other wells.
  - b. Enter the same sample name for duplicate samples. **The calculated DNA quantities for samples with the same sample name will be averaged in the Forensics report** (Section 8.D).
  - c. Names can be copied from a Microsoft® Excel spreadsheet, provided that the cells are arranged in the same array as your samples (e.g., 8 × 12). Highlight the sample names in the spreadsheet, and select “Copy”. In the Edit menu of the Plexor® Analysis Software, 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.

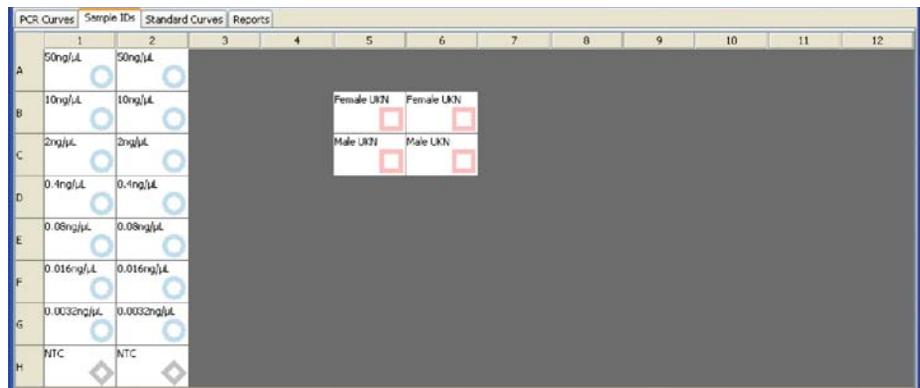


Figure 26. The Sample IDs tab.

## 8.B. Adjusting the Expected Target Melt Temperature

Set the expected target melt temperature and 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 reports (Sections 8.D and 8.H). The expected melt temperature range must be adjusted for all three dye channels (FAM, CO560 and CR610).

The average expected target melt temperature values are instrument-dependent. However, the average expected target melt temperature for the autosomal target (FAM) is generally in the range of 79–81°C, and the average expected target melt temperature for the Y-chromosomal target (CO560) is generally in the range of 81–83°C. The average expected target melt temperature for the IPC (CR610) is generally in the range of 79–81°C, but for some samples, the IPC  $T_m$  value can fall outside this range by as much as 2°C. Adjust the lower bounds of the expected melt range to encompass these samples, if desired. Amplification data, in particular the  $C_t$  value, are the primary means of analyzing IPC data.

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  $\pm 1.5^\circ\text{C}$ .
2. Select wells containing the DNA standards. The  $T_m$  for each selected sample will be displayed in a table to the right of the graph (Figure 27). 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 these standards.
3. In the melt curves window, move the mouse so that the arrow is over the expected target melt temperature line, and drag it to the midpoint of the melt curves. Alternatively, double-click on the line, and enter the desired temperature. See Notes 1–6.

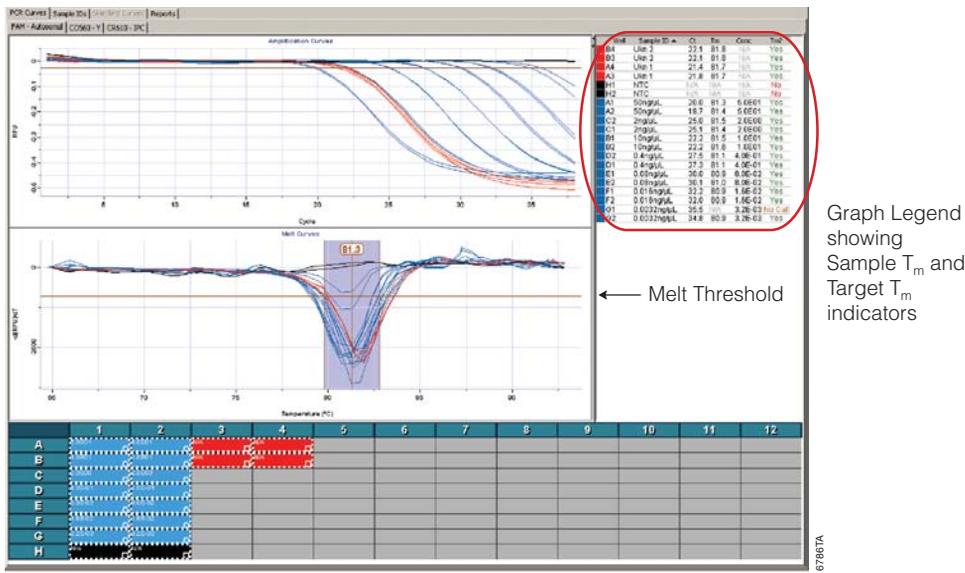


Figure 27. The target melt temperature is displayed in a table to the right of the graph.

## 8.B. Adjusting the Expected Target Melt Temperature (continued)

### Notes:

1. 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.
2. 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. See Section 10.E for advanced options to set the melt threshold.
3. A “Yes” or “No” in the  $T_m?$  column indicates whether a sample  $T_m$  is within the expected target melt temperature range. A “No Call” in this column indicates that the melt curve displays the expected target melt temperature, but there is insufficient amplification product to cause the melt curve to cross the melt threshold.
4. The “ $T_m\#$ ” 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.
5. An increase in  $T_m$  for the unknowns relative to that of the DNA standards may be the result of impurities remaining after DNA extraction. To remove these impurities, increase the number of wash steps or comparable steps.
6. The default display of the melt curve has not been processed beyond the melt curve derivation. In some cases it may be helpful to view a smoothed melt curve using a Gaussian fit of the raw melt curve data to deemphasize minor variability in the melt data. To change the display, right-click on the Melt Curves window, choose “Select Melt Data”, then select “Gaussian Fit”. Note that any melt curve that does not meet the melt threshold will be rendered as a straight line ( $y$  intercept = 0) in this view.

## 8.C. Generating a Standard Curve and Determining Concentrations of Unknowns

Amplification results from the dilution series of the DNA standard are used to generate a standard curve. This standard curve is used to determine the DNA concentration of unknown samples. Samples for generating the standard curve must be designated as standards (Section 8.A). Standard curves must be generated for the autosomal (FAM) and Y (CO560) channels.

The Plexor® Analysis Software performs a linear regression to the standard dilution series data and calculates the equation for the best-fit line (the standard curve). The equation is in the form of  $y = mx + b$ , where  $x = \log$  concentration and  $y = C_t$  (if using the software default settings). The  $R^2$  value is a measure of the fit of the data points to the regressed line. The slope ( $m$  in the equation) is an indication of the PCR efficiency. A slope of  $-3.3$  indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). The  $y$  intercept ( $b$  in the equation) is defined as the  $y$  value ( $C_t$ ) when  $x$  ( $\log$  concentration) equals 0. Therefore,  $b$  gives the  $C_t$  for the  $1\text{ng}/\mu\text{l}$  standard if concentrations are defined as described in Figure 25 ( $\log_{10}(1) = 0$ ). If the value for  $b$  changes significantly from run to run without a change in the slope or  $R^2$  value of the standard curve, then this suggests the DNA standard was not sufficiently mixed before use or has degraded.

In general, the standard curve for the autosomal target (FAM) has an average slope (m) in the range of -3.1 to -3.7 and an  $R^2$  value  $\geq 0.990$ . The standard curve for the Y-chromosomal target (CO560) generally has an average slope (m) in the range of -3.0 to -3.6 and an  $R^2$  value  $\geq 0.990$ .

Low  $R^2$  values ( $R^2 \leq 0.98$ ) may be due to variability in the amplification results for the replicate samples of the 0.0032ng/ $\mu$ l dilution. The 0.0032ng/ $\mu$ l dilution may exhibit increased variability compared to the other dilutions of the DNA standard, most notably in the autosomal reactions. If a low  $R^2$  value is observed, change the sample type for the 0.0032ng/ $\mu$ l standards to “unknown”. The software will automatically incorporate this change into the standard curves. If this does not improve the  $R^2$  value, proceed to Section 9 and review the comments for “Variability in signal among replicate samples”, “Nonlinear standard curve, low  $R^2$  values  $\leq 0.98$ ” and “Slope is outside of the range specified in Section 8.C”.

1. In the autosomal channel (FAM tab), select all samples and DNA standards. Select “Add Standard Curve” to generate a standard curve and determine DNA concentrations of the unknowns based on the standard curve.



2. Repeat Step 1 for the Y channel (CO560 tab).
3. Select the Standard Curves tab to view the standard curves (Figure 28). One subtab shows the autosomal (FAM) standard curve, and a second subtab displays the Y (CO560) standard curve. The default display shows the log concentration on the X axis and cycle threshold on the Y axis.

