Quantifiler™ Trio Automated DNA Quantification Kit

USER GUIDE

Catalog Number A58787

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Revision A00

For Research, Forensic, or Paternity Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Life Technologies Ltd | 7 Kingsland Grange | Woolston, Warrington WA1 4SR | United Kingdom
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN1000066 A00 (English)

Revision	Date	Description
A00	15 March 2024	New document for the Quantifiler™ Trio Automated DNA Quantification Kit.

The information in this guide is subject to change without notice.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The Quantifiler™ Trio Automated DNA Quantification Kit (Cat. No. A58787) quantifies the total amount of amplifiable human DNA in a sample. The kit contains all the necessary reagents for the amplification, detection, and quantification of three human-specific DNA targets. The kit is designed for liquid handling automation platforms and includes an enhancer to reduce foaming during automated pipetting.

The kit uses the same TaqMan™ quantitative real-time PCR technology as the Quantifiler™ HP DNA Quantification Kit and the Quantifiler™ Trio DNA Quantification Kit. For information about validation experiments performed using the Quantifiler™ HP and Trio DNA Quantification Kits, see the *Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits User Guide* (Pub. No. 4485354).

The assay chemistry is optimized for more efficient multiplexing, faster PCR cycle times (1 hour), and better inhibitor tolerance. The overall performance improvements allow the kit to better match the enhanced performance of newer short tandem repeat (STR) kits that accommodate more challenging samples, for example, the NGM Detect™, VeriFiler™ Plus , NGM SElect™, GlobalFiler™, and GlobalFiler™ IQC PCR Amplification Kits.

For details on the assay chemistry, see Appendix A, "Supplemental information".

Quantification targets

The Quantifiler™ Trio Automated DNA Quantification Kit uses multiple-copy target loci for improved detection sensitivity.

- The human-specific target loci (Small Autosomal, Large Autosomal, and Y-chromosome targets) each consist of multiple copies dispersed on various autosomal chromosomes (Small Autosomal and Large Autosomal), or multiple copies on the Y-chromosome.
- The primary quantification targets (Small Autosomal and Y) consist of relatively short amplicons (75–80 bases) to improve the detection of degraded DNA samples. The Large Autosomal target is a longer amplicon (>200 bases) that helps to determine if a DNA sample is degraded.

To maximize the consistency of quantification results, genomic targets were selected with conserved primer- and probe-binding sites within individual genomes and also with minimal copy number variability between different individuals and population groups.

About DNA quantification before STR analysis

DNA quantification can be used to determine the following:

- If the sample contains sufficient human DNA and/or human male DNA to proceed with short tandem repeat (STR) analysis.
- The amount of sample to use in STR analysis applications.
- *(When using the Quantifiler™ Trio Automated DNA Quantification Kit)* The relative quantities of human male and female DNA in a sample. The relative quantities can guide selection of the appropriate STR chemistry.
- If PCR inhibitors are present in a sample. If inhibitors are present, the sample may require additional purification before proceeding to STR analysis.
- The DNA quality, regarding the inhibition level and the DNA degradation level. The quality metric is useful for determining the likelihood of recovery of STR loci with larger amplicon sizes.

Note: Highly degraded samples that cannot be recovered by STR analysis with capillary electrophoresis can be analyzed with the Precision ID NGS System and Panels. Optimized for degraded samples, the Precision ID Identity Panel provides discrimination of individuals similar to STR genotype match probabilities. Also, the Precision ID Ancestry Panel infers biogeographical ancestry for investigative leads.

Contents and storage

The Quantifiler™ Trio Automated DNA Quantification Kit contains materials sufficient to perform 960 reactions at a 20-µL reaction volume.

Table 1 Quantifiler™ Trio Automated DNA Quantification Kit (Cat. No. A58787)

Item	Amount	Description	Storage[1]
Quantifiler™ THP PCR Reaction Mix	10 x 1 mL	Contains dNTPs, buffer, enzyme, MUSTANG PURPLE™ passive reference standard, and stabilizers.	–25°C to –15°C upon receipt. 2°C to 8°C after initial use. Protect from light.[2]
Quantifiler™ Trio Primer Mix	10 x 0.8 mL	Contains target-specific primers, ABY™, JUN™, VIC™, and FAM™ dye-labeled probes, and Internal PCR Control (IPC) template.	
Quantifiler™ THP DNA Dilution Buffer	6 x 1.8 mL	Contains genomic DNA standard dilution buffer.	–25°C to –15°C upon receipt. 2°C to 8°C after initial use.
Quantifiler™ THP DNA Standard	2 x 0.12 mL	Contains genomic DNA standard formulated at 100 ng/µL to generate standard curves.	
Quantifiler™ Automation Enhancer	1 x 0.2 mL	Contains defoaming agent.	15°C to 30°C upon receipt and after initial use.

[1] See the expiration date on the package. Do not use expired product.

[2] Excessive exposure to light can affect the fluorescent probes and the passive reference dye.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier. Catalog numbers that appear as links open the web pages for those products.

Item	Source
Real-time PCR instrument, one of the following:	
7500 Real-Time PCR Instrument	Contact your local sales office.
QuantStudio™ 5 Real-Time PCR System	
Automation system, one of the following configurations:	
HID NIMBUS® QNA System	A55771
HID NIMBUS® Presto QNA System	A55769

(continued)

Item	Source
Consumables	
(Optional) Tabletop centrifuge with 96-well plate adapters	MLS
Pipettors and pipette tips	MLS
Nonstick, RNase-free Microfuge Tubes, 1.5 mL	AM12450
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	4306737
MicroAmp™ Optical Adhesive Film	4311971
MicroAmp™ Splash-Free 96-Well Base	4312063

Instruments and software compatibility

System	Description
7500 Real-Time PCR System for Human Identification	• **HID Real-Time PCR Analysis Software v1.3**—Designed for human identification laboratories that perform DNA quantitation; simplifies assay setup, data review, and dilution and reaction setup for downstream STR analysis. • **7500 Real-Time PCR Instrument**—For more information, see Appendix B, "7500 Real-Time PCR System for Human Identification". **IMPORTANT!** The 7500 Fast Real-Time PCR System is not supported for use with the Quantifiler™ Trio Automated DNA Quantification Kit.
QuantStudio™ 5 Real-Time PCR System for Human Identification	• **HID Real-Time PCR Analysis Software v1.3 or later**—Designed for human identification laboratories that perform DNA quantification; simplifies assay setup, data review, and dilution and reaction setup for downstream STR analysis. • **QuantStudio™ 5 Real-Time PCR Instrument**—For more information, see Appendix C, "QuantStudio™ 5 Real-Time PCR System for Human Identification".
HID NIMBUS® QNA systems	• **HID NIMBUS® System Software v2.0**—Designed and validated for human identification laboratories that perform DNA quantification; simplifies PCR plate map setup and streamlines sample tracking. • **HID NIMBUS® QNA systems**—For more information, see the *Automated DNA Quantification, Normalization, and Amplification Setup User Bulletin* (Pub. No. MAN1000064).

Workflow

Set up and run the plate

Set up the software

1. Start the HID Real-Time PCR Analysis Software (page 12)
2. Create an experiment (page 13)
3. Save an experiment template (page 19)

Prepare, then run the reactions

1. Prepare the DNA quantification standards (page 21)
2. Prepare the reaction plate with the HID NIMBUS® QNA systems (page 22)
3. Load the plate, then start the run (page 38)

Analyze the experiment and view results

1. Analyze the experiment (page 39)
2. View results (page 41)

Review the results

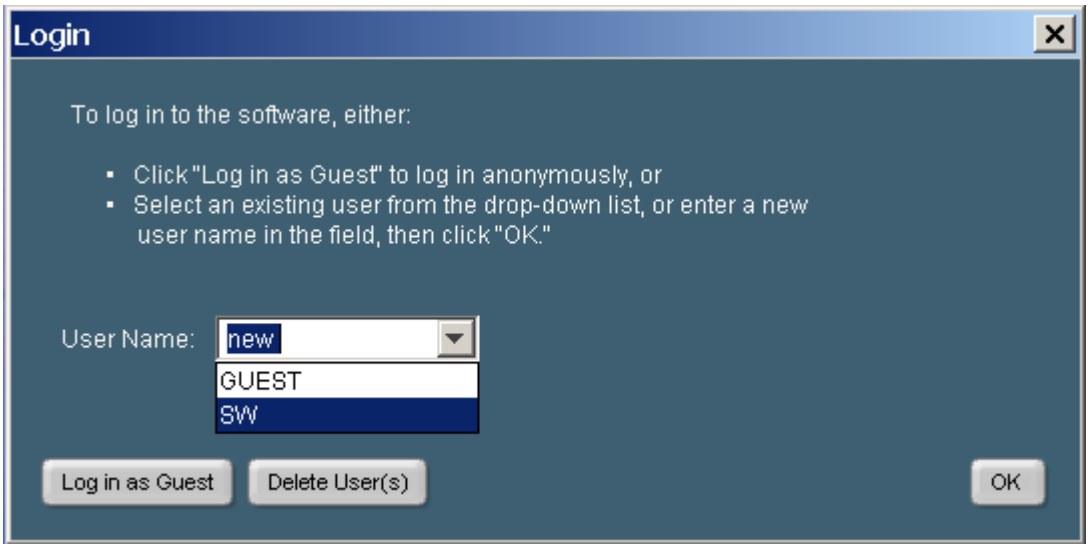
To evaluate the results using various metrics, see Review the results (page 46).

2 Set up the software

Start the HID Real-Time PCR Analysis Software

IMPORTANT! Before running samples using the Quantifiler™ Trio Automated DNA Quantification Kit for the first time, ensure that the instrument has been calibrated as described in "Calibrate the 7500 instrument" on page 79 and "Calibrate the QuantStudio™ 5 Instrument" on page 83.

1. On the computer desktop, double-click or select **Start ▸ All Programs ▸ Applied Biosystems ▸ HID Real-Time PCR Analysis Software ▸ HID Real-Time PCR Analysis Software**. The **Login** screen should open within 1 minute.



2. In the **User Name** field, enter your user name or select it from the dropdown list.

   You can log in as a guest, but only users logged in with a user name can perform the following functions:

   - Edit the names of folders for experiment information import, information export, or data.
   - Enable or disable the requirement to enter a user name to start the software.
   - Set a plate layout as the default layout.
   - Configure how data is displayed.

For more information about user functions, see the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

3. Click **OK** to open the **Home** screen.

Create an experiment

This section contains brief procedures. For more information, see the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

Note: Alternatively, you can generate a plate setup file using the HID NIMBUS® System Software. For more information, see Chapter 3, "Prepare and run the reactions".

1. In the **Home** screen, click the icon for your application.

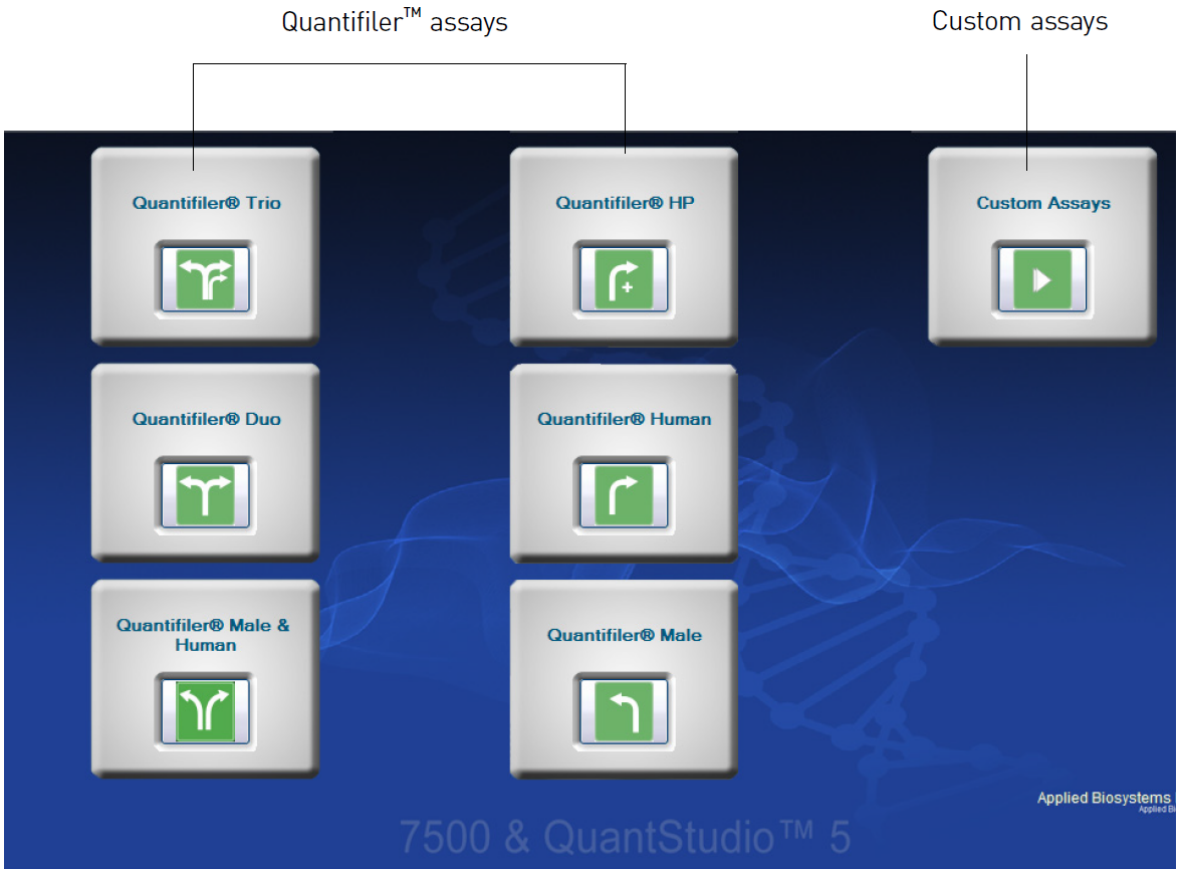


Figure 1 Home screen

2. In the **Experiment Properties** screen, enter a name for the experiment. All other settings on this screen are automatically set for your application or are optional.