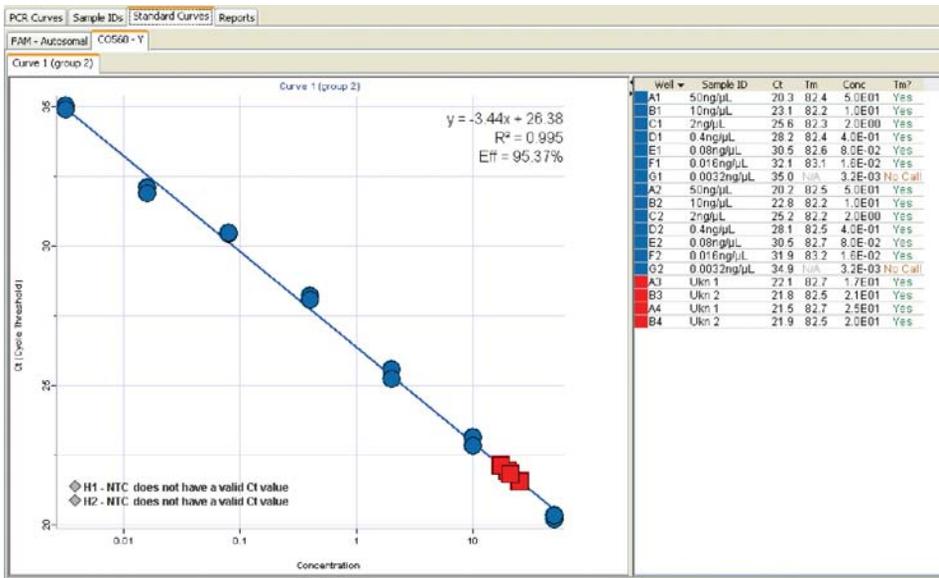


Figure 28. The Standard Curves tab.

## 8.C. Generating a Standard Curve and Determining Concentrations of Unknowns (continued)

4. View DNA concentrations for all samples, including the unknown samples, in the table next to the standard curve graph (Figure 28). The calculated concentrations can also be viewed in the sample details report (Section 8.H).

**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.

## 8.D. Normalization of DNA Quantitation Results for Input into STR Reactions and Setup of IPC Parameters

After generating standard curves and quantitating unknowns for autosomal and Y-chromosomal DNA, the quantitation results can be used to prepare short tandem repeat (STR) amplifications. A normalization tool has been incorporated into the Plexor® Analysis Software to help determine a) which samples are suitable for analysis, b) which samples require dilution, c) which samples may need repurification for accurate quantitation, and d) how much sample should be added to your autosomal STR amplifications (e.g., PowerPlex® 16 reactions) or Y-chromosomal STR amplifications (e.g., PowerPlex® Y reactions).

DNA concentrations determined by autosomal and Y-chromosomal quantitation may differ. Quantitative PCR results and the copy number of the target sequences can vary. You may see small differences in the quantitation results for the same sample. The preferred DNA amount for subsequent STR analysis should be defined through laboratory validation of the STR assays in conjunction with Plexor® HY quantitation.

**Note:** Quantitation results for samples with identical sample ID names will be averaged by the normalization tool.

Following normalization, a new Forensics subtab will be displayed under “Reports”.

In situations where multiple people use the same computer, the parameters entered in Steps 2–8 will be retained for each Windows® user account when the Plexor® Analysis Software is closed and reopened, so there is no need to repeat Steps 2–8 for subsequent runs unless the parameters change.

1. Select “Set Normalization and IPC Parameters” in the Forensics menu.
2. In the dialog box that appears (Set STR Normalization and IPC Parameters; Figure 29), be sure that the box next to “disable volume normalization (show concentrations and  $C_t$  only)” is not checked. If this box is checked, STR normalization data will not be displayed in the subsequent forensic report. See Section 8.F.
3. **Optional:** If the DNA standards were defined in pg/ $\mu$ l (Section 8.A, Step 1), select the check box to set units as picograms. The default display is nanograms.
4. Select the Autosomal STRs tab.
5. Enter the preferred DNA quantity per STR amplification reaction.

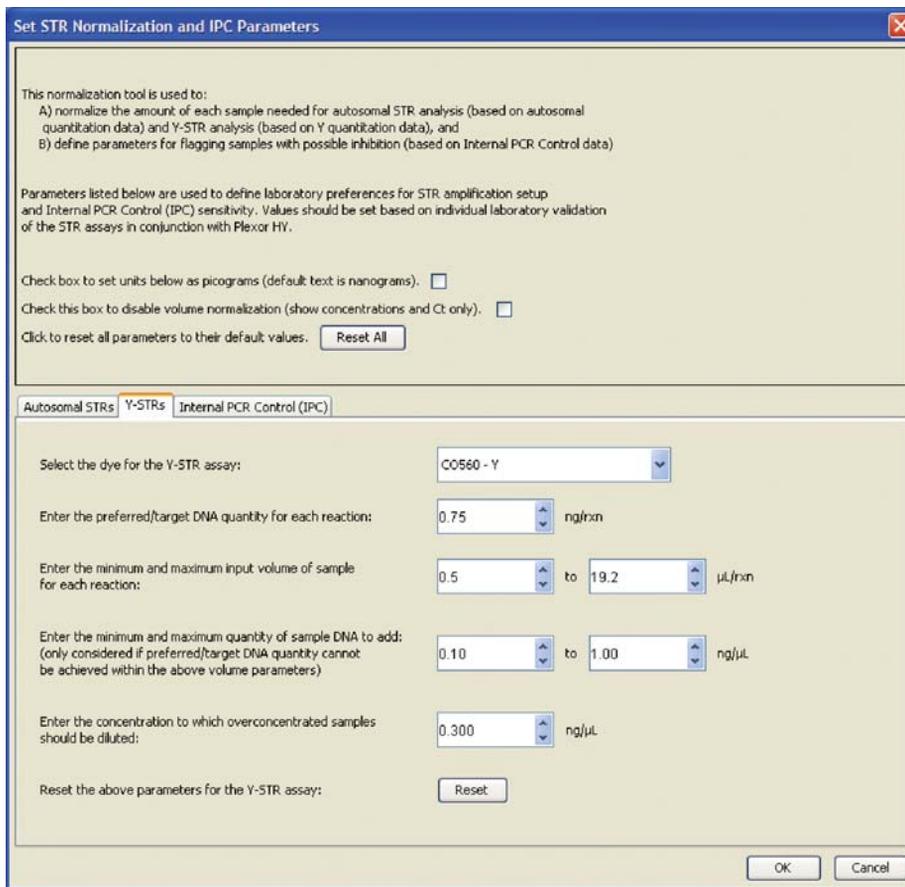


Figure 29. The Set STR Normalization and IPC Parameters window.

6. Enter the minimum and maximum input volumes of sample (in  $\mu\text{L}$ ) for each STR reaction. The limits for pipetting of sample should be defined in your laboratory. Minimum volume is generally defined by the limits of the pipettes being used and laboratory guidelines. Maximum volume will often be defined by the STR reaction volume or laboratory guidelines.

If a single sample volume for STR amplification setup is desired, narrow this range by entering the same volume for the minimum and maximum volumes. A broad range of input target quantities and volumes will limit the number of samples requiring dilution.

7. Enter the minimum and maximum quantity of sample DNA (in  $\text{ng}/\text{reaction}$ , or  $\text{pg}/\text{reaction}$  if selected above), indicating the lowest and highest amount of DNA known to produce acceptable STR profiles, as demonstrated through laboratory validation of the STR assays in conjunction with the Plexor<sup>®</sup> HY System.
8. Enter the preferred concentration (in  $\text{ng}/\mu\text{L}$ , or  $\text{pg}/\mu\text{L}$  if selected above) to which overconcentrated samples will be diluted. The concentration entered must achieve the preferred DNA quantity per reaction entered in Step 5 within the range of the minimum and maximum input volumes chosen in Step 6.

### 8.D. Normalization of DNA Quantitation Results for Input into STR Reactions and Setup of IPC Parameters (continued)

9. Select the Y-STRs tab, and repeat Steps 5-8.
10. Select the Internal PCR Control (IPC) tab (Figure 30). If desired, modify the IPC  $C_t$  threshold, which defines the acceptable difference between the IPC  $C_t$  values in DNA standards and samples. The default value is 2 cycles. If the IPC  $C_t$  value in a sample differs from the IPC  $C_t$  value in the DNA standard by more than 2 cycles, "Check IPC" will be displayed in the Forensics report for that sample. See Section 8.E for more details about interpreting IPC results and modifying this value.

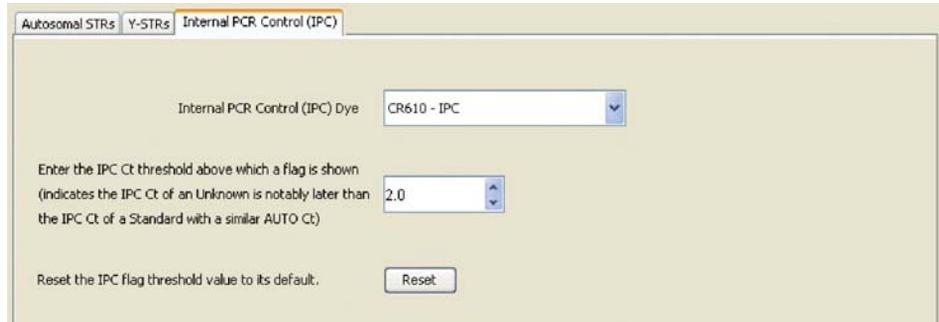
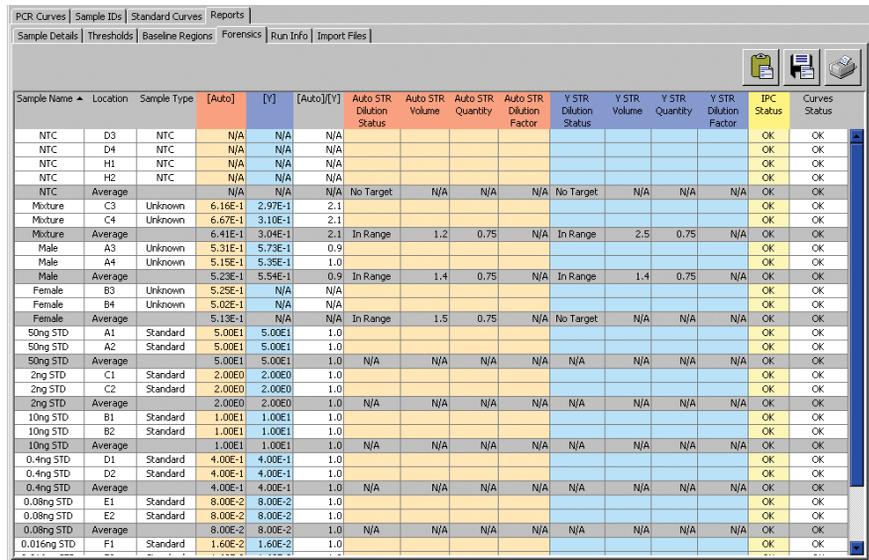


Figure 30. The Internal PCR Control (IPC) tab.

11. After values for all three tabs [i.e., Autosomal STRs, Y-STRs and Internal PCR Control (IPC)] have been entered, select "OK".
12. The Forensics report will be displayed (Figure 31).



Sample Name	Location	Sample Type	[Auto]	[Y]	[Auto][Y]	Auto STR Dilution Status	Auto STR Volume	Auto STR Quantity	Auto STR Dilution Factor	Y STR Dilution Status	Y STR Volume	Y STR Quantity	Y STR Dilution Factor	IPC Status	Curves Status
NTC	D3	NTC	N/A	N/A	N/A									OK	OK
NTC	D4	NTC	N/A	N/A	N/A									OK	OK
NTC	H1	NTC	N/A	N/A	N/A									OK	OK
NTC	H2	NTC	N/A	N/A	N/A									OK	OK
NTC	Average		N/A	N/A	N/A	No Target	N/A	N/A	N/A	No Target	N/A	N/A	N/A	OK	OK
Mixture	C3	Unknown	6.16E-1	2.97E-1	2.1									OK	OK
Mixture	C4	Unknown	6.67E-1	3.10E-1	2.1									OK	OK
Mixture	Average		6.41E-1	3.04E-1	2.1	In Range	1.2	0.75	N/A	In Range	2.5	0.75	N/A	OK	OK
Male	A3	Unknown	5.31E-1	5.73E-1	0.9									OK	OK
Male	A4	Unknown	5.15E-1	5.35E-1	1.0									OK	OK
Male	Average		5.23E-1	5.54E-1	0.9	In Range	1.4	0.75	N/A	In Range	1.4	0.75	N/A	OK	OK
Female	B3	Unknown	5.25E-1	N/A	N/A									OK	OK
Female	B4	Unknown	5.02E-1	N/A	N/A									OK	OK
Female	Average		5.13E-1	N/A	N/A	In Range	1.5	0.75	N/A	No Target	N/A	N/A	N/A	OK	OK
50ng STD	A1	Standard	5.00E1	5.00E1	1.0									OK	OK
50ng STD	A2	Standard	5.00E1	5.00E1	1.0									OK	OK
50ng STD	Average		5.00E1	5.00E1	1.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	OK	OK
2ng STD	C1	Standard	2.00E0	2.00E0	1.0									OK	OK
2ng STD	C2	Standard	2.00E0	2.00E0	1.0									OK	OK
2ng STD	Average		2.00E0	2.00E0	1.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	OK	OK
10ng STD	B1	Standard	1.00E1	1.00E1	1.0									OK	OK
10ng STD	B2	Standard	1.00E1	1.00E1	1.0									OK	OK
10ng STD	Average		1.00E1	1.00E1	1.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	OK	OK
0.4ng STD	D1	Standard	4.00E-1	4.00E-1	1.0									OK	OK
0.4ng STD	D2	Standard	4.00E-1	4.00E-1	1.0									OK	OK
0.4ng STD	Average		4.00E-1	4.00E-1	1.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	OK	OK
0.08ng STD	E1	Standard	8.00E-2	8.00E-2	1.0									OK	OK
0.08ng STD	E2	Standard	8.00E-2	8.00E-2	1.0									OK	OK
0.08ng STD	Average		8.00E-2	8.00E-2	1.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	OK	OK
0.015ng STD	F1	Standard	1.60E-2	1.60E-2	1.0									OK	OK

Figure 31. The Forensics report.

## Definitions and Forensic Report Interpretation

**[Auto]:** Concentration of total human autosomal DNA in a sample in ng/ $\mu$ l (or pg/ $\mu$ l if DNA concentrations were entered as pg/ $\mu$ l).

**[Y]:** Concentration of human male DNA in a sample in ng/ $\mu$ l (or pg/ $\mu$ l if DNA concentrations were entered as pg/ $\mu$ l).

**[Auto]/[Y]:** Ratio of total human autosomal DNA concentration to male (Y) DNA concentration. A very high ratio is indicative of a "male/female" mixture with minimal male DNA. If the male contributor is of interest, samples with a very high ratio may benefit from Y-STR analysis in addition to, or instead of, autosomal STR analysis. Laboratory validation of the Plexor® HY System in conjunction with the preferred autosomal and Y-STR systems is required to determine a meaningful value upon which to base this decision.

**Note:** Due to some individual-to-individual variation in copy number of the autosomal target and Y-chromosomal target, a single male DNA may not have an [Auto]/[Y] value of 1.0. Additionally, quantitative real-time PCR has inherent variation, especially with samples that have higher  $C_t$  values (i.e., samples with lower DNA concentrations). This variation can be reduced by performing replicate analysis. [Auto]/[Y] values in the range of 0.4 to 2 are commonly observed in single-source male samples.

The [Auto]/[Y] ratio is not intended to identify minor female contribution in a male sample. This ratio should be used to form guidelines to suggest when Y-STR analysis may be a beneficial supplement to autosomal STR analysis (i.e., [Auto]/[Y] is between approximately 5 and 20) and when only Y-STR analysis is likely to produce a meaningful STR profile (i.e., [Auto]/[Y]  $\geq$  20). Laboratory validation of the Plexor® HY System, in conjunction with STR analysis, should be performed to determine these interpretation guidelines.

**STR Dilution Status (Autosomal or Y):** An indication of whether a sample contains DNA of adequate concentration to use directly in STR analysis or whether dilution is necessary. The status terms listed below are established by the values indicated in the Set STR Normalization and IPC Parameters window (Figure 29). Auto STR columns use autosomal quantitation results and are intended to guide setup of autosomal STR reactions. Y-STR columns use Y-chromosomal quantitation results and are intended to guide setup of Y-STR reactions.

- "No Target" indicates no DNA was detected.
- "Underconc." indicates that DNA was detected, but the minimum acceptable DNA quantity (ng or pg per reaction) cannot be achieved with the maximum volume that has been defined.
- "Below Target" indicates that the specified maximum volume of sample will deliver an acceptable quantity of DNA but less input DNA than the indicated preferred DNA quantity.
- "In Range" indicates that the preferred DNA quantity is achievable within the acceptable volume range.
- "Above Target" indicates that the specified minimum volume of sample will deliver an acceptable quantity of DNA but will deliver more input DNA than the preferred DNA quantity.
- "Overconc." indicates that the maximum acceptable DNA quantity (ng or pg per reaction) cannot be achieved with the minimum volume that has been defined. Samples will need dilution.