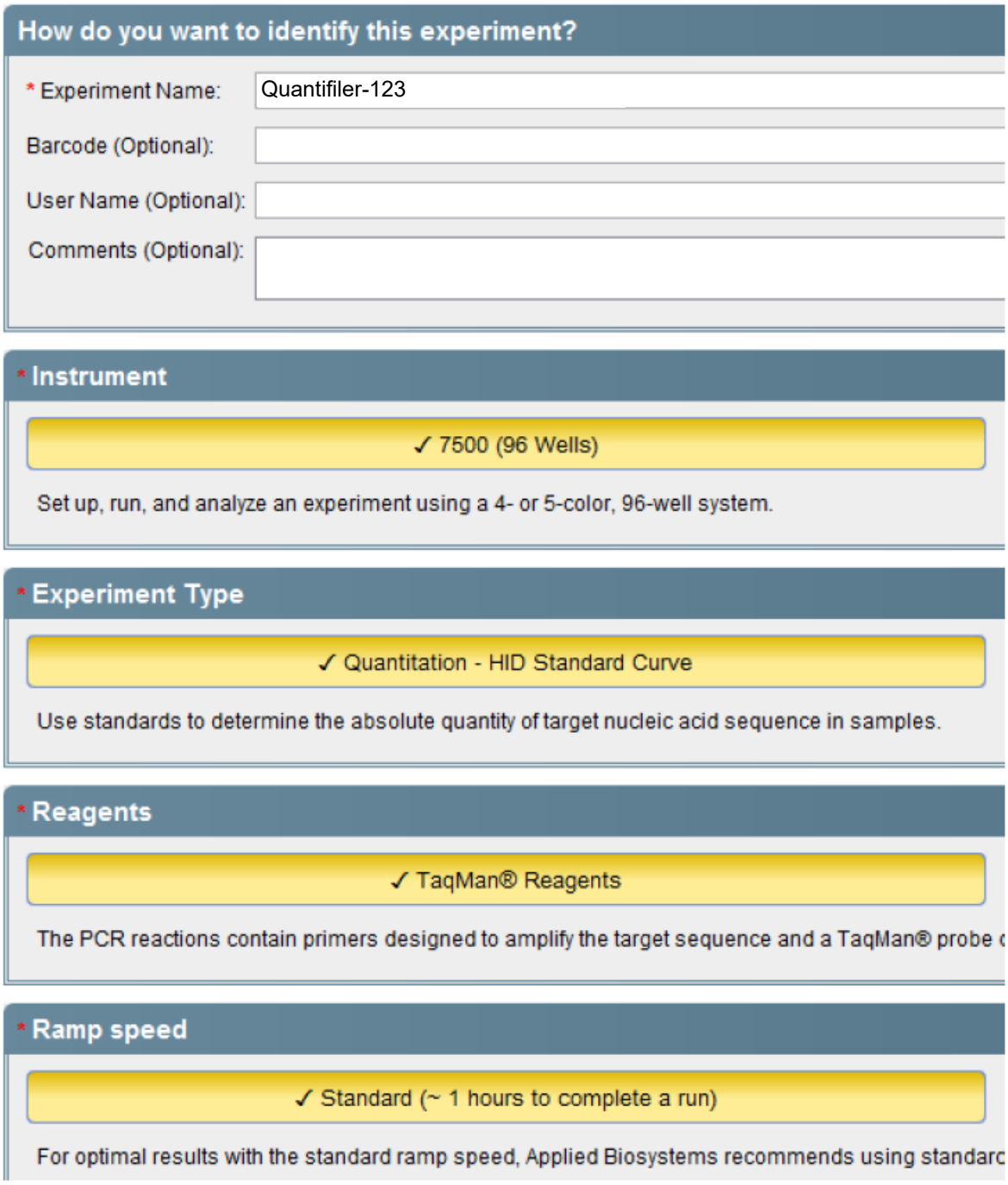


Figure 2 Experiment Properties screen

3. In the left navigation pane, click **Setup** ▸ **Plate Setup**.

Note: If you set up a quantification plate using the HID NIMBUS® System Software, it generates an import plate map with the sample information and plate locations. After the automated run on the HID NIMBUS® system is complete, select the **Import Plate map** option to import the file. For more information, see Chapter 3, “Prepare and run the reactions”.

Figure 3 Setup menu

Targets are automatically specified for your application.

Defined Targets

Target Name	Reporter	Quencher
T.Large Autosomal	ABY	QSY7
T.Small Autosomal	VIC	NFQ-MGB
T.IPC	JUN	QSY7
T.Y	FAM	NFQ-MGB

Figure 4 Defined Targets pane

4. Define samples.

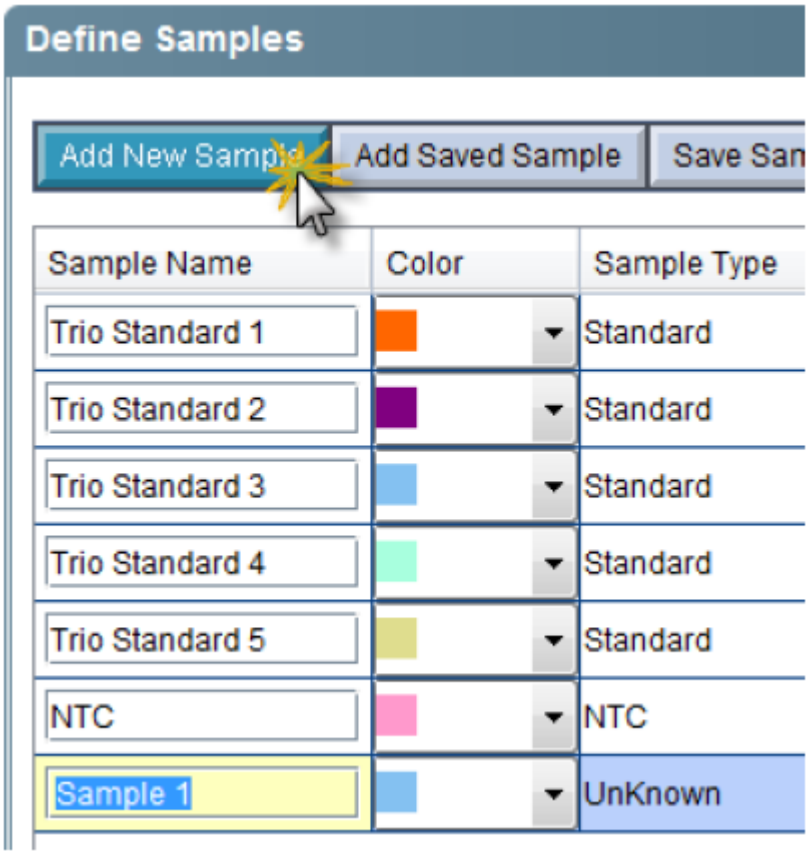


Figure 5 Add New Sample tab

   a. in the **Define Samples** pane, click **Add New Sample**

   b. In a text field within the **Sample Name** column, enter the sample name.

   c. Repeat substep 4a and substep 4b for the remaining samples.

5. Click **Assign Targets and Samples**.
   Targets are automatically assigned, and the standard quantities are automatically specified.
   For an example of the standard setup for the Quantifiler™ Trio Automated assay, see Figure 6.

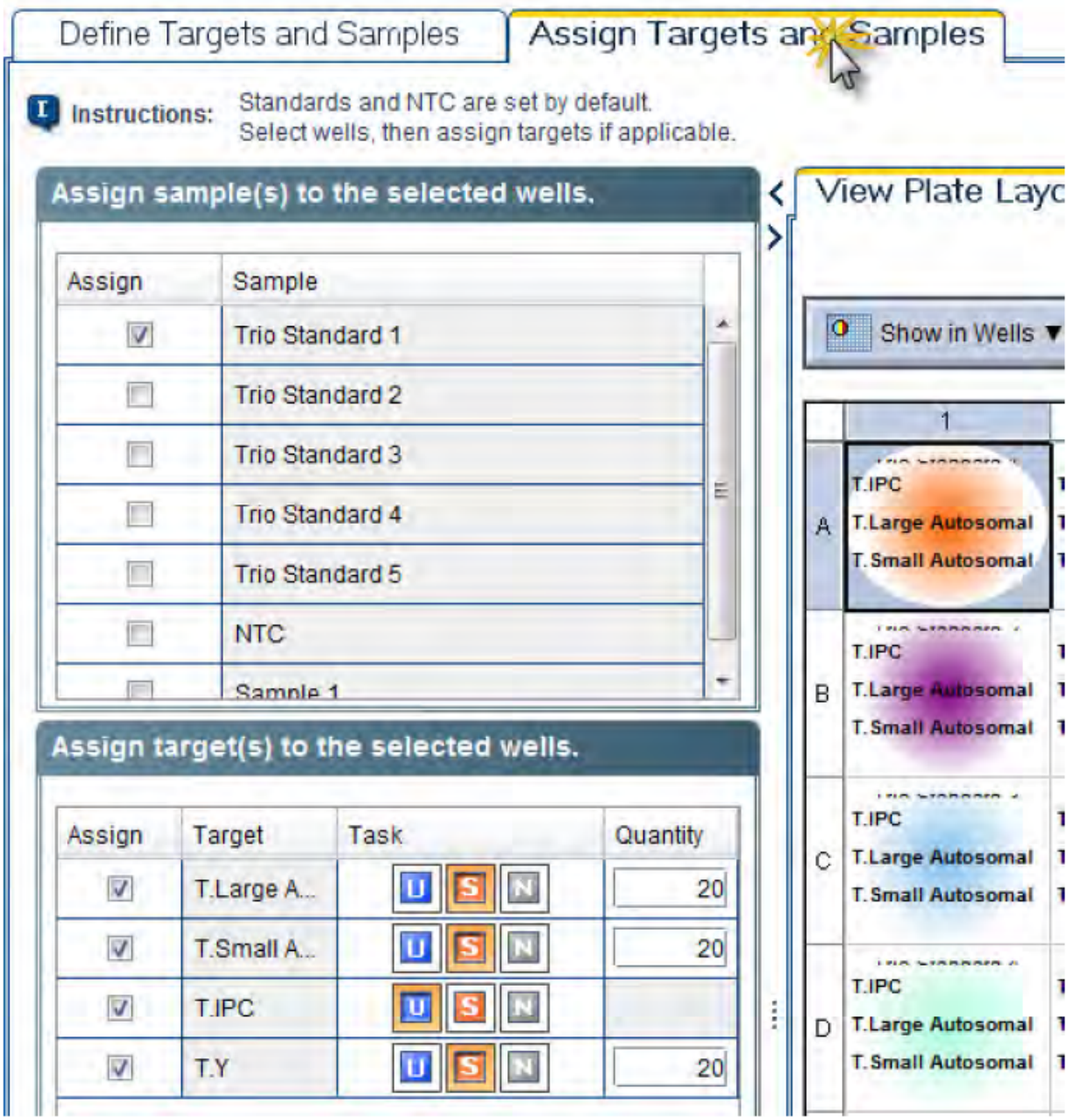


Figure 6 Assign Targets and Samples screen

6. Assign the samples to the plate wells.
   a. To select wells, compete the tasks below according to the desired well selection:

Well selection	Task
Single well	Click the well.
Row of wells	Click a letter on the side of the layout.
Column of wells	Click a number at the top of a column.
More than one well, row, or column	Drag the pointer over the wells, letters, or columns to select.

b. In the **Assign sample(s) to the selected wells** pane, in the **Assign** column, select the checkbox for the sample of interest.

   The target for each sample is set by default.

c. Repeat substep 6a and substep 6b for the remaining samples.

7. Reassign a sample to a different well.
   a. Click the well.
   b. In the **Assign sample(s) to the selected wells** pane, deselect the sample.
   c. Click the new well.
   d. In the **Assign sample(s) to the selected wells** pane, select the sample.

8. In the left navigation pane, click **Setup** ▸ **Run Method** to view the parameters. The parameters are automatically specified.

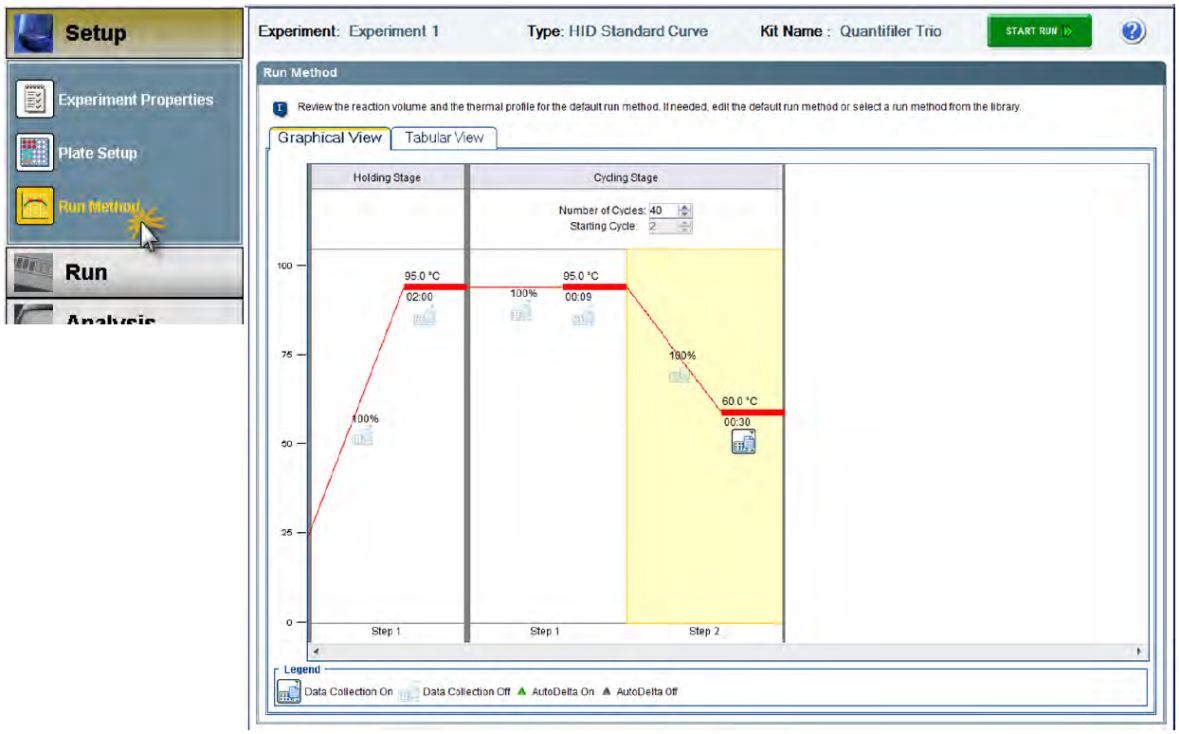


Figure 7 Run Method screen

9. Click **Save**.

Save an experiment template

Template settings

In addition to assay settings, templates can contain the following:

- Assay-specific detectors
- Well assignments for quantification standards, with targets, tasks, and quantity
- Well assignments for unknown samples, with targets and tasks
- Instrument settings: reaction volume settings and 9600 Emulation setting

Create and use a template

1. Select **File** ▸ **New Experiment**, then select the application for the template.
2. Specify settings and plate layout as needed.
3. Select **Save** ▸ **Save As Template**.
   Templates are saved as EDT files. The default location is `C:\Applied Biosystems\7500\Experiments`
4. Click **Open**, then navigate to the template of interest.