## 8.D. Normalization of DNA Quantitation Results for Input into STR Reactions and Setup of IPC Parameters (continued)

**STR Volume (Autosomal or Y):** The volume ( $\mu\text{l}$ ) of sample that should be used in STR analysis. Auto STR columns use autosomal quantitation results and are intended to guide setup of autosomal STR reactions. Y-STR columns use Y-chromosomal quantitation results and are intended to guide setup of Y-STR reactions.

**STR Quantity (Autosomal or Y):** The quantity (ng, or pg if selected) of sample that will be input into the STR reaction using the indicated "STR Volume". Auto STR columns use autosomal quantitation results and are intended to guide setup of autosomal STR reactions. Y-STR columns use Y-chromosomal quantitation results and are intended to guide setup of Y-STR reactions.

**STR Dilution Factor (Autosomal or Y):** The dilution factor necessary to achieve the desired concentration indicated in the Set STR Normalization and IPC Parameters window (Section 8.D, Step 7). Only samples indicated as "Overconc." will have a dilution factor listed. Auto STR columns use autosomal quantitation results and are intended to guide setup of autosomal STR reactions. Y-STR columns use Y-chromosomal quantitation results and are intended to guide setup of Y-STR reactions.

**IPC Status:** An indication of whether the difference between the sample's  $C_t$  values for the IPC and DNA standard is greater than the designated threshold in the Set STR Normalization and IPC Parameters window (Figure 29). A difference of less than the designated threshold is indicated as "OK"; a difference greater than the threshold is indicated as "Check IPC".

**Curves Status:** "OK" indicates the following:

- The sample, if defined as a standard, shows amplification.
- The sample, if defined as a no-template control, shows no amplification.
- If a melt peak is present, the  $T_m$  is within the expected range.

"Check STD", "Check NTC" or "Check Melts" will be displayed if these criteria are not met.

**Note:** It is acceptable for the 0.0032ng/ $\mu\text{l}$  DNA standard to display "No" or "No Call" in the "Tm?" column. Verify that if any subthreshold peak is present in the melt curve, this peak is within the expected target melt temperature range.

## 8.E. Analysis of the Internal PCR Control (IPC)

The Internal PCR Control (IPC) is amplified and detected in the CR610 dye channel under PCR Curves. These data can provide an indication of potential inhibition that may have affected the quantitation data (FAM and CO560 data). By comparing IPC  $C_t$  values for unknown samples with IPC  $C_t$  values for DNA standards, inferences regarding the presence of inhibitors can be made. Decreasing the IPC threshold above which a flag is shown (Figure 30) will increase the sensitivity to differences in the  $C_t$  values, while increasing the threshold will necessitate greater IPC  $C_t$  value differences to present the Check IPC flag.

**Note:** Data from samples are compared to data from DNA standards of similar autosomal  $C_t$  values. The IPC amplification is designed to be the most inhibition-sensitive or least robust amplification in the triplex.

- If the IPC  $C_t$  value of an unknown is several cycles higher than that of DNA standards with similar total DNA amounts, inhibition may have occurred and the quantitation data are in doubt. Consider repurifying and requantitating the sample DNA.

**Note:** High levels of total human DNA ( $\geq 10\text{ng}/\mu\text{l}$ ) can cause a slight delay in the IPC crossing the cycle threshold (1–2 cycles).

- IPC  $C_t$  value differences can be used to automatically flag potential inhibition in the Forensics report as described in Section 8.D, Step 10.

Laboratory validation with relevant inhibitors should be performed to determine criteria for detecting inhibition.

## 8.F. Forensic Report Without Normalization

The quantitation results can be reported without normalization of volumes. This report shows quantitation results and IPC status and curves status columns.

1. Select “Set Normalization and IPC Parameters” in the Forensic menu.
2. Check the box to disable volume normalization (show concentrations and  $C_t$  values only).
3. Select the Forensic tab in the Reports menu.

## 8.G. Data Export in a 96-Well Format

The quantitation results can be exported in a 96-well format. The exported file is an Excel spreadsheet, which can be used for automated PCR setup. This export is only possible after the instructions in Section 8.D are performed.

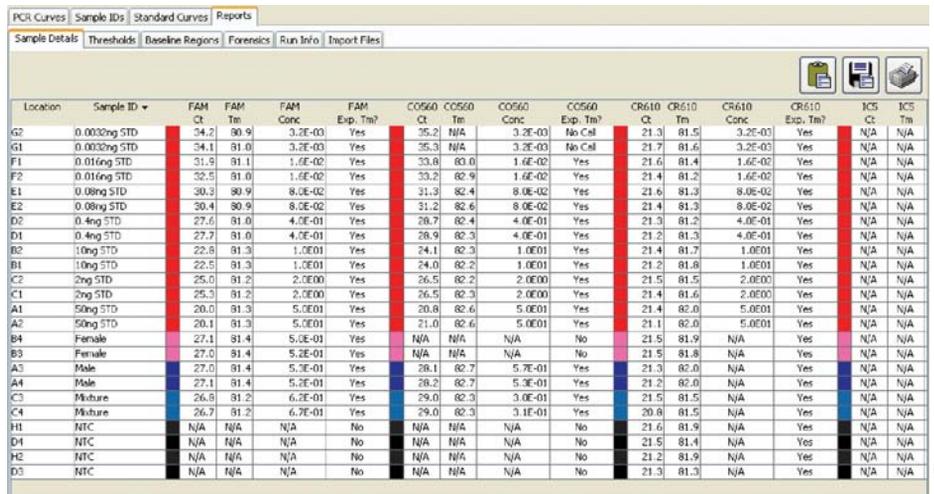
1. Select “Export 96-well Concentration” in the Forensics menu.
2. Select “Autosomal Concentrations” for the autosomal results, enter a file name and select “Save”.
3. Select “Y Concentrations” for the Y results, enter a file name and select “Save”.

## 8.H. Supplemental Reports

In addition to the Forensics report described in Section 8.D, 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.

**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 32).

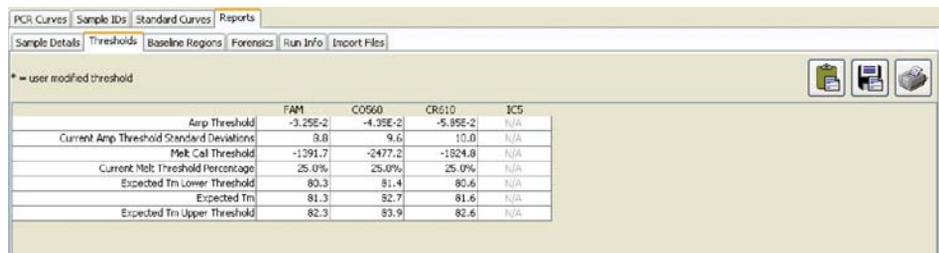
A sample with a  $C_t$  value of “N/A” has an amplification curve that did not cross the amplification threshold.



Location	Sample ID	FAM Ct	FAM Tm	FAM Conc	FAM Exp. Tm	CO560 Ct	CO560 Tm	CO560 Conc	CO560 Exp. Tm	CR610 Ct	CR610 Tm	CR610 Conc	CR610 Exp. Tm	ICS Ct	ICS Tm
G2	0.0030ng STD	34.2	80.9	3.2E-03	Yes	35.2	N/A	3.2E-03	No Call	21.3	81.5	3.2E-03	Yes	N/A	N/A
G1	0.0030ng STD	34.1	81.0	3.2E-03	Yes	35.3	N/A	3.2E-03	No Call	21.7	81.6	3.2E-03	Yes	N/A	N/A
F1	0.016ng STD	31.5	81.1	1.6E-02	Yes	33.8	83.8	1.6E-02	Yes	21.6	81.4	1.6E-02	Yes	N/A	N/A
F2	0.016ng STD	32.5	81.0	1.6E-02	Yes	33.2	82.9	1.6E-02	Yes	21.4	81.2	1.6E-02	Yes	N/A	N/A
E1	0.08ng STD	30.3	80.9	8.0E-02	Yes	31.3	82.4	8.0E-02	Yes	21.6	81.3	8.0E-02	Yes	N/A	N/A
E2	0.08ng STD	30.4	80.9	8.0E-02	Yes	31.2	82.6	8.0E-02	Yes	21.4	81.3	8.0E-02	Yes	N/A	N/A
D2	0.4ng STD	27.6	81.0	4.0E-01	Yes	28.7	82.4	4.0E-01	Yes	21.3	81.2	4.0E-01	Yes	N/A	N/A
D1	0.4ng STD	27.7	81.0	4.0E-01	Yes	28.9	82.3	4.0E-01	Yes	21.2	81.3	4.0E-01	Yes	N/A	N/A
B2	10ng STD	22.8	81.3	1.0E01	Yes	24.1	82.3	1.0E01	Yes	21.4	81.7	1.0E01	Yes	N/A	N/A
B1	10ng STD	22.5	81.3	1.0E01	Yes	24.0	82.2	1.0E01	Yes	21.2	81.8	1.0E01	Yes	N/A	N/A
C2	2ng STD	25.0	81.2	2.0E00	Yes	26.5	82.2	2.0E00	Yes	21.5	81.5	2.0E00	Yes	N/A	N/A
A1	2ng STD	25.3	81.2	2.0E00	Yes	26.5	82.3	2.0E00	Yes	21.4	81.6	2.0E00	Yes	N/A	N/A
A2	50ng STD	20.0	81.3	5.0E01	Yes	20.8	82.6	5.0E01	Yes	21.4	82.0	5.0E01	Yes	N/A	N/A
A2	50ng STD	20.1	81.3	5.0E01	Yes	21.0	82.6	5.0E01	Yes	21.1	82.0	5.0E01	Yes	N/A	N/A
B4	Female	27.1	81.4	5.0E-01	Yes	N/A	N/A	N/A	No	21.5	81.9	N/A	Yes	N/A	N/A
B3	Female	27.0	81.4	5.0E-01	Yes	N/A	N/A	N/A	No	21.5	81.8	N/A	Yes	N/A	N/A
A3	Male	27.0	81.4	5.0E-01	Yes	28.1	82.7	5.7E-01	Yes	21.3	82.0	N/A	Yes	N/A	N/A
A4	Male	27.1	81.4	5.0E-01	Yes	28.2	82.7	5.3E-01	Yes	21.2	82.0	N/A	Yes	N/A	N/A
C3	Mixture	26.8	81.2	6.2E-01	Yes	29.0	82.3	3.0E-01	Yes	21.5	81.5	N/A	Yes	N/A	N/A
C4	Mixture	26.7	81.2	6.7E-01	Yes	29.0	82.3	3.1E-01	Yes	20.8	81.5	N/A	Yes	N/A	N/A
H1	NTC	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.6	81.9	N/A	Yes	N/A	N/A
D1	NTC	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.5	81.4	N/A	Yes	N/A	N/A
H2	NTC	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.2	81.9	N/A	Yes	N/A	N/A
D3	NTC	N/A	N/A	N/A	No	N/A	N/A	N/A	No	21.3	81.3	N/A	Yes	N/A	N/A

Figure 32. The Sample Details tab.

**Thresholds:** The thresholds report includes the numerical values for the thresholds in the current analysis (Figure 33). 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 10.G for more information about creating an analysis template.



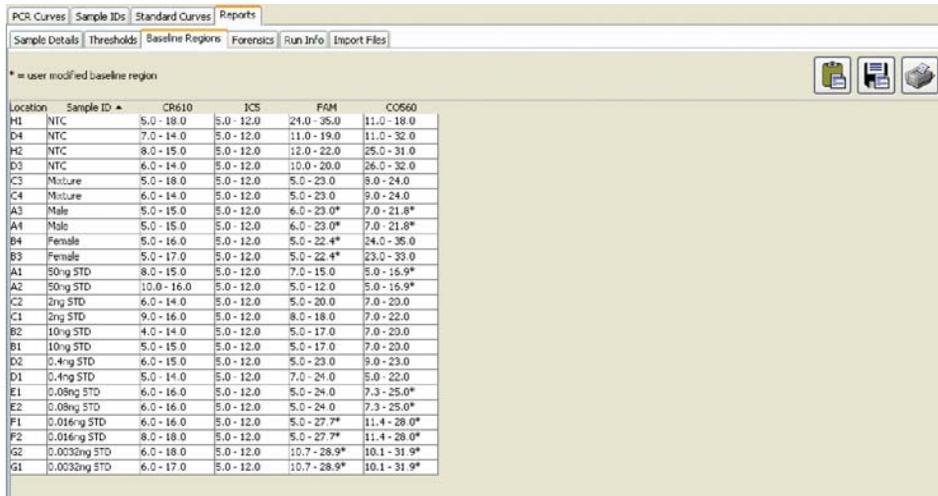
	FAM	CO560	CR610	ICS
Ampl Threshold	-3.25E-2	-4.39E-2	-5.95E-2	N/A
Current Ampl Threshold Standard Deviations	9.8	9.6	10.0	N/A
Melt Call Threshold	-1391.7	-2477.2	-1524.8	N/A
Current Melt Threshold Percentage	25.0%	25.0%	25.0%	N/A
Expected Tm Lower Threshold	80.3	81.4	80.6	N/A
Expected Tm	81.3	82.7	81.6	N/A
Expected Tm Upper Threshold	82.3	83.9	82.6	N/A

Figure 33. The Thresholds tab.

**Baseline Regions:** The baseline regions report includes the cycle number values that define the region used to calculate the baseline value for each sample (Figure 34).

**Run Info:** The run info report includes information from the data import, such as instrument and date of run (Figure 35).

**Import Files:** The import files report includes information on the data import files, such as dye names and file names (Figure 36).



Location	Sample ID	CR610	ICS	FAM	CO660
H1	NTC	5.0 - 18.0	5.0 - 12.0	24.0 - 35.0	11.0 - 18.0
D4	NTC	7.0 - 14.0	5.0 - 12.0	11.0 - 19.0	11.0 - 32.0
H2	NTC	8.0 - 15.0	5.0 - 12.0	12.0 - 22.0	25.0 - 31.0
D3	NTC	6.0 - 14.0	5.0 - 12.0	10.0 - 20.0	26.0 - 32.0
C3	Mixture	5.0 - 18.0	5.0 - 12.0	5.0 - 23.0	8.0 - 24.0
C4	Mixture	6.0 - 14.0	5.0 - 12.0	5.0 - 23.0	9.0 - 24.0
A3	Male	5.0 - 15.0	5.0 - 12.0	6.0 - 23.0*	7.0 - 21.8*
A4	Male	5.0 - 15.0	5.0 - 12.0	6.0 - 23.0*	7.0 - 21.8*
B4	Female	5.0 - 16.0	5.0 - 12.0	5.0 - 22.4*	24.0 - 35.0
B3	Female	5.0 - 17.0	5.0 - 12.0	5.0 - 22.4*	23.0 - 33.0
A1	50ng STD	8.0 - 15.0	5.0 - 12.0	7.0 - 15.0	5.0 - 16.9*
A2	50ng STD	10.0 - 16.0	5.0 - 12.0	5.0 - 12.0	5.0 - 16.9*
C2	2ng STD	6.0 - 14.0	5.0 - 12.0	5.0 - 20.0	7.0 - 20.0
C1	2ng STD	9.0 - 16.0	5.0 - 12.0	8.0 - 18.0	7.0 - 22.0
B2	10ng STD	4.0 - 14.0	5.0 - 12.0	5.0 - 17.0	7.0 - 20.0
B1	10ng STD	5.0 - 15.0	5.0 - 12.0	5.0 - 17.0	7.0 - 20.0
D2	0.4ng STD	6.0 - 15.0	5.0 - 12.0	5.0 - 23.0	9.0 - 23.0
D1	0.4ng STD	5.0 - 14.0	5.0 - 12.0	7.0 - 24.0	5.0 - 22.0
E1	0.08ng STD	6.0 - 16.0	5.0 - 12.0	5.0 - 24.0	7.3 - 25.0*
E2	0.08ng STD	6.0 - 16.0	5.0 - 12.0	5.0 - 24.0	7.3 - 25.0*
F1	0.016ng STD	6.0 - 16.0	5.0 - 12.0	5.0 - 27.7*	11.4 - 28.0*
F2	0.016ng STD	8.0 - 18.0	5.0 - 12.0	5.0 - 27.7*	11.4 - 28.0*
G2	0.0032ng STD	6.0 - 18.0	5.0 - 12.0	10.7 - 28.9*	10.1 - 31.9*
G1	0.0032ng STD	6.0 - 17.0	5.0 - 12.0	10.7 - 28.9*	10.1 - 31.9*

Figure 34. The Baseline Regions tab.

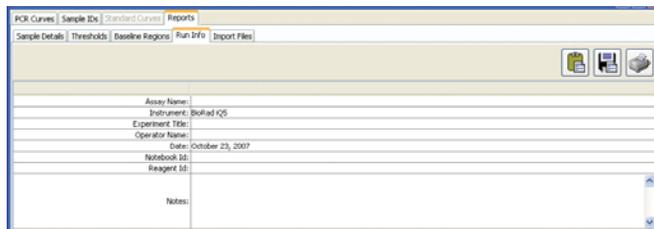


Figure 35. The Run Info tab.



Contents	File Name
AMP_FAM	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-FAM-amp.txt
AMP_CO660	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-CO660-amp.txt
AMP_CH610	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-CH610-amp.txt
AMP_ICS	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-ICS-amp.txt
MEL1_FAM	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-FAM-mel.txt
MEL1_CO660	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-CO660-mel.txt
MEL1_CH610	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-CH610-mel.txt
MEL1_ICS	I:\Prometh\126\1M\PHN-10-07\PHN-10-07-ICS-mel.txt

Figure 36. The Import Files tab.