3 Prepare and run the reactions

Prepare the enhanced reaction mix

1. Thaw the Quantifiler™ THP PCR Reaction Mix and the Quantifiler™ Automation Enhancer.

   IMPORTANT! Excessive exposure to light can affect the fluorescent probes and the passive reference dye. Protect the Quantifiler™ THP PCR Reaction Mix from light.

   IMPORTANT! Thawing is required only during first use of the kit. After first use, reagents are stored at 2–8°C, and therefore, do not require subsequent thawing. Do not refreeze the reagents.

2. Before opening the tubes, remove droplets from the caps by centrifuging the tubes briefly.

3. Add 1 µL of the Quantifiler™ Automation Enhancer to 1 mL of the Quantifiler™ THP PCR Reaction Mix (1:1,000 dilution)

   Follow the standard kit setup for the PCR reactions and the 7500 Real-Time PCR Instrument or the QuantStudio™ 5 Real-Time PCR System.

4. Gently invert the reaction mix tube 10 times, then centrifuge the tube briefly.

5. Mark the cap of the enhanced reaction mix with a (+) to indicate that the Quantifiler™ Automation Enhancer was added.

Store the enhanced reaction mix at 2–8°C for up to 6 months.

Importance of minimizing bubbles

Bubbles in reaction wells can cause noise in the fluorescence signal and can affect results.

The Quantifiler™ Automation Enhancer assists with preventing bubbles from being introduced during robotic mixing and pipetting procedures.

Prepare the DNA quantification standards

Prepare the standards using one of the following methods:

- "Prepare the DNA quantification standards using the HID NIMBUS® QNA systems" on page 21
- "Manually prepare the DNA quantification standards" on page 21

General guidelines

- You can store the diluted DNA quantification standards for up to 2 weeks at 2–8°C. Longer term storage is not recommended.
- Store diluted DNA standards in low-bind tubes (for example, Applied Biosystems™ Non-Stick RNase-free Microfuge Tubes, 1.5 mL, Cat. No. AM12450).
- We recommend a ten-fold dilution series with 5 concentration points. If needed, add 100 ng/µL of the standard point.

Note: There may be an increase in the IPC C_T. For more information, see "Use the internal PCR control system" on page 57.

Prepare the DNA quantification standards using the HID NIMBUS® QNA systems

The DNA quantification standards can be prepared using the HID NIMBUS® QNA System or the HID NIMBUS® Presto QNA System. For information about the automated preparation of DNA quantification standards, see the *Automated DNA Quantification, Normalization, and Amplification Setup User Bulletin* (Pub. No MAN1000064).

Manually prepare the DNA quantification standards

Procedural guidelines

- The quality of pipettors and tips, use of low-binding DNA tubes for dilutions, and the care used in measuring and mixing dilutions affects the accuracy of a run.
- To help ensure accuracy of manual pipetting, we recommend a minimum input DNA volume of 10 µL for dilutions.

Prepare the DNA quantification standards

For recommended volumes, see "Standard dilution series example" on page 22.

1. Label five microcentrifuge tubes: S1, S2, S3, S4, S5.
2. Add Quantifiler™ THP DNA Dilution Buffer (diluent) to each tube.
   For recommended diluent volumes, see "Standard dilution series example" on page 22.

3. Prepare the S1 standard:
   a. Vortex the Quantifiler™ THP DNA Standard for 3–5 seconds.

      For recommended Quantifiler™ THP DNA Standard volume, see "Standard dilution series example" on page 22.

   b. Using a new pipette tip, add the Quantifiler™ THP DNA Standard to the S1 tube.

   c. Vortex the tube for 3–5 seconds, then briefly centrifuge to remove drops inside the lid.

4. Prepare the remaining standards:
   a. Using a new pipette tip, add 10 µL of the prepared standard to the tube for the next standard.

   b. Vortex the tube for 3–5 seconds, then briefly centrifuge to remove drops inside the lid.

   c. Repeat substep 4a and substep 4b for each subsequent standard in the dilution series.

Standard dilution series example

Table 2 Standard dilution series example

Standard	Volume		Concentration	Dilution factor
	Diluent (Quantifiler™ THP DNA Dilution Buffer)	Standard[1]		
S1	10 µL	10 µL (100 ng/µL) of Quantifiler™ THP DNA Standard	50 ng/µL	2X
S2	90 µL	10 µL of S1	5 ng/µL	10X
S3	90 µL	10 µL of S2	0.5 ng/µL	10X
S4	90 µL	10 µL of S3	0.05 ng/µL	10X
S5	90 µL	10 µL of S4	0.005 ng/µL	10X

[1] To help ensure manual pipetting accuracy, pipet a minimum volume of 10 µL.

Note: When 2 µL of a sample at the lowest concentration (0.005 ng/µL) is loaded in a reaction, the well contains approximately 1.5 diploid human genome equivalents.

Prepare the reaction plate with the HID NIMBUS® QNA systems

For more information about the automated plate setup for quantification on the HID NIMBUS® QNA System and the HID NIMBUS® Presto QNA System using the Applied Biosystems™ HID NIMBUS® System Software, see the *Automated DNA Quantification, Normalization, and Amplification Setup User Bulletin* (Pub. No MAN1000064).

Required materials

- Quantifiler™ Trio Automated DNA Quantification Kit
- 1.5 mL low-binding DNA tubes
- 96-well optical reaction plate
- Extracted DNA samples
- DNA quantification standards dilutions series
- HID NIMBUS® QNA System or HID NIMBUS® Presto QNA System
- Optical adhesive cover

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier. Catalog numbers that appear as links open the web pages for those products.

Table 3 Automated quantification

Item	Source
Tips, Conductive Non-sterile Filter, 50 µL	A58978
MicroAmp™ Optical 96-Well Reaction Plate (recommended)	N8010560
MicroAmp™ Optical Adhesive Film (recommended)	4311971
RNase-Free Microfuge Tube (1.5 mL) (recommended)	AM12450
Quantifiler™ Trio Automated DNA Quantification Kit	A58787

Guidelines for the automated quantification run

- The Quantifiler™ Trio Automated DNA Quantification Kit is sufficient for ≤960 samples, depending on the batch size.
- The HID NIMBUS® QNA System and HID NIMBUS® Presto QNA System are only compatible with 1.5 mL microcentrifuge tubes, 96-well PCR plates, and KingFisher™ 96-well deep-well plates for purified sample input.
- Before loading the instrument deck, remove air bubbles from the surface of reagents and samples. Bubbles can lead to incorrect liquid detection and pipetting errors.
- When loading tubes on the deck, ensure that the tip will not come into contact with any objects, including the tube caps, to prevent false liquid detection. Firmly press down on all labware placed on the instrument deck.
- We do not recommend a reagent or sample volume <35 µL in microcentrifuge tubes. If the eluate volume from purification is <35 µL, use a MicroAmp™ PCR plate as the input labware.
- For information on barcode positioning, see the *NIMBUS HD Operator's Manual* (Pub. No. 98538-01).
- Before the first run of the day, we recommend that you perform daily maintenance. See *NIMBUS HD Operator's Manual* (Pub. No. 98538-01) for instructions.

Workflow

Prepare automated DNA quantification plate

- **Create a worklist** (page 24)
- **Enter the run settings** (page 27)
- **Load the deck and start the run** (page 31)
- **Remove the quantification plate setup, unused reagents, and labware** (page 36)
- **View the output files** (page 37)

Create a worklist

For a quantification run, we recommend using a worklist for the samples in XLS, XLSX, or CSV format. Templates are installed on the provided laptop computer.

Alternatively, use the worklist generated from the run-tracking report of the purification workflow. For more information about the purification workflow, see *PrepFiler™ and PrepFiler™ BTA Automated Forensic DNA Extraction Kits: Automated DNA Purification on the HID NIMBUS® Presto Systems User Bulletin* (Pub. No. MAN0019368).

1. Before each run, navigate to the **ExampleWorklists** folder on the system computer:

   ```
   C:\Program Files (x86)\Hamilton\HID Nimbus
   System\QuantNormAmp\Resources\Example Worklists
   ```

   Note: If you cannot find the worklist templates on the computer, contact your local Thermo Fisher Scientific representative.

2. Copy the required template, then save it with a new file name.

3. In the saved copy, enter information for each sample, as shown in Table 4, Figure 8, and Figure 9.

IMPORTANT! The worklist must include the required information shown in Table 4. Do not change the column headings or leave cells blank. The system only reads XLS, XLSX, and CSV formats.

Table 4 Worklist information for each sample

Required column order	Required column heading	Action
1	**Sample ID**	Enter the sample name or ID associated with the sample. The sample ID must be unique. The following characters are valid in the worklist: • 0–9 • a–z (uppercase and lowercase) • _ (underscore) • - (hyphen) We recommend that you do not use the period character because it causes issues in the overall target adjustment in the amplification workflow.
2	**Eluate Position**	For tubes, use numeric values. For plates, use alphanumeric values. **Note:** The eluate position on the instrument deck refers to the loading position, regardless of your choice to remove samples from the run during quantification or amplification.
3	**Eluate Barcode**	If you are using barcodes, enter the expected eluate barcode for each sample lysate tube or for the MicroAmp™ PCR plate or KingFisher™ deep-well plate. • **Tubes**—Each tube has a unique barcode. • **Plates**—All sample wells of the plate have the same barcode.

IMPORTANT! To proceed with plate setup, ensure the barcode information on the worklist matches the barcodes loaded on the instrument in the same positions. You must include barcode information in all sample lines. If barcode information is absent in one or more sample lines, you will receive a read error on import. For plates, use the same barcode for each sample. If barcodes are not used, fill the input and eluate barcode cells with ***No Barcode***.

Sample ID	Eluate Position	Eluate Barcode
Sample 1	1	No Barcode
Sample 2	2	No Barcode
Sample 3	3	No Barcode
Sample 4	4	No Barcode
Sample 5	5	No Barcode
Sample 6	6	No Barcode
Sample 7	7	No Barcode
Sample 8	8	No Barcode
Sample 9	9	No Barcode
Sample 10	10	No Barcode
Sample 11	11	No Barcode
Sample 12	12	No Barcode
Sample 13	13	No Barcode
Sample 14	14	No Barcode
Sample 15	15	No Barcode
Sample 16	16	No Barcode
Sample 17	17	No Barcode
Sample 18	18	No Barcode
Sample 19	19	No Barcode
Sample 20	20	No Barcode

Figure 8 Quantification worklist example for tubes

Sample ID	Eluate Position	Eluate Barcode
Sample 1	A1	No Barcode
Sample 2	B1	No Barcode
Sample 3	C1	No Barcode
Sample 4	D1	No Barcode
Sample 5	E1	No Barcode
Sample 6	F1	No Barcode
Sample 7	G1	No Barcode
Sample 8	H1	No Barcode
Sample 9	A2	No Barcode
Sample 10	B2	No Barcode
Sample 11	C2	No Barcode
Sample 12	D2	No Barcode
Sample 13	E2	No Barcode
Sample 14	F2	No Barcode
Sample 15	G2	No Barcode
Sample 16	H2	No Barcode
Sample 17	A3	No Barcode
Sample 18	B3	No Barcode
Sample 19	C3	No Barcode
Sample 20	D3	No Barcode

Figure 9 Quantification worklist example for plates

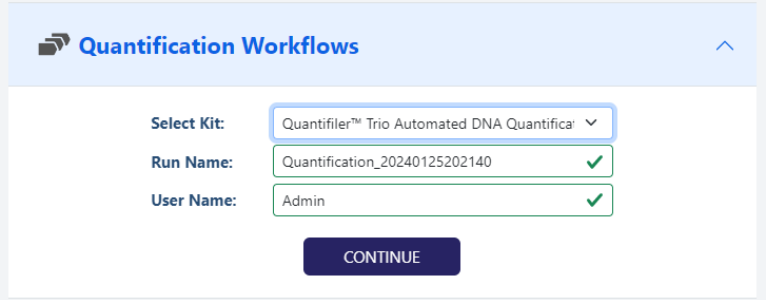
Enter the run settings

1. On the desktop, click **Applied Biosystems HID NIMBUS System Software**.

   Note: If the shortcut is not present on the desktop, navigate to `C:\Program Files (x86)\Hamilton\HID Nimbus System\Application`

2. *(Optional)* Expand the **Input Catalog Number** pane, then scan or type the Quantifiler™ Trio Automated DNA Quantification Kit catalog number.

3. Expand the **Quantification Workflows** pane, then enter the workflow information.



   a. Use the **Select Kit** dropdown list to select a kit.

b. *(If needed)* Change the run name, then enter a user name.

IMPORTANT!

- The run name and user name fields cannot include special characters other than - (hyphen) and _ (underscore).
- The run name and user name must be 1–30 characters in length.

Note: The run name is prepopulated with the workflow type, followed by the date and time (format: Quantification_YYYYMMDDhhmmss). The input run name determines the folder where the run tracking and instrument import files are saved. Use the run name field to organize files across purification, quantification, and amplification workflow runs on the HID NIMBUS® systems.

4. Click **CONTINUE**.

The settings window is displayed.