## 8.I. 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 analysis (Section 10.G).

## 9. Troubleshooting

Symptoms	Causes and Comments
Flat amplification curve in the amplification curves window for autosomal and Y channels but not the IPC. The IPC C <sub>t</sub> value of the sample in question was similar to the C <sub>t</sub> value of the DNA standards (no apparent amplification for the autosomal and Y targets)	Template was degraded or of insufficient quantity. Verify the integrity of the DNA template by electrophoresis.
	Be sure that the reactions were assembled correctly
	Thermal cycler was programmed incorrectly. Verify cycle times and temperatures (Section 6.A).
	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. Be sure the selected detectors are appropriate for the fluorescent dyes used.
	The passive reference was set incorrectly. Be sure the passive reference is set as “IC5” (Section 7.B, Step 7).
	The Plexor® HY 2X Master Mix may have lost activity. Be sure to store the Plexor® HY System at -20°C to avoid loss of enzyme activity. Confirm instrument settings, and review the DNA standard amplification data to determine if there is a problem with the Plexor® HY System reagents.
Flat amplification curve in the amplification curves window for all channels (i.e., no C <sub>t</sub> values) or for autosomal and Y DNA with higher or no C <sub>t</sub> values for the IPC	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.
	Amplification inhibitor is present in the DNA template. Reduce the concentration or volume of the template in the reaction. Repeat the DNA purification if necessary
	Sample well(s) did not receive reaction mix. Examine the plate to identify any empty wells. Return to the Plate Setup Editor (Figure 10), and redefine the samples, taking care not to define any empty wells. Reexport and reanalyze the data (Section 7).
	The scale of the Y axis is 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.
	The Plexor® HY 2X Master Mix may have lost activity. Be sure to store the Plexor® HY System at -20°C to avoid loss of enzyme activity.
The Plexor® HY 20X Primer/IPC Mix may have been degraded. Minimize the number of freeze-thaw cycles.	

<u>Symptoms</u>	<u>Causes and Comments</u>
Increasing fluorescence over time	<p>The passive reference was set incorrectly. Be sure the passive reference is set as "IC5" (Section 7.B, Step 7)</p> <p>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 10.C).</p> <p>The baseline region was set too close to the signal change. Manually adjust the baseline region (Section 10.C).</p>
Two or more distinct melt curves or one broad melt curve in the melt curves window	<p>Check for signal bleedthrough. Calibrate the instrument as instructed in Section 4.</p>
No melt curve observed in the melt curves window	<p>Poor amplification. See causes and comments for "Flat amplification curve in the amplification curves window" symptoms above.</p> <p>Problems with data export or instrument analysis have occurred. Review the instructions for data export and instrument setup.</p> <p>Data collection settings were incorrect. Verify that the thermal cycling program and data collection settings are correct (Section 6.A).</p> <p>Incorrect files were imported. Be sure to import the proper files containing related amplification data and dissociation data.</p> <p>Instrument was programmed incorrectly. Verify that the thermal cycling program is correct (Section 6.A).</p> <p>Sample well(s) did not receive reaction mix. Examine the plate to identify any empty wells. Return to the Plate Setup Editor (Figure 10), and redefine the samples, taking care not to define any empty wells. Reexport and reanalyze the data (Section 7).</p> <p>Melt Curve display has been changed to "Gaussian Fit" mode, and selected samples do not meet the melt threshold. If "Gaussian Fit" mode is selected, the Plexor® Analysis Software draws as straight lines (zero slope, zero y-intercept) those curves that did not meet the melt threshold at the time of import, at the time of opening the *.aan file, or at the time of switching from "Raw Data" view to the "Gaussian Fit". This indicates that these curves were not considered for melt peak calling (Exp Tm?). Switch the melt curve view to "Raw Data".</p>
Variability in signal among replicate samples	<p>Calibrate your pipettes to minimize variability when pipetting. Small volumes are difficult to pipet accurately. Do not pipet volumes &lt;1µl; we recommend using a sample volume of 2µl or higher. If necessary, dilute the template so that larger volumes are pipetted.</p> <p>Some variation is normal. A difference of 1-2 cycles for the C<sub>t</sub> values of replicates is within the normal variation associated with an exponential amplification reaction. C<sub>t</sub> variation should be &lt;2 cycles for replicates.</p> <p>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 dilute samples with few target molecules.</p> <p>Mixing was inadequate. Vortex reagents to mix well prior to pipetting.</p>

## 9. Troubleshooting (continued)

Symptoms	Causes and Comments
Variability in signal among replicate samples (continued)	<p>Plate performance can differ from manufacturer to manufacturer. Use plasticware recommended by the instrument manufacturer.</p> <p>Instrument was improperly calibrated. Calibrate instrument as instructed by the manufacturer. Perform a background check as directed by the manufacturer.</p> <p>Viscous samples (e.g., high-molecular-weight genomic DNA) are difficult to pipet accurately. Dilute the DNA template.</p> <p>Store the DNA at 4°C overnight to improve sampling consistency.</p> <p>The plate was not completely sealed. Carefully seal the plates to avoid evaporation.</p> <p>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 10.C).</p>
Fluorescence decrease observed in the no-template control reaction	<p>Reactions were contaminated with target DNA. Human DNA contamination will produce a melt product similar to that of the DNA standard amplifications. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Take precautions to prevent contamination. Use separate bench spaces and pipettes when handling DNA.</p> <p>Decrease the cycle number to reduce accumulation of nonspecific amplification products.</p> <p>An improperly calibrated instrument can lead to erratic fluorescence readings. Calibrate the instrument as instructed by the manufacturer.</p>
Vertical fluorescence spikes or significant “noise” in the amplification curve	<p>Consult the instrument manufacturer’s user’s guide for information about potential instrument problems that can cause spikes or noise.</p> <p>No amplification or poor amplification for the entire plate. Poor amplification can lead to improper data scaling, making fluorescence measurements appear erratic. See possible causes and comments for “Flat amplification curve in the amplification curves window” symptoms above.</p> <p>Instrument was improperly calibrated. Calibrate instrument as instructed by the manufacturer.</p> <p>The passive reference was set incorrectly. Be sure the passive reference is set as “IC5” (Section 7.B, Step 7).</p> <p>Sample well(s) did not receive reaction mix. Examine the plate to identify any empty wells. Return to the Plate Setup Editor (Figure 10), and redefine the samples, taking care not to define any empty wells. Reexport and reanalyze the data (Section 7).</p>
Small signal change in amplification and melt curves	<p>No amplification or poor amplification. See causes and comments for “Flat amplification curve in the amplification curves window” symptoms above.</p> <p>Incorrect dye was selected. Verify the presence of the appropriate dye.</p>

<b>Symptoms</b>	<b>Causes and Comments</b>
Small signal change in amplification and melt curves (continued)	<p>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 10.F.</p> <p>The passive reference was set incorrectly. Be sure the passive reference is set as "IC5" (Section 7.B, Step 7).</p> <p>Sample well(s) did not receive reaction mix. Examine the plate to identify any empty wells. Return to the Plate Setup Editor (Figure 10), and redefine the samples, taking care not to define any empty wells. Reexport and reanalyze the data (Section 7).</p>
Nonlinear standard curve, low R <sup>2</sup> values ≤0.98	<p>Be sure that the DNA standard is completely thawed and mixed well before use. Store the DNA standard at 4°C overnight to improve sampling consistency.</p> <p>Be sure dilutions of the DNA standard are well mixed before removing each aliquot for the serial dilution. Change pipette tips between each dilution step.</p> <p>Calibrate your pipettes to minimize variability when pipetting. Small volumes are difficult to pipet accurately. Do not pipet volumes &lt;1µl; we recommend using a sample volume of 2µl or higher. If necessary, dilute the template so that larger volumes are pipetted.</p> <p>Adjust the baseline region. The baseline region can be manually adjusted for each reaction. See Section 10.C.</p> <p>Some variation is normal. Perform duplicate or triplicate reactions for the standard curve to minimize the effect of this variation.</p> <p>There will be statistical variation in the amount of template in a reaction with the DNA target present at low copy number. Perform duplicate or triplicate reactions for the standard curve. Remove the 0.0032ng/µl dilution of the DNA standard from the standard curve by changing the sample type to "unknown".</p> <p>An error was made during dilution of the DNA standard. Verify all calculations, and repeat dilution of the DNA standard. Do not pipet volumes &lt;1µl.</p> <p>Incorrect concentration values were entered in the Plexor® Analysis Software. Verify the concentrations for all samples used to generate the standard curve.</p> <p>Reactions were contaminated with target DNA. Clean workstations and pipettes with a mild bleach solution before and after use. Use new reagents and solutions. Take precautions to prevent contamination.</p> <p>The plate was not completely sealed. Carefully seal the plates to avoid evaporation.</p> <p>Aberrant fluorescence can be caused by writing on plates, contamination, fingerprints, etc. Do not write on the plate. Use caution when handling plates. Wear gloves. Do not place plates on surfaces that might be contaminated with fluorescent material. If you suspect contamination of the benchtop, thermal cycler block or any other area, clean it thoroughly.</p>