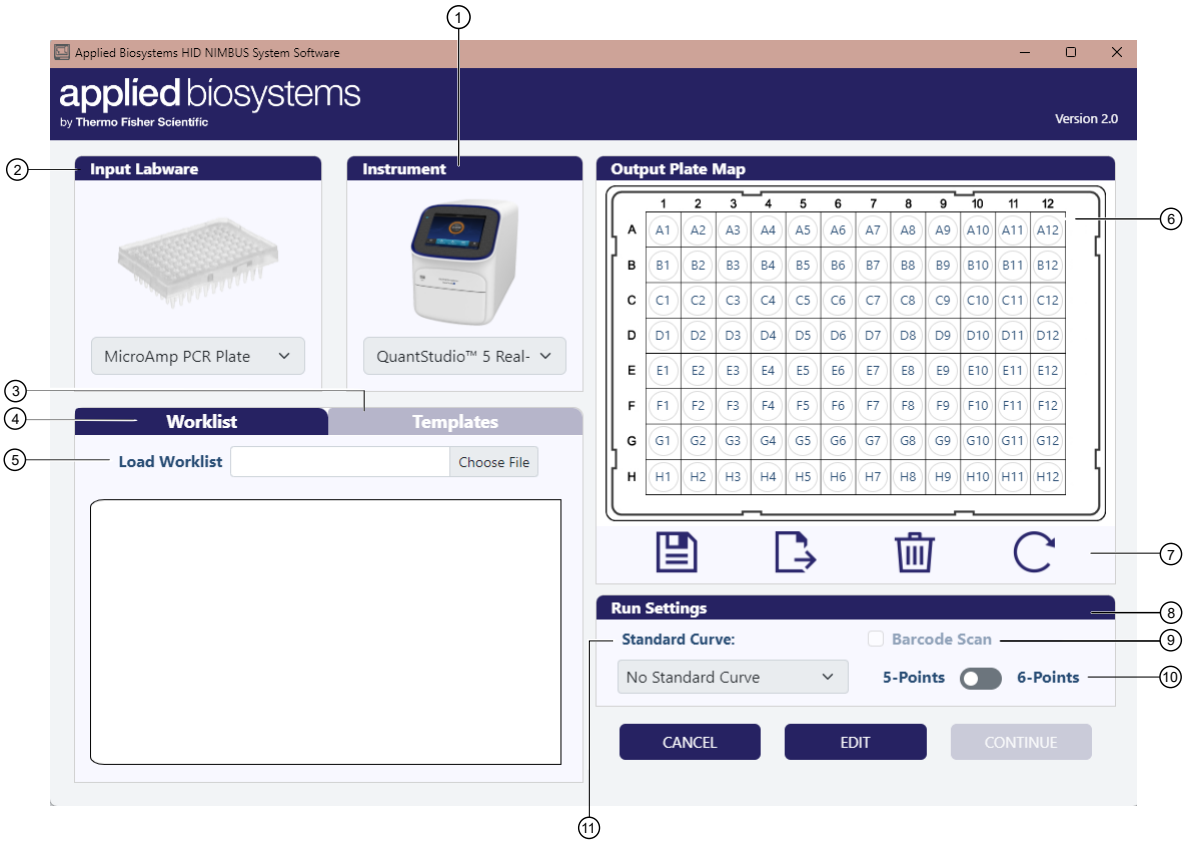


Figure 10 Settings window

1. Select an instrument
2. Select the input labware
3. Select a template
4. **Worklist** tab
5. Import a worklist
6. Plate map
7. Plate map tools
8. **Run Settings** pane
9. Enable or disable barcode scanning
10. Points toggle
11. Select a standard curve option

5. Select the input labware and the instrument for the quantification experiment.
   a. Click **EDIT** to unlock the user input interface.
   b. In the **Parameter Modification** window, click **CONTINUE**.
   c. Use the dropdown list in the **Input Labware** pane to select an option.
      - **MicroAmp PCR plate**
      - **Microcentrifuge Tubes**
      - **KingFisher Deep Well Plate**
   d. Use the dropdown list in the **Instrument** pane to select an option.
      - **QuantStudio™ 5 Real-Time PCR System**
      - **7500 Real-Time PCR System**
   e. Click **SAVE** to confirm the selection.
      Anytime that you edit the parameters, the edits are tracked in the GUI log file, located at `C:\Program Files (x86)\Hamilton\LogFiles`
6. In the **Worklist** tab, import a worklist.
   a. In the **Worklist** pane, click **Choose File**, or click within the blank file upload field.
   b. Select the prepared worklist or select purification run tracking file.

      Note: Before uploading, ensure that the worklist matches the input labware. For example, if you are using a MicroAmp™ PCR plate, ensure that the worklist is not intended for 1.5-mL microcentrifuge tubes.
   c. *(Optional)* To change a worklist after it has been uploaded, click **Choose File**, or click within the blank file upload field. In the **Changing Worklist** window, click **CONTINUE**.

   After the worklist is imported, the samples and controls appear in the **Worklist** pane.
7. In the **Run Settings** pane, use the **Standard Curve** dropdown list to select an option.
   - **Create New Standard Curve**
   - **Use Existing Standard Curve**
   - **No Standard Curve**

   Note: If a template was applied, a standard curve option is preselected. To remove the standard curve, in the **Run Settings** pane, use the **Standard Curve** dropdown list to select **No Standard Curve**. Do not drag-and-drop standards to **(Remove Target)**.
8. In the **Run Settings** pane, use the toggle to select **5-Points** or **6-Points**.

   IMPORTANT! If a template was applied and you select standard curve points that differ from the template, the standards are removed from the plate map. For example, if you select the **Default - 5 Point Duplicates** template but manually change the number of points to **6-Points**, the standards are removed from the plate map.

9. *(Optional)* Enable barcode scanning.
   a. Click **EDIT** to unlock the user input interface.
   b. In the **Run Settings** pane, select the **Barcode Scan** checkbox.
   c. Click **SAVE** to confirm the selection.

Note: When barcode scanning is on, you are prompted to manually scan the barcodes of the labware and carriers. Your worklist must contain the relevant barcode information to avoid scanning errors.

---

10. Select an action to set up the plate map.
    - Drag-and-drop each sample from the **Worklist** tab to the desired location of the **Output Plate Map**.

      Note: The positive control and no template control samples dragged onto the **Output Plate Map** are ignored when importing the quantification data file in the amplification workflow.
    - In the **Templates** tab, use the arrows to scroll, select the desired template, then click **APPLY**.

Note: Samples in the worklist that are not included in the plate map blink red in the worklist tab. Click **(Export Worklist)** to export a worklist for the remaining samples. The file is saved and exported to `C:\Program Files (x86)\Hamilton\Local Outputs\Run Name`, where the run name is the input selected in step 3.

---

11. *(Optional)* Create and save a template.
    a. Drag-and-drop samples, standards, and controls from the **Worklist** tab to the desired location of the **Output Plate Map**.
    b. Click **(Save Plate Map)**.
    c. In the **Save Current Plate Map** window, enter a template name.
    d. Use the **Template ID** dropdown list to select **Create a new template**.
    e. Click **SUBMIT**.
12. *(Optional)* To reset the plate map, click **(Restart Plate Map)**.
13. *(Optional)* To remove a sample or control from the plate map, drag-and-drop a desired target to **(Remove Target)**.

    The target is immediately removed from a plate map. To add a target back, drag-and-drop the desired target from the **Worklist** tab onto the **Output Plate Map**.

Note: To remove the standard curve, in the **Run Settings** pane, use the **Standard Curve** dropdown list to select **No Standard Curve**. Do not drag-and-drop standards to **(Remove Target)**.

14. Click **CONTINUE**.
    The method calculates the required number of tips and reagent volumes according to the number of samples to be processed and the information you provided in the settings.

Load the deck and start the run

IMPORTANT! Due to reagent stability at room temperature, load the deck and start the run within 3 hours.

1. Place all reagents according to the diagram in the deck summary screen (see Figure 12 and Figure 13).
   a. Prepare the master mix off-deck, then place the quantification reagents on the deck with the specified volumes.

   Note: If the master mix requirement is >1,500 µL, we recommend that you prepare the master mix in a single tube, then equally split the master mix into two 1.5 mL microcentrifuge tubes (Cat. No. AM12450) before placing the tubes on the deck. Click the **Master Mix** row to view a diagram of the tubes and their contents.

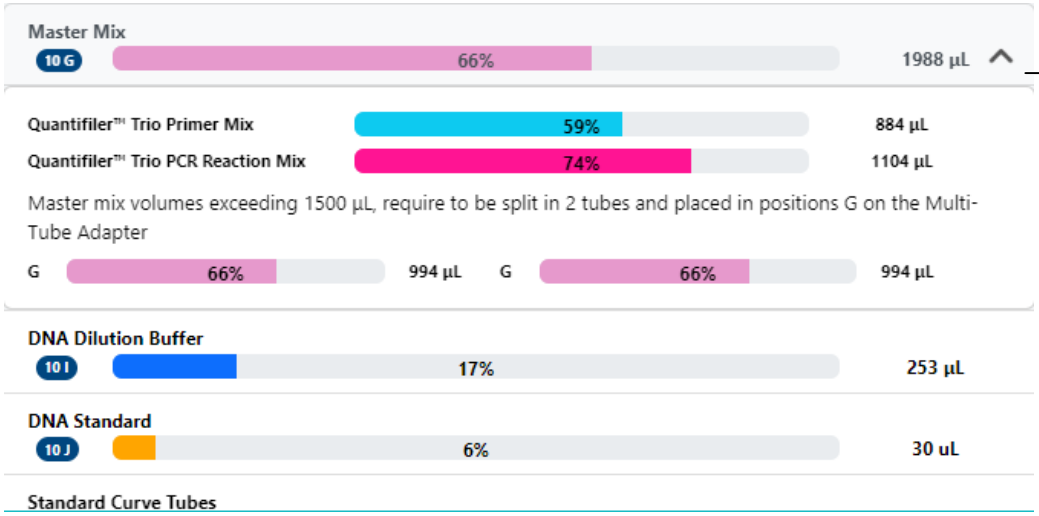


Figure 11 Reagent volumes

① Master Mix row (expanded)

Note: Reagent percentages are calculated based on the labware on the deck.

   b. Place the pointer over each position number on the deck layout to show tips with instructions.

c. *(Optional)* Click **Guidance Mode** to follow prompts for how to set up the deck.
Click **NEXT** to navigate through the videos. Click **FINISH** to close **Guidance Mode**.

Note: Due to the unavailability of videos for all scenarios, the positions shown in the guidance mode videos are not specific to every use case. To help ensure appropriate guidance for each deck position, read the instructions provided below each video.

d. Follow the color-coding and letters to load the labware into the correct carriers and positions. When loading the labware, start from the carriers at the back.

IMPORTANT! To avoid a potential collision hazard with the instrument arm and channels, do not leave any objects on the instrument deck that are not specifically called out in the deck loading instructions. This includes de-capping tubes before clicking **CONTINUE**.

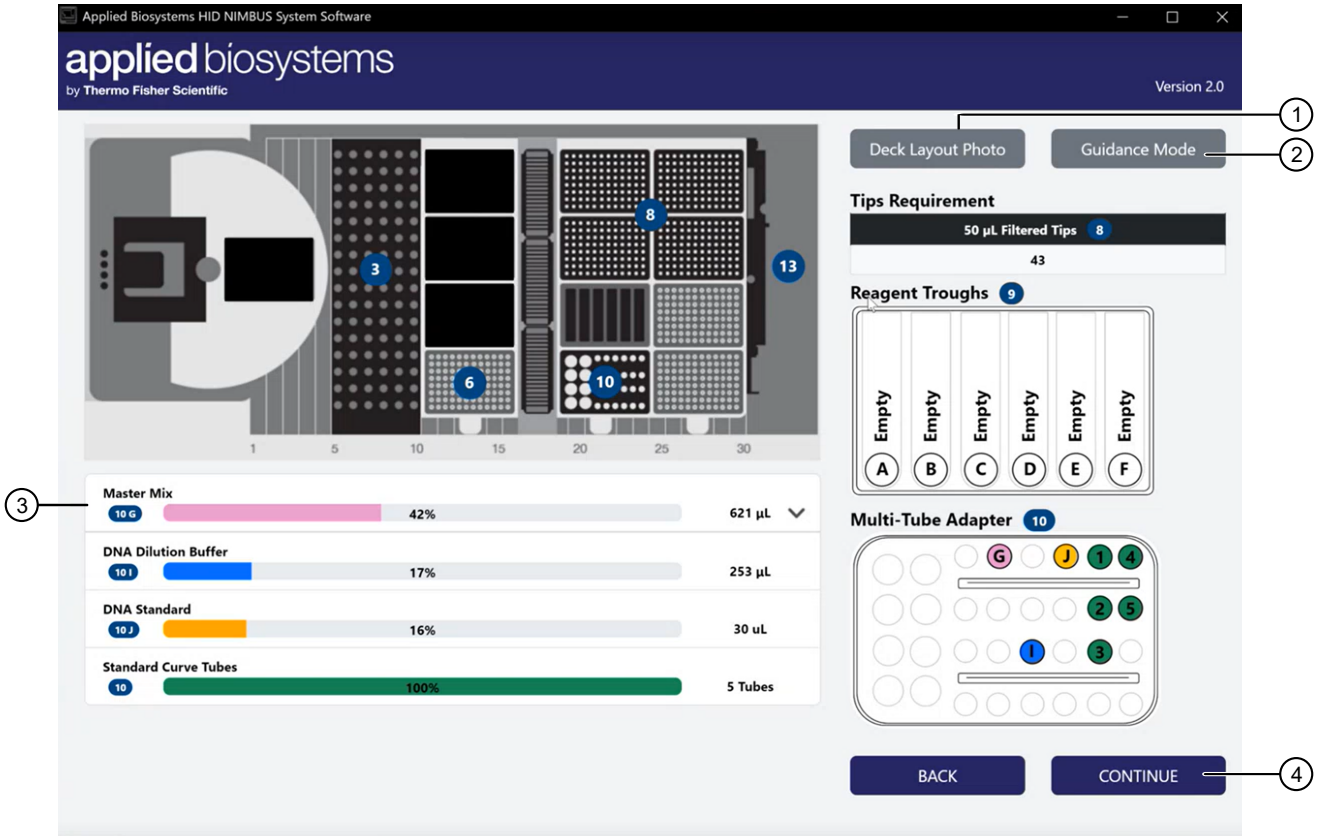


Figure 12 Example quantification deck layout with tubes as the input labware

① Change the deck layout image
② Access instructional videos
③ **Master Mix** row (expandable)
④ Continue to the next screen

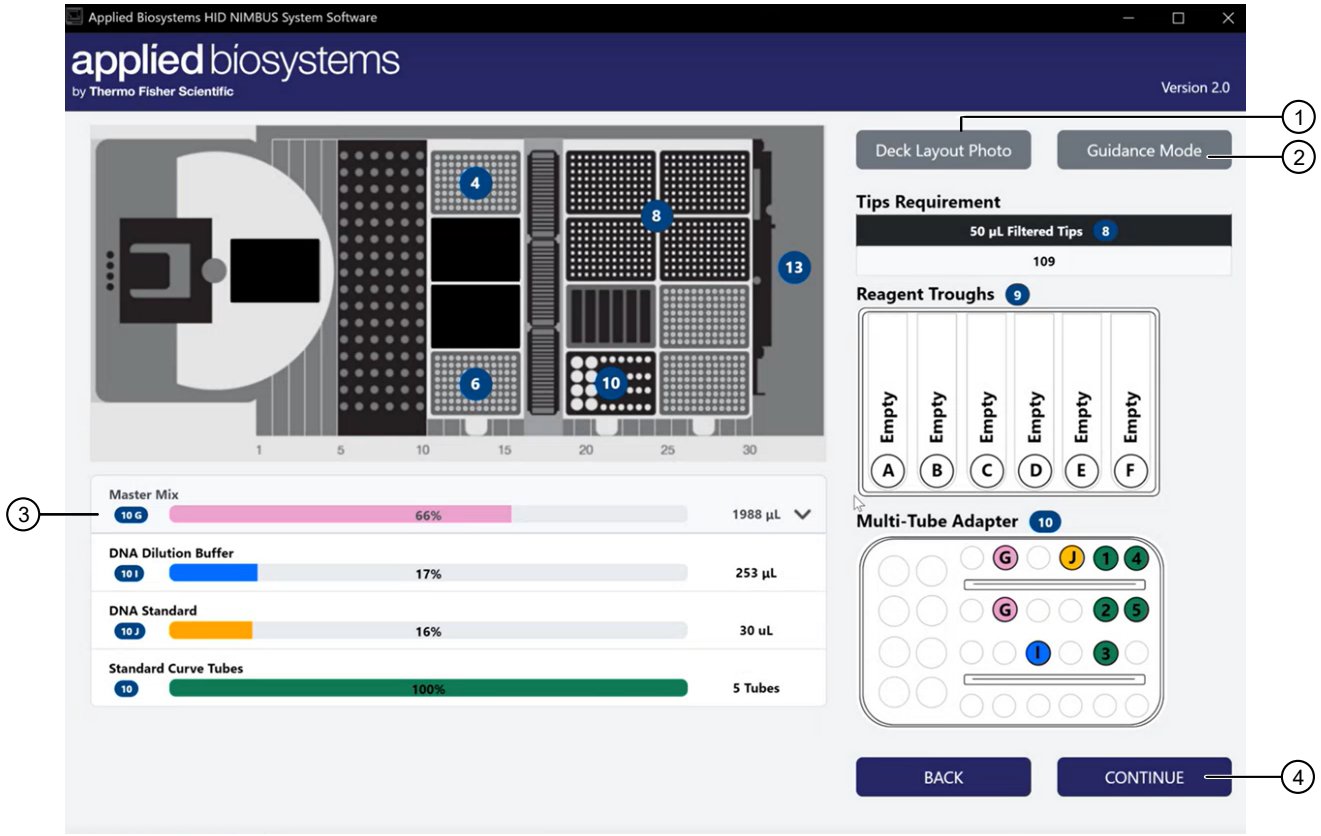


Figure 13 Example quantification deck layout with a plate as the input labware

① Change the deck layout image
② Access instructional videos
③ **Master Mix** row (expandable)
④ Continue to the next screen

2. *(Optional)* If you are using tubes as input labware, load the tubes in the appropriate order: back to front, then left to right, starting with the first carrier at track 5.

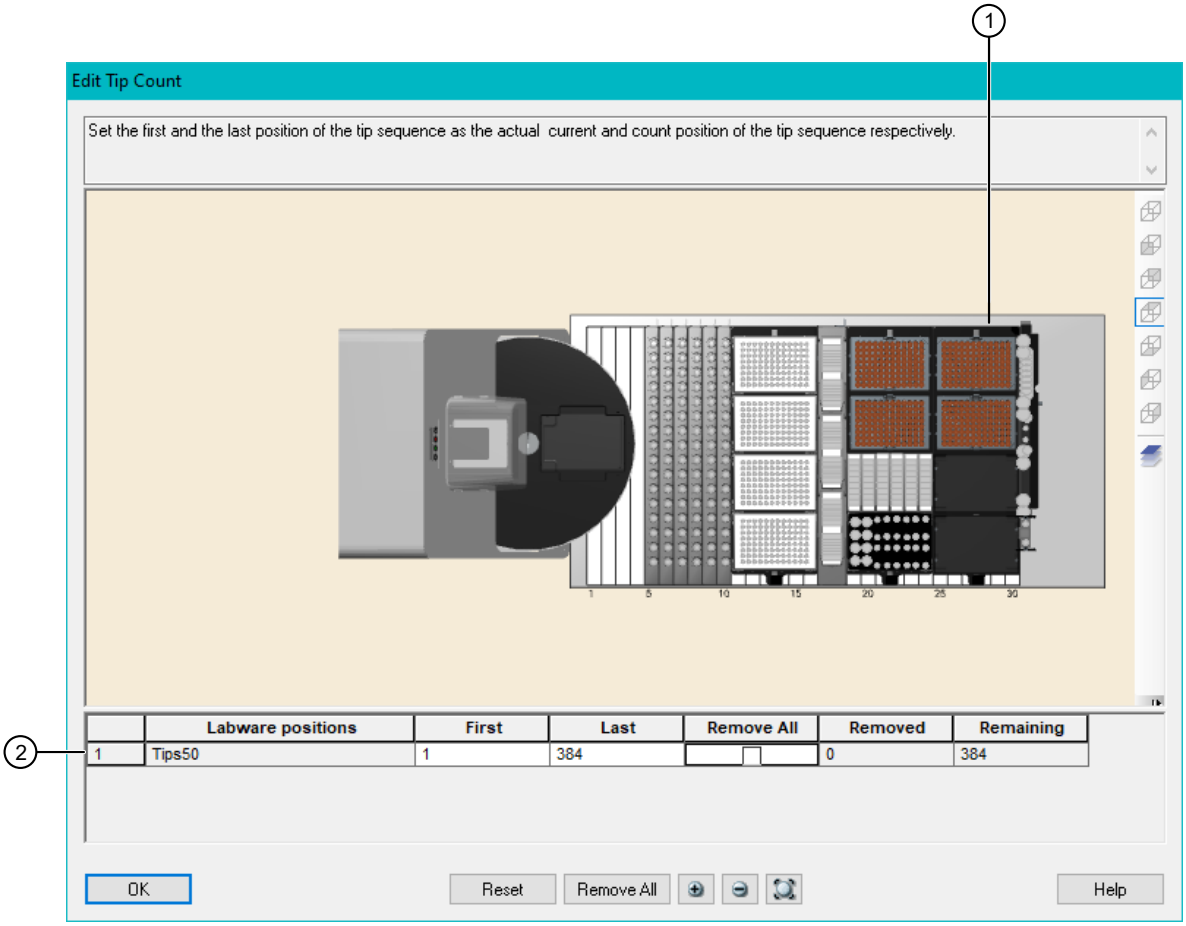
3. *(Optional)* Click **Deck Layout Photo** to change the image format.

   IMPORTANT! Follow the numbered deck layout image when loading the deck. The numbered deck layout image shows deck positions clearly and provides tips with instructions.

4. Click **CONTINUE**.

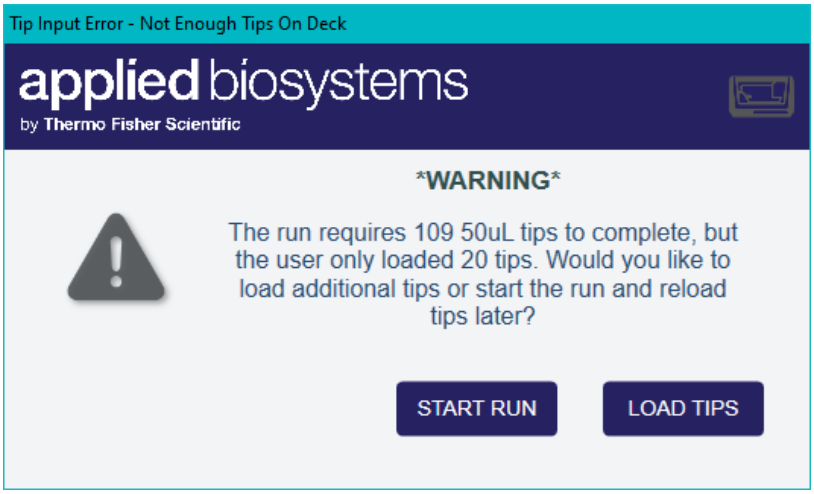
5. *(If needed)* Edit the tip counts for the 50 µL tips (position 8) to reflect the number and placement of tips on each rack. Select an action to edit the tip counts.
   - Click each position to select/deselect the appropriate wells in the diagram. Gray indicates that the well does not have a tip; orange indicates that the well does have a tip.
   - In the **First** and **Last** fields, enter the first and last tip positions.

     IMPORTANT! The total number of 50 µL tips is 384. The top left rack contains tips 1–96, the bottom left rack contains tips 97–192, the top right rack contains tips 193–288, and the bottom right rack contains tips 289–384. See Figure 12 and Figure 13.



① Diagram
② Text fields

Note: If you do not load enough tips to complete the run, you will be warned to load additional tips or start the run at your own risk. We recommend you click **LOAD TIPS** to complete the run. This will avoid pausing midway through the run to load additional tips.



6. *If barcode scanning is on,* scan the carrier and labware barcodes.

Note: The carriers should already be loaded in the correct positions on the deck.

a. At the prompt, slide the specified carrier out of position so that the barcodes can be scanned by the on-deck barcode scanner, then click **Start Scan**.

Note: The scanner reads the carrier and labware barcodes at the same time. You do not need to remove the labware from the carrier.

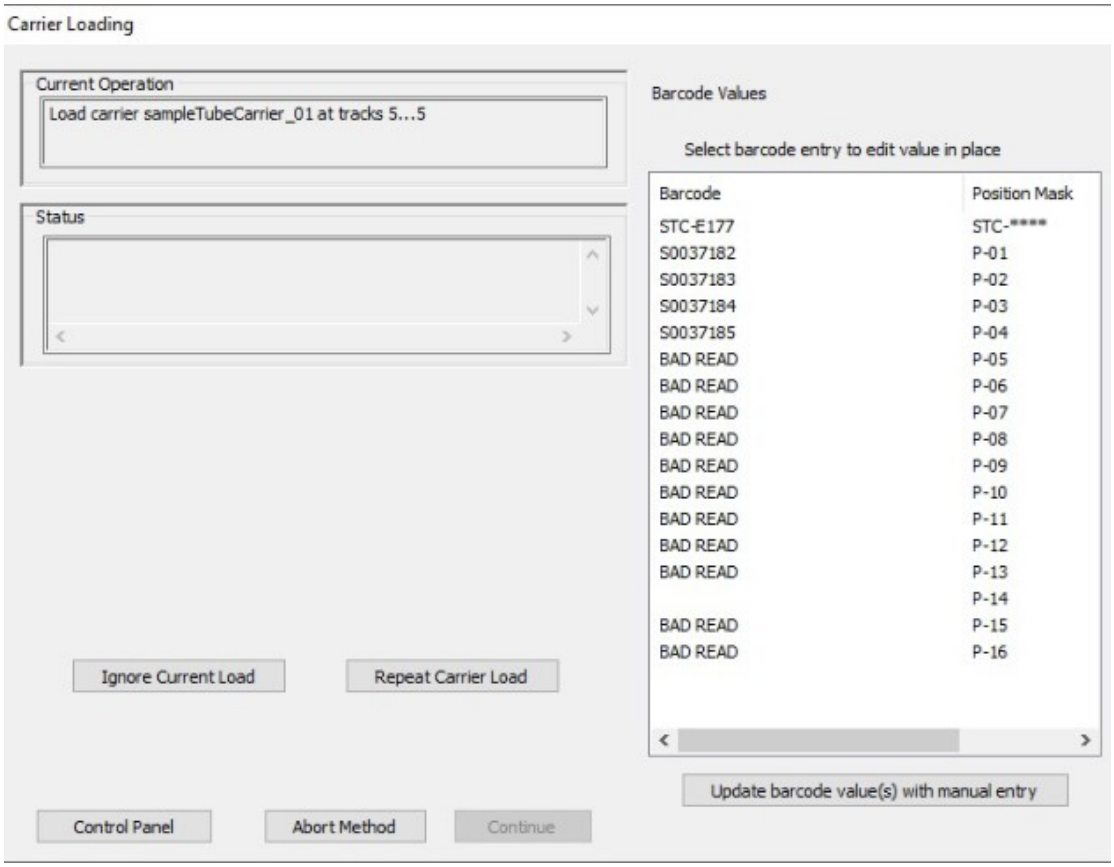


Figure 14 Barcode scanning in progress

b. Push the carrier all the way back into position, then click **Finish Scan**.

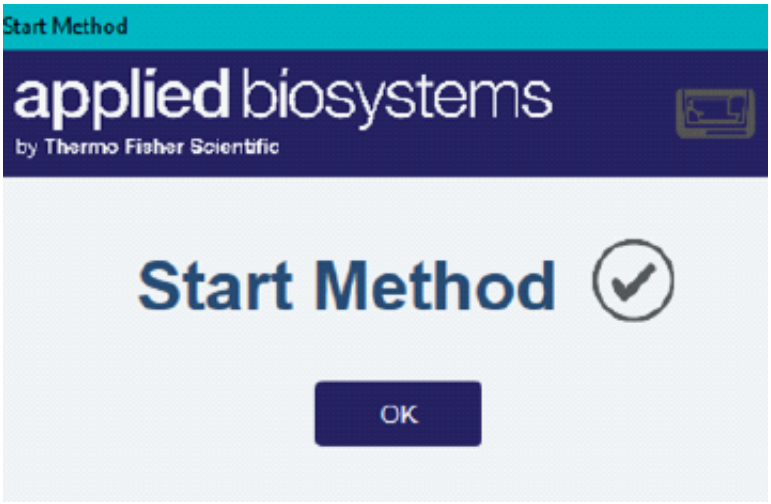
c. Repeat substep 6a and substep 6b for each carrier.