## 9. Troubleshooting (continued)

Symptoms	Causes and Comments
Slope is outside of the range specified in Section 8.C	<p>No amplification or poor amplification. See causes and comments for "Flat amplification curve in the amplification curves window" symptoms above.</p> <p>Nonspecific amplification can become a problem in later amplification cycles for samples containing small amounts of target template. Decrease the number of amplification cycles.</p> <p>An error was made during dilution of the DNA standard. Verify all calculations, and repeat dilution of the DNA standard. Do not pipet volumes &lt;1<math>\mu</math>l.</p> <p>There will be statistical variation in the amount of template in a reaction with the DNA target present at low copy number. Perform duplicate or triplicate reactions for the standard curve. Remove the 0.0032ng/<math>\mu</math>l dilution of the DNA standard from the standard curve by changing the sample type to "unknown".</p> <p>Incorrect concentration values were entered in the Plexor<sup>®</sup> Analysis Software (Section 8.A). Verify the concentrations for all samples used to generate the standard curve.</p> <p>There was variability or inaccuracy when pipetting reaction components. Calibrate pipettes to improve accuracy.</p>
Unable to import saved data files (Section 7.C). Encountered "Expecting NEWLINE, found" or "Unexpected Token Error" message	<p>The data have 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.</p>
Data display appears abnormal in the Plexor <sup>®</sup> Analysis Software (the screen appears compressed, lines are replaced with dots, etc.)	<p>Be sure that the display settings for the computer are set to 32-bit color, rather than 16-bit color, when using the Plexor<sup>®</sup> Analysis Software.</p>
Standard curve is not linear	<p>An incorrect amplification or melt data set was selected during import into Plexor<sup>®</sup> Analysis Software. If data import was via tab-delimited text files (Section 7.C), review the Import Files Report (Section 8.H). If data were imported by pasting directly into the Plexor<sup>®</sup> Analysis Software (Section 7.B), raw data may be re-imported into a new Plexor<sup>®</sup> file to double-check this step.</p>
All samples show an [Auto]/[Y] ratio of 1.0	<p>See causes and comments for "Standard curve is not linear" symptoms above.</p>
Melt peaks for all samples within a dye channel have the wrong $T_m$	<p>See causes and comments for "Standard curve is not linear" symptoms above.</p>

## 10. Appendix

### 10.A. Plexor<sup>®</sup> Analysis Software Operating System Compatibility

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

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

## 10.B. Quantitation Using Large Sample Volumes

You may wish to increase input volumes to increase sensitivity or match the input volume of subsequent STR assays. Laboratory validation is required for any protocol modifications. The Plexor® HY System allows up to 9µl of input material per reaction. The sample volume can be increased as long as the final reaction volume remains constant. **The reaction volumes must be the same for both the DNA standards and unknown samples.** This allows the DNA standards to be considered as a concentration (in ng/µl) instead of input amount in ng (ng/µl × volume).

Table 4 shows the necessary serial dilutions to perform an alternative standard curve using 5µl input volumes and DNA standard concentrations in the range of 1.28pg/µl to 20ng/µl.

**Table 4. Serial Dilution of the Plexor® HY Genomic DNA Standard for the Alternative Standard Curve (assumes 5µl of DNA per reaction).**

Concentration	Volume of DNA	Volume of TE <sup>-4</sup> Buffer
20ng/µl	Use 10µl of DNA	15µl
4ng/µl	10µl of 20ng/µl dilution	40µl
0.8ng/µl	10µl of 4ng/µl dilution	40µl
0.16ng/µl	10µl of 0.8ng/µl dilution	40µl
0.032ng/µl	10µl of 0.16ng/µl dilution	40µl
0.0064ng/µl	10µl of 0.032ng/µl dilution	40µl
0.00128ng/µl	10µl of 0.0064ng/µl dilution	40µl

### Reaction Setup

Prepare the reaction mix by combining the Water, Amplification Grade, Plexor® HY Master Mix and Plexor® HY Primer/IPC Mix as indicated in Table 5.

If the sample volume is increased, decrease the volume of water added to each reaction so that the final reaction volume is constant.

Proceed with reaction setup as described in Section 5.B, but dispense 15µl of reaction mix to wells, rather than 18µl. Add 5µl of sample DNA or DNA standard to each reaction. Add 5µl of TE<sup>-4</sup> buffer to the NTC reactions.

**Table 5. Preparation of Reaction Mix for Quantitation Assays (5µl of template per reaction).**

Component	Volume (Per Reaction)
Plexor® HY 2X Master Mix	10µl
Water, Amplification Grade	4µl
Plexor® HY 20X Primer/IPC Mix	1µl
Final volume	15µl

## 10.B. Quantitation Using Large Sample Volumes (continued)

### Data Analysis

During sample definition (Section 8.A), the dilution series must be modified to reflect the change in concentration of the DNA standards. When using 5µl input volumes, note that the dilution series is a decreasing, fivefold dilution series with “20” as a starting concentration or an increasing fivefold dilution series starting with “0.00128”, as appropriate for the plate layout used.

## 10.C. Adjusting the Baseline Region (Optional)

The Plexor® Analysis Software automatically sets the baseline region for each sample during import. The baseline is set in a flat region of the amplification curve before product accumulation (i.e., the downward inflection). Manual adjustment of the baseline region is possible for each sample but rarely necessary. Manual adjustment might be required for runs where the baseline region is set during or after amplification (i.e., not within the flat part of the amplification curve; Figures 37 and 38).

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. Figures 37 and 38 show examples of data with improperly set baseline regions that should be adjusted manually. In Figure 37, the upper limit of the baseline region (21.1) is within the visible signal change. The  $C_t$  value has been erroneously increased to accommodate this baseline region. In general, the upper bound of the baseline region is set several cycles before the amplification curve crosses the

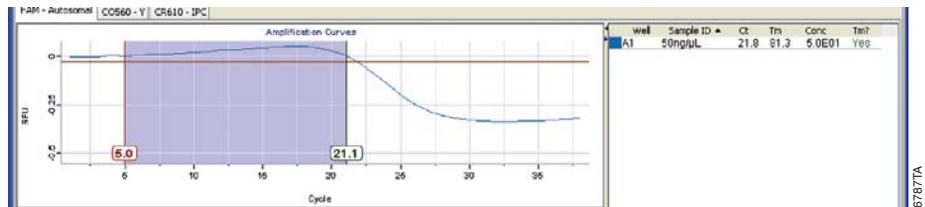
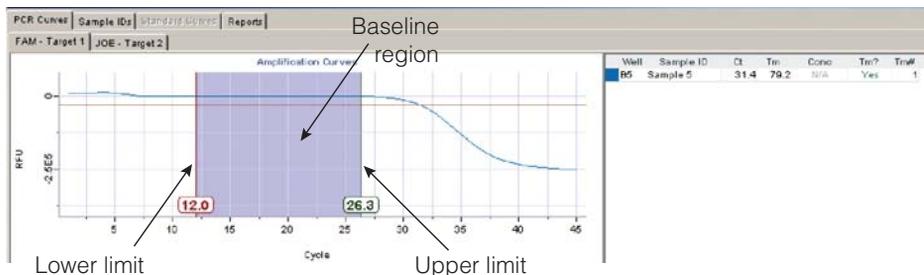


Figure 37. An amplification window showing an improper baseline region with an improper baseline upper limit.



Figure 38. An amplification window showing an improper baseline region with improper upper and lower limits.

threshold. In this example, the difference between the upper baseline region bound and  $C_t$  value is only 0.7 cycles. In Figure 38 the lower and upper limits of the baseline region (32.0 and 35.0, respectively) are set after the visible signal change. The baseline region must precede the visible amplification. A properly set baseline region with appropriate lower and upper limits is shown in Figure 39.



**Figure 39. An amplification window showing the proper baseline region and baseline upper and lower limits.**

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 39). The baseline is set independently for each dye.
4. Adjust the upper limit of the baseline region for each sample so that 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 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 10.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.

## 10.C. Adjusting the Baseline Region (Optional) (continued)

3. 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”.
4. The baselines for all samples can be reset to the automatic setting by selecting the Reset Baselines and Amp Thresholds icon: 

## 10.D. Adjusting the Amplification Threshold (Optional)

The amplification threshold is used to determine the  $C_t$  value for the samples (Figure 40). The default amplification threshold is based on the variation (noise) in the baseline regions of all samples. It is determined by calculating 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:** 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.