Note: If you are using an input worklist preloaded with barcode information, the system checks that your plates and/or tubes are loaded in the same positions as the worklist. If the loaded positions do not match the worklist, you are prompted to adjust the load positions or abort the run and restart with the correct worklist information.

The HID NIMBUS® System Software performs the following functions:

- **For the microcentrifuge tubes input labware**—Cross-references the scanned barcodes against the expected barcodes supplied in the worklist.
- **For the plate input labware**—Cross-references the scanned barcode against the expected barcode supplied in the worklist.

7. Close the instrument door.
   The **Start Method** dialog box is displayed.



8. Click **OK**.
   The door automatically locks and the run starts.

Remove the quantification plate setup, unused reagents, and labware

1. Open the instrument door.
2. Remove the plate that contains the quantification plate setup, seal it with an optical adhesive film, then proceed to setting up the real-time PCR instrument.

   Note: The import file for the real-time instrument is generated by the HID NIMBUS® System Software. To locate the import file, navigate to:
   `C:\Program Files (x86)\Hamilton\Local Outputs\Quantification_YYYYMMDDHHMMSS`
3. Cap and store the remaining Quantifiler™ THP DNA Dilution Buffer.
4. If the **Create New Standard Curve** option was selected, cap and store the remaining Quantifiler™ THP DNA Standard.
5. Store the low-binding tubes containing the serial dilutions of the standard curve if still within the usable range (14 days stored at 2–8°C).

6. Properly dispose of any unused master mix in the microcentrifuge tubes.

   IMPORTANT! Do not reuse the leftover master mix. Dispose of any unused volumes.

7. Empty the waste container that contains used tips.

IMPORTANT! To properly dispose of all used reagents and labware, see Appendix E, "Safety".

WARNING! Do not use cleaning or disinfecting solutions which contain hypochlorite, such as bleach, on the instrument.

Clean the HID NIMBUS® QNA systems after each use. For detailed instructions, see the *NIMBUS HD Operator's Manual* (Pub. No. 98538-01).

View the output files

The output files for each run include a run-tracking report and a log file. Both files are given software-generated unique identifiers.

On the HID NIMBUS® QNA system or HID NIMBUS® Presto QNA system computer, open the output file of interest, based on the run date.

Output file	File name and location	Description
Run-tracking report	QuantificationRunTracking_Quantification_Run Name.xls, located at `C:\Program Files (x86)\Hamilton\Local Outputs\Run Name`	An Excel™ file that includes the following information: • Run Information • Lot Tracking • Run Status List • Worklist • Final Sample List • Pipette Tracking • Pipette Error Report
Real-time PCR import file	Quantification_Run Name_RT.txt, located at `C:\Program Files (x86)\Hamilton\Local Outputs\Run Name`	A TXT file that includes detailed information and is used as an input for the real-time PCR instrument software.
Log file	`<unique identifier>`_Trace.trc, located at `C:\Program Files (x86)\Hamilton\LogFiles`	A TRC file that includes detailed information for each robotic step that was performed during the run.
	Quantification_Run Name_GUILog.txt, located at `C:\Program Files (x86)\Hamilton\LogFiles`	A TXT file that includes information for each instance the edit function is used to modify parameters.

Seal the plate and remove bubbles

1. Seal the reaction plate with the optical adhesive cover.
2. Remove bubbles: Tap the base on the benchtop to bring the bubbles to the liquid surface. Lift the plate, then inspect each well for bubbles; tap each well with a marker, pen, or gloved fingertip.

   IMPORTANT! This step is critical to avoid noise in the fluorescence signal that bubbles can cause.
3. Centrifuge the plate at 3,000 rpm for about 20 seconds in a tabletop centrifuge with plate holders to remove any bubbles.

   Note: If a tabletop centrifuge with 96-well plate adapters is not available, visually inspect the plate for bubbles, and lightly tap the plate to remove bubbles in wells.

Proceed to load the plate and start the quantification run. See “Load the plate, then start the run” on page 38.

Load the plate, then start the run

IMPORTANT! If the computer that performs the run is on a network, avoid excess use of the network during a run.

1. Load the reaction plate into the real-time PCR instrument.

   For more information, see the user documentation for your instrument.
2. In the HID Real-Time PCR Analysis Software, open the experiment that you set up for the run (see Chapter 2, “Set up the software”), or import the file generated by the HID Nimbus System located at `C:\ Program Files (x86)\ Hamilton\Local Outputs\Quantification_Run Name`.

   Note: Instrument import files from the HID NIMBUS® QNA systems are only compatible with HID Real-Time PCR Analysis Software v1.3 or later.
3. In the **Experiment Menu**, select **Setup**, select any screen, then click **START RUN** at the top-right corner.

   Alternatively, click **Run**, select any screen, then carefully click **START RUN** at the top-left corner.

The green **START RUN** button becomes a red **STOP RUN** button, and the run begins.

Note: If you double-click the **START RUN** button, it may not become a **STOP RUN** button, but the run proceeds normally.

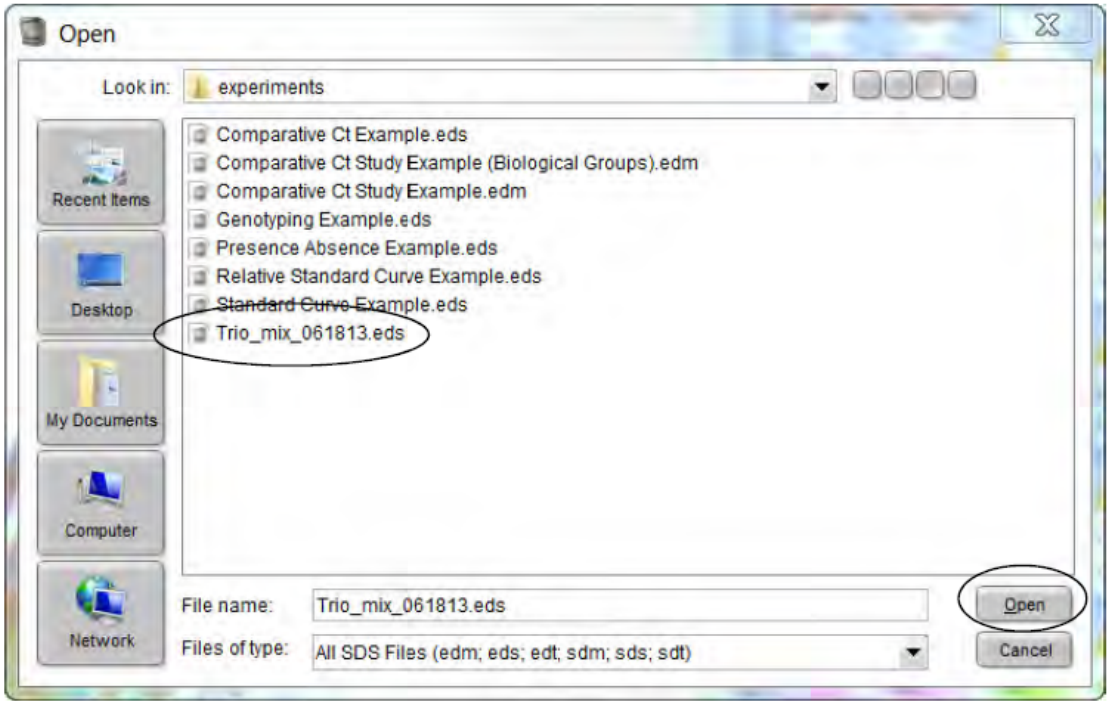
4 Analyze the experiment and view results

Analyze the experiment

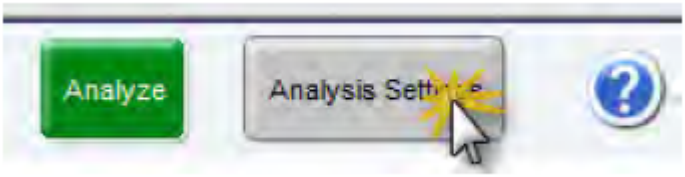
Analyze a run after it is complete and reanalyze after you make any changes to the experiment, such as sample names.

1. Open the experiment for analysis.
   - Navigate to the folder where the run file is stored, then double-click the run file.

     or
   - Launch the software from the shortcut on your desktop:
     a. Double-click the HID Real-Time PCR Analysis Software icon,
     b. Select **File** ▸ **Open**,
     c. Navigate to the run file and click **Open** (or double-click the run file).



2. Verify the analysis settings.
   a. Click **Analysis Settings** in the upper-right corner of the window.



   b. Click the **C_T Settings** tab to view the C_T settings.

   c. Ensure that the C_T settings are configured based on the values specified in Table 5.
      - If the analysis settings are correct, click **Apply Analysis Settings**.
      - If the analysis settings are not correct, change them to match the settings. To change the settings, click **Edit Default Settings**. In the **Edit Default C_T settings** dialog box, enter the new values, then click **Save Changes**. In the **C_T Settings** tab, click **Apply Analysis Settings.**

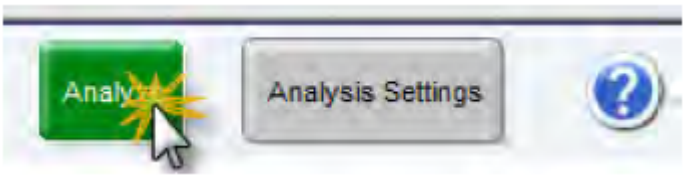
Table 5 Target, threshold, and baseline settings

Target	Threshold	Baseline start	Baseline end
T.IPC	0.1	3	15
T.Large Autosomal	0.2	3	15
T.Small Autosomal	0.2	3	15
T.Y	0.2	3	15

Note: The Quantifiler™ Trio Kit has been validated using the Manual Baseline method. Studies were also performed applying the Automatic Baseline method and the Manual Baseline method to evaluate potential differences between the methods for concentrations from 5–0.005 ng/µL. No statistically significant differences were observed within this range for C_T values generated using the Automatic Baseline and Manual analysis methods.

A value of 0.1 was used for the IPC Threshold during the developmental validation studies. Before using alternative baseline methods, (for example, automatic) or thresholds, perform the appropriate internal validation studies.

3. Click **Analyze**.



View results

Overview

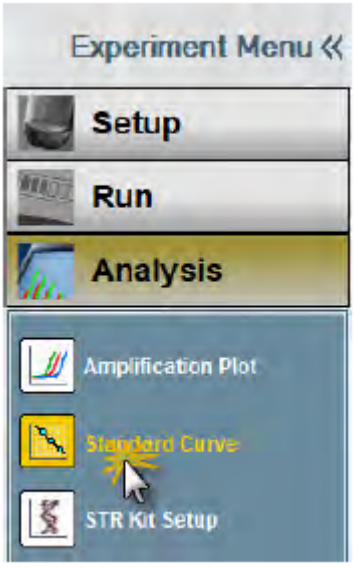
Viewing the results of data analysis can involve one or more of the following:

- "View the standard curve" on page 41
- "View the amplification plot" on page 43
- "Export the results" on page 44

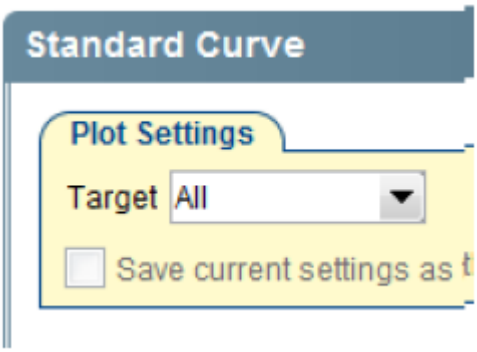
View the standard curve

For information about interpreting and troubleshooting the standard curve, see "Examine the standard curve" on page 53 and "Troubleshoot the standard curve" on page 56.

1. In the left navigation pane, click **Analysis** ▸ **Standard Curve**.



2. In the **Target** dropdown list, select **All**.



3. View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.

An example of standard curve plots is shown in Figure 15. The gap between the Small Autosomal, Large Autosomal, and Male C_T values may vary depending on the relative slopes of the targets and the instrument.

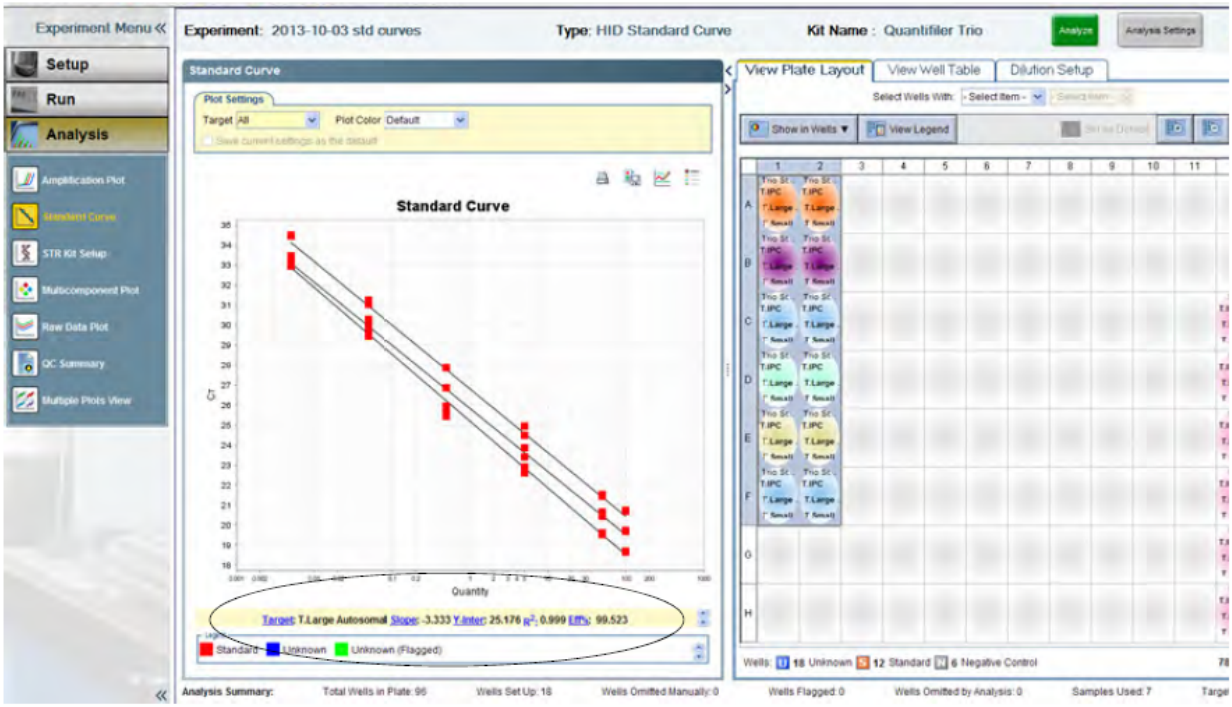


Figure 15 Standard curve plots

Amplification plot results

The amplification plot can display one of the following:

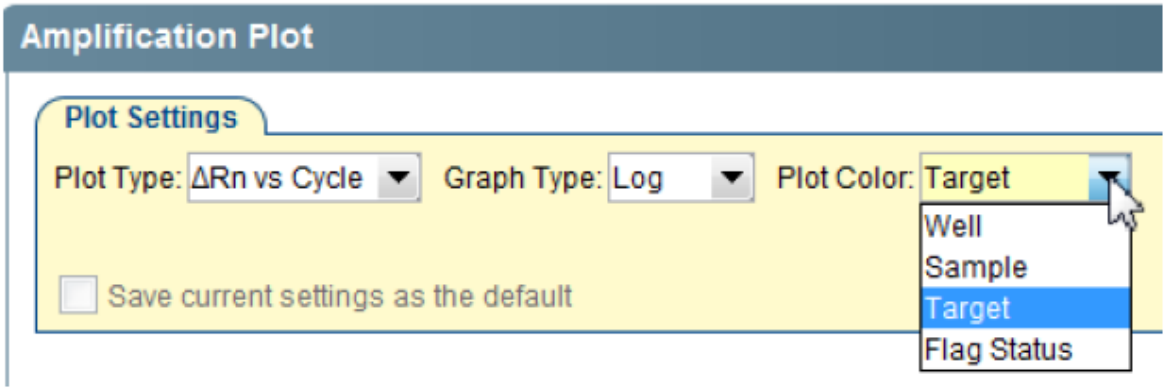
- C_T versus well position view
- Plot of normalized reporter signal (R_n) versus cycle (linear view)

For more information about the amplification plot, see “Real-time data analysis” on page 76 or the *Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve* (Pub. No. 4347825).

View the amplification plot

1. In the left navigation pane, click **Analysis** ▸ **Amplification Plot**.

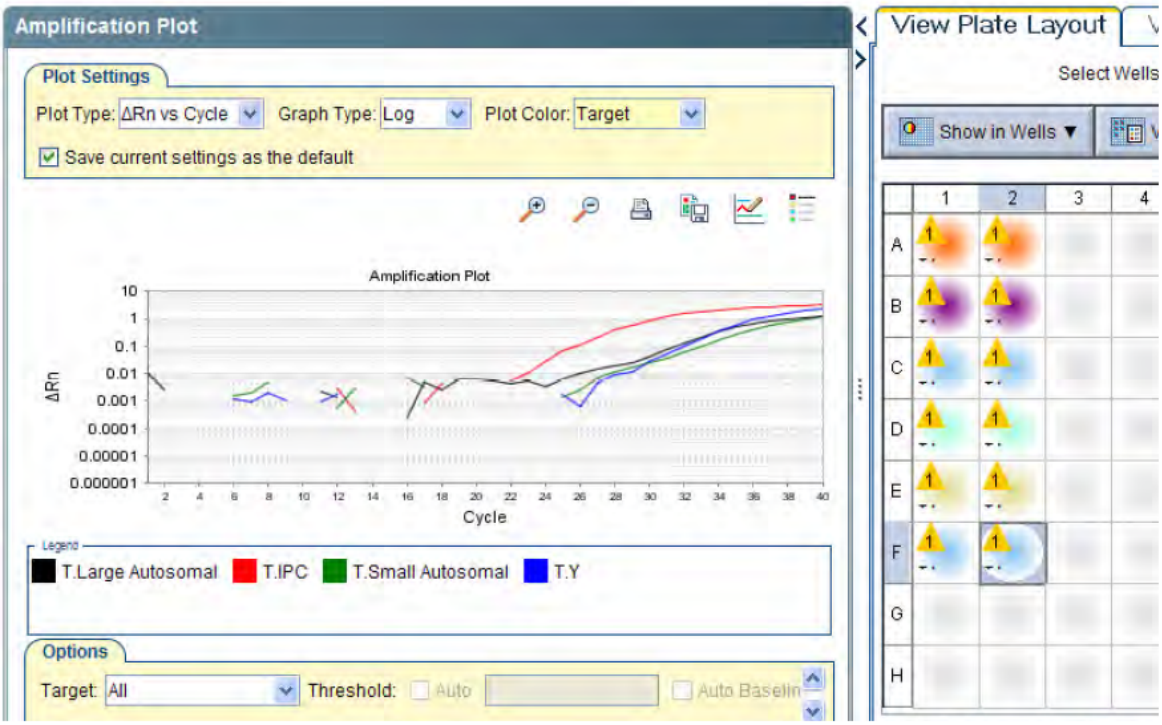
2. Select a plot color in the drop-down list:
   - Well
   - Sample
   - Target
   - Flag Status



3. Select the targets to view in dropdown list located under the amplification plots.
   - Select **All** to view all targets simultaneously

     or
   - Select a single Quantifiler™ Trio target:
     - T.IPC
     - T.Large Autosomal
     - T.Small Autosomal
     - T.Y

4. Select the applicable samples in the plate layout. The example below displays all targets for a single sample using target plot colors.



5. If you selected a single target in step 3, repeat step 3 and step 4 for the remaining targets.

For troubleshooting information, see “Troubleshoot amplification plots” on page 62.

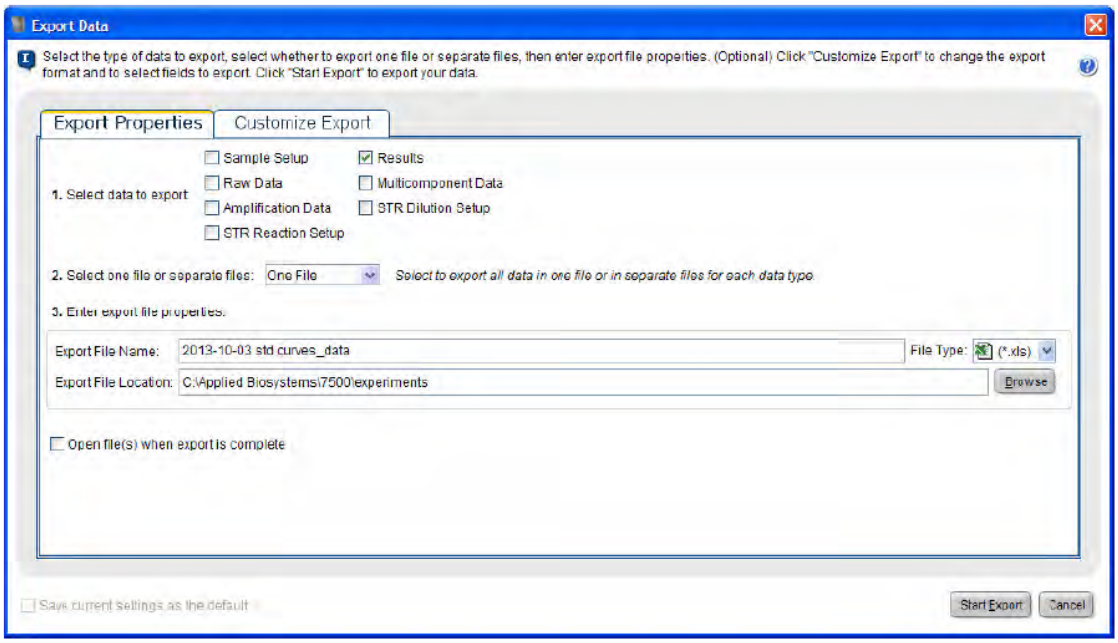
Export the results

You can export numeric data into text files, which can then be imported into spreadsheet applications such as Microsoft™ Excel™ software.

1. In the Experiment Menu, click **Analysis**. Click any analysis screen, then click **View Plate Layout** or **View Well Table**.

2. Select the wells to export.

3. Complete the **Export** dialog box and export the data.
   a. In the toolbar, click **Export**.
   b. Select **Results** as the type of data to export.
   c. Select **Separate Files** or **One File** in the drop-down list.
   d. Enter a file name and export location.
   e. Click **Start Export** to export the data to the files that you selected.



4. When the export is complete, select an action.
   - Click **Export More Data** to export different types of data for the same samples.
   - Click **Close Export Tool**.

The exported results are used as an import file for normalization and amplification.

For more information about exporting data, see the *HID Real-Time PCR Analysis Software User Guide* (Pub. No. MAN0009819).

5 Review the results

Typical plots obtained with the Quantifiler™ Trio Automated assay

Examples of typical male, female, and no template control (NTC) amplification plots for the Quantifiler™ Trio Automated assay are shown in Figure 16, Figure 17, and Figure 18.

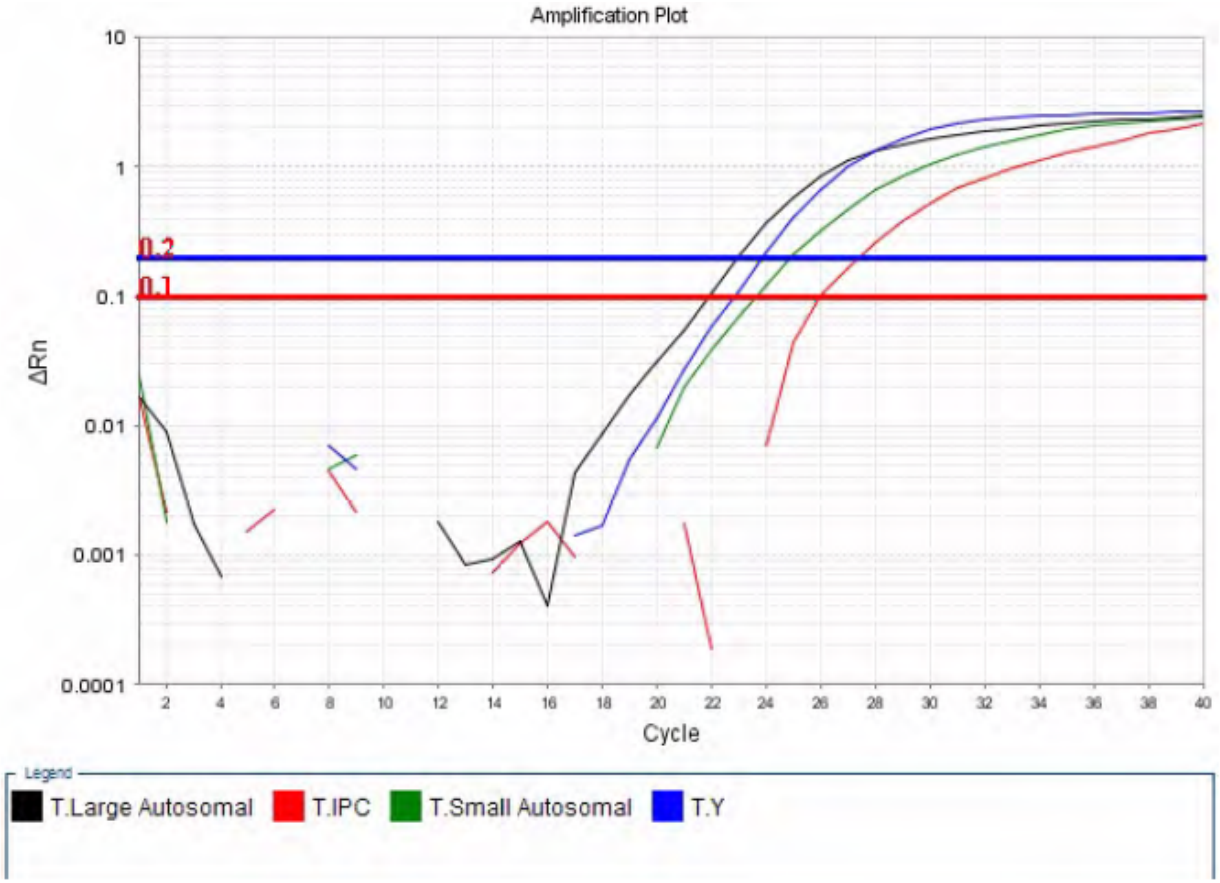


Figure 16 Typical male amplification plot (horizontal blue line indicates the C_T threshold for small autosomal, large autosomal and male targets; horizontal red line indicates the C_T threshold for IPC)

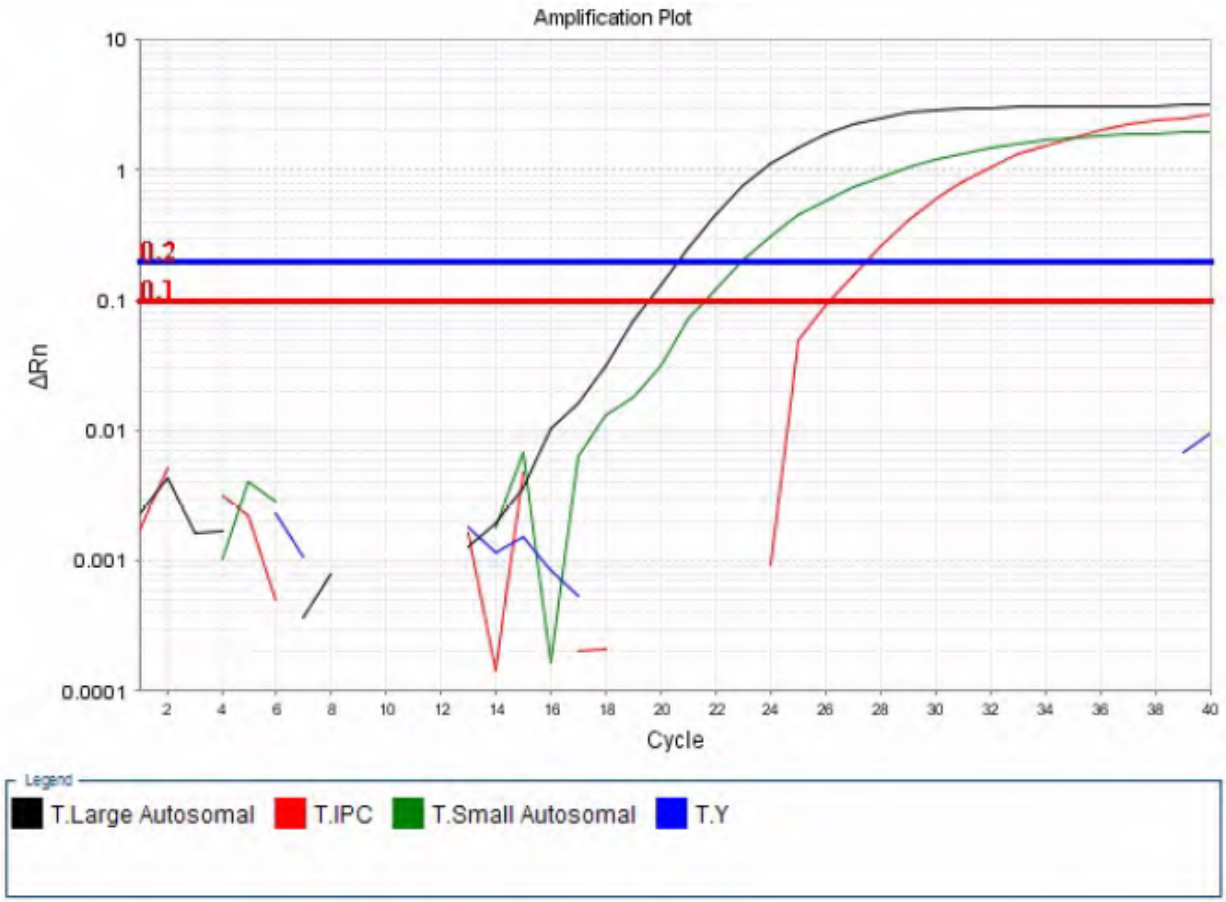


Figure 17 Typical female amplification plot (horizontal blue line=C_T threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)