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.

3. **Optional:** The amplification threshold is based on noise within the baseline region for all samples. When manual baseline adjustments are complete, consider recalculating the amplification threshold for all samples. See Notes 1 and 2.

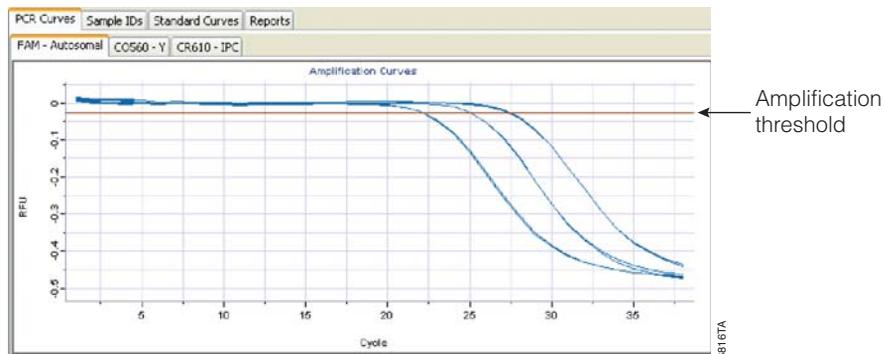


Figure 40. The amplification threshold.

**Notes:**

1. The amplification threshold can be reset based on all samples. Select all samples, and in the Edit menu, select “Set Amp Threshold from Selected Samples”.
2. The amplification threshold and baselines for all samples can be reset to the automatic setting by selecting the Reset Baselines and Amp Thresholds icon:

**10.E. Adjusting the Melt Threshold (Optional)**

**Optional:** The melt threshold may be reset to change the sensitivity in detecting the amplification product. See Notes 1-4. 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 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.
2. A “Yes” or “No” in the  $T_m?$  column indicates whether a sample  $T_m$  is within the expected target melt temperature range. A “No Call” in this column indicates that 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.
3. The “ $T_m\#$ ” is the number of peaks that cross the melt threshold line. More than one peak indicates heterogeneous amplification products or improper calibration. This may be due to nonspecific amplification.
4. 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.

## 10.F. 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.

## 10.G. Templates for Routine Setups (Optional)

At the File Import screen, Section 7.B (Figure 17) or Section 7.C (Figure 22), 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 DNA concentrations of standards (Figure 41). If you routinely use the same setup for plates of standards and unknowns, a run template can be created, stored and applied to subsequent runs.

1. Select “Run Template”.
2. Assign colors, sample types and DNA concentrations to the standards in the Plate Setup tab.



Figure 41. A Run Template.

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”.

**Note:** To import an existing \*.rtp file that contains a saved plate configuration, select “Import” and browse to that file.

**Analysis Template and Definition of Analysis Functions:** The analysis template is used to optimize the analysis settings for the experiment. A template can be set up to include the expected target melt temperature for each reaction. The expected target melt temperature should be determined for each real-time instrument. We recommend the default settings for all other analysis parameters.

1. Select “Analysis Template”.
2. Enter the desired values for the “Expected Target Melt Temperature ( $T_m$ )” for each dye used (Figure 42).

**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 (Section 7.C, Step 18).



Figure 42. The Analysis Defaults tab.

## 10.G. Templates for Routine Setups (Optional) (continued)

### **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  $C_t$  values are called. This threshold value is based on the variation (noise) in the baseline regions of all samples and is determined by calculating the mean and standard deviation of all RFUs in baseline regions. The threshold is set at 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 the Set Amp Threshold from Selected Samples option in the Edit menu (Section 10.D).

**Default Melt Threshold  $-d(\text{RFU})/dT$  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 and are present within the expected melt temperature range. The melt threshold represents the  $-d(\text{RFU})/dT$  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)  $-d(\text{RFU})/dT$  value.

The default melt threshold  $-d(\text{RFU})/dT$  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  $-d(\text{RFU})/dT$  value for the sample with the lowest  $-d(\text{RFU})/dT$  value in the data set.

See Section 10.E for instructions to adjust the melt threshold.

**Expected Target Melt Temperature:** The expected target melt temperature is the melt temperature of the correct PCR product (Section 8.B). 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 +1.5°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 -1.5°C.

## 10.H. 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 wells using the well selector, then select this button to choose the desired color for the selected samples. 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 wells 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 if a  $C_t$  value is present.



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 wells 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 concentration of sample in the no-template control reaction will be calculated and reported if a  $C_t$  value is present.



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 wells 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 if a  $C_t$  value is present.



Assign Standard (shortcut = “r”)

The Assign Standard function allows you to assign the sample type “Standard” to all selected samples. Select one or more wells 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 DNA standards 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.). See Section 8.A. The software does not accept commas in the concentration assignments. Standards are displayed as circles in the well selector and standard curve graphs. They are labeled “Standard” in reports.

## 10.H. Icon Definitions (continued)



### Create Dilution Series (shortcut = "f")

The Create Dilution Series function creates a full dilution series within a row or column of wells. Select the wells that contain a dilution series of the DNA standard, then select "Create Dilution Series". You must enter the initial DNA concentration of the series, the dilution factor and whether the series is increasing or decreasing (Figure 25). 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 wells will be assigned the sample type "Standard" with a corresponding concentration. This function can only be performed with DNA standards in adjacent rows or columns. Using this function produces the same result as selecting each well 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 standard curves.



### Add Standard Curve (shortcut = "d")

The Add Standard Curve function fits the experimentally measured  $C_t$  values and user-entered concentration values for DNA standards to a straight line using the least mean squares method. It will calculate the DNA 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 amplification threshold, so the concentration of that sample cannot be calculated.

Select all of the samples you wish to use as standards, as well as all other samples for which you wish to calculate concentrations. Choose "Add Standard Curve" from the Edit menu, and 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 10.C.



### 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 Sections 10.C and 10.D.

## 10.I. References

1. Sherrill, C.B. *et al.* (2004) Nucleic acid analysis using an expanded genetic alphabet to quench fluorescence. *J. Am. Chem. Soc.* **126**, 4550–6.
2. Johnson, S.C. *et al.* (2004) A third base pair for the polymerase chain reaction: Inserting isoC and isoG. *Nucleic Acids Res.* **32**, 1937–41.
3. Moser, M.J. and Prudent, J.R. (2003) Enzymatic repair of an expanded genetic information system. *Nucleic Acids Res.* **31**, 5048–53.

## 10.J. Composition of Buffers and Solutions

### 10.K. Related Products

#### TE<sup>-4</sup> buffer (10mM Tris-HCl, 0.1mM EDTA [pH 8.0])

2.21g      Tris base  
0.037g     EDTA (Na<sub>2</sub>EDTA • 2H<sub>2</sub>O)

Dissolve Tris base and EDTA in 900ml deionized water. Adjust to pH 8.0 with HCl. Bring the volume to 1 liter with deionized water.

Product	Size	Cat.#
PowerPlex® 16 System	100 reactions	DC6531
	400 reactions	DC6530
PowerPlex® Y System	50 reactions	DC6761
	200 reactions	DC6760
PowerPlex® 16 BIO System	100 reactions	DC6541
	400 reactions	DC6540
PowerPlex® ES System	100 reactions	DC6731
	400 reactions	DC6730
PowerPlex® 1.1 System	100 reactions	DC6091
	400 reactions	DC6090
PowerPlex® 2.1 System	100 reactions	DC6471
	400 reactions	DC6470
Water, Amplification Grade	5 × 1,250µl	DW0991

## 10.K. Related Products (continued)

### Sample Preparation Systems

Product	Size	Cat.#
DNA IQ™ System	100 reactions	DC6701
	400 reactions	DC6700
Maxwell® 16 Instrument	1 each	AS2000
Standard Conversion Kit for Maxwell® 16 LEV	1 each	AS1200
LEV Conversion Kit for Maxwell® 16	1 each	AS1250
DNA IQ™ Reference Sample Kit for Maxwell® 16	48 preps	AS1040
DNA IQ™ Casework Sample Kit for Maxwell® 16	48 preps	AS1210
Slicprep™ 96 Device	10 pack	V1391

### ART® Aerosol-Resistant Tips

Product	Volume	Size (tips/pack)	Cat.#
ART® 10 Ultramicro Pipet Tip	0.5–10µl	960	DY1051
ART® 20E Ultramicro Pipet Tip	0.5–10µl	960	DY1061
ART® 20P Pipet Tip	20µl	960	DY1071
ART® GEL Gel Loading Pipet Tip	100µl	960	DY1081
ART® 100 Pipet Tip	100µl	960	DY1101
ART® 100E Pipet Tip	100µl	960	DY1111
ART® 200 Pipet Tip	200µl	960	DY1121
ART® 1000E Pipet Tip	1,000µl	800	DY1131

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