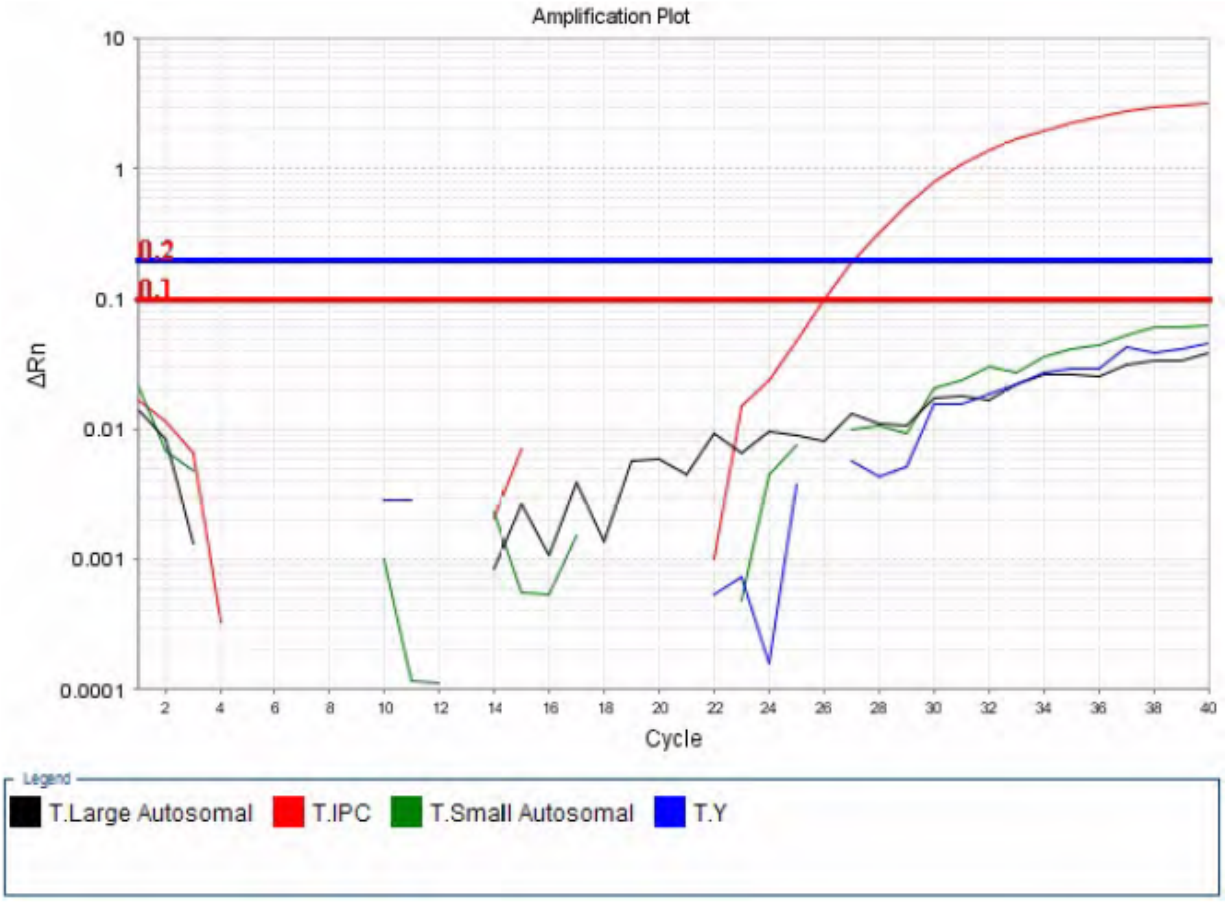


Figure 18 Typical no template control (NTC) amplification plot (horizontal blue line=CT threshold for small autosomal, large autosomal and male targets; horizontal red line=C_T threshold for IPC)

Examine the multicomponent plot to check for noise

The multicomponent plot displays the fluorescence data for each target in the quantification assay plotted against cycle number. Samples in a normal multicomponent plot generally demonstrate a flat line for at least the first 15–20 cycles, before exponential growth of the PCR product can be detected. If using the default settings for the Quantifiler™ Trio kit, the flat line between cycles 3–15 is used to calculate the baseline for the sample.

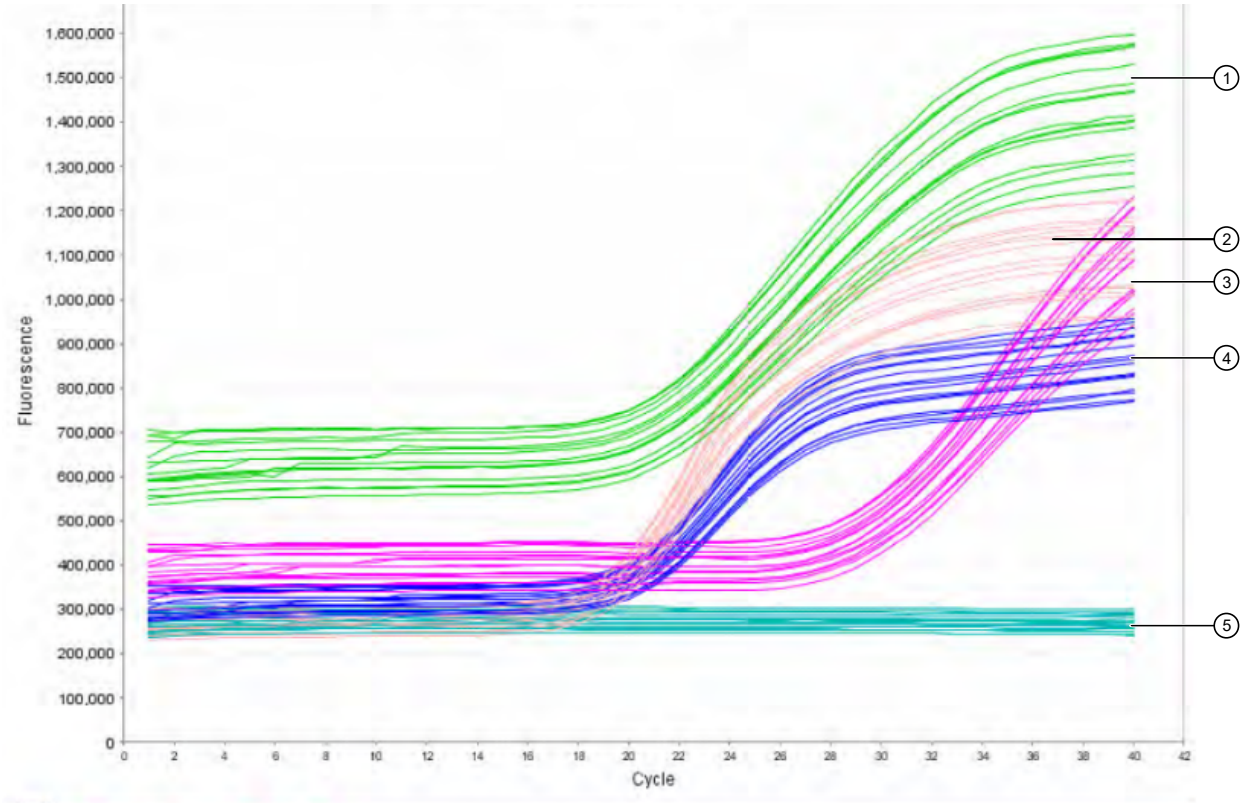


Figure 19 Typical Quantifiler™ Trio kit multicomponent plot

① VIC™ dye, small autosomal target
② ABY™ dye, large autosomal target
③ JUN™ dye, IPC
④ FAM™ dye, male target
⑤ MUSTANG PURPLE™ (MP) dye, passive reference

Samples with an abnormal multicomponent plot may exhibit short or long dips or rises in the fluorescence readings. When this noise occurs between cycles 3–15, it may affect the baseline calculation for the sample, which can in turn affect the C_T value calculated for the DNA targets.

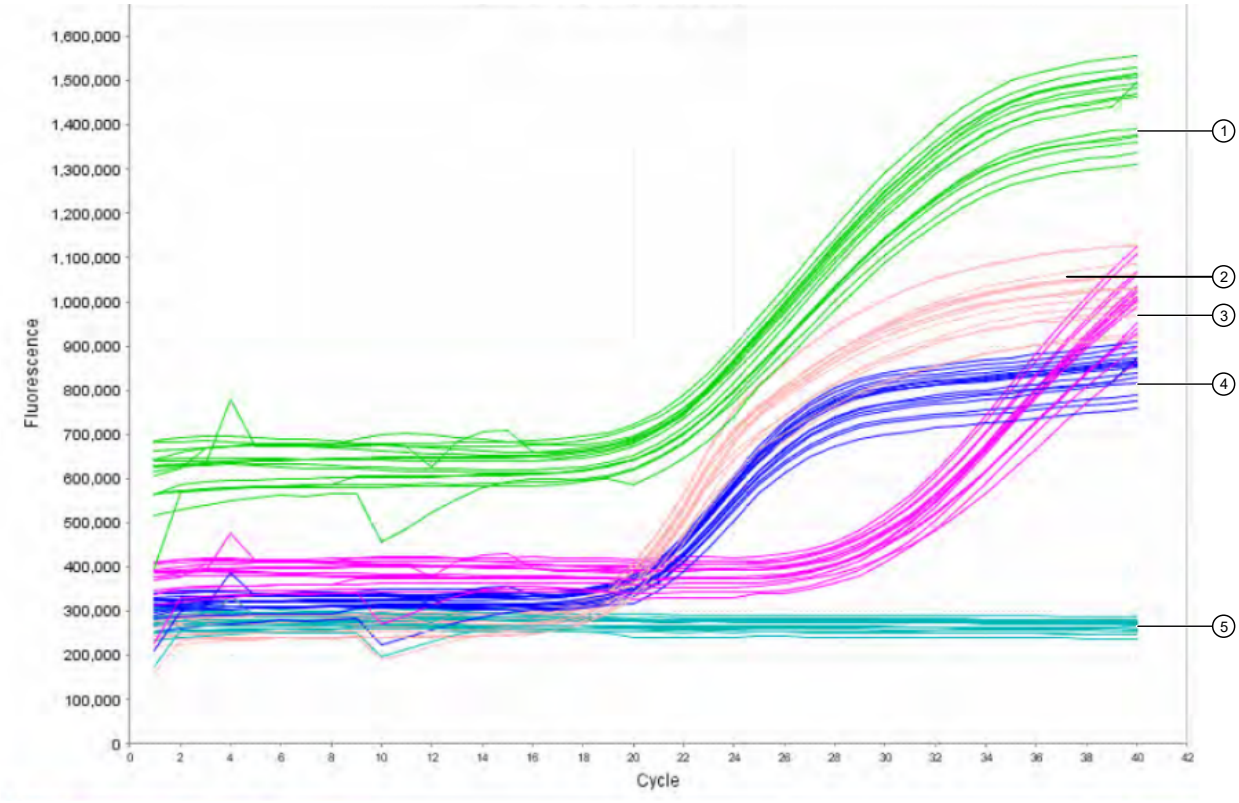


Figure 20 Multicomponent plot with noise between cycles 3-15

① VIC™ dye
② ABY™ dye
③ JUN™ dye
④ FAM™ dye
⑤ MUSTANG PURPLE™ (MP) dye

Impact of fluorescence noise on quantification and STR results

Fluorescence noise may alter the C_T value calculated for the DNA targets, but this effect is difficult to observe unless multiple replicates of a sample have been quantified. In our observations, results for the small autosomal target are the most susceptible to the effects of fluorescent noise.

Three replicates of sample F–C have been quantified in the example shown in Figure 21. The first two replicates had small autosomal target concentrations of 0.884 ng/µL and 0.873 ng/µL, but the third replicate had a small autosomal target concentration of 0.446 ng/µL.

Well	Sam...	Targ...	CT	Quantity
F-C1				
D6	F-C1	T.IPC	27.543	
D6	F-C1	T.Larg...	25.017	1.202
D6	F-C1	T.Sma...	27.339	0.884
D6	F-C1	T.Y	Undetermined	
F-C2				
E6	F-C2	T.IPC	27.579	
E6	F-C2	T.Larg...	25.098	1.141
E6	F-C2	T.Sma...	27.357	0.873
E6	F-C2	T.Y	Undetermined	
F-C3				
F6	F-C3	T.IPC	27.057	
F6	F-C3	T.Larg...	25.323	0.988
F6	F-C3	T.Sma...	28.347	0.446
F6	F-C3	T.Y	Undetermined	

Figure 21 C_T results for F-C example data

The multicomponent plot for these samples shows a dip in fluorescence between cycle 8 and cycle 9 that corresponds to F6, the well that contains the outlier replicate.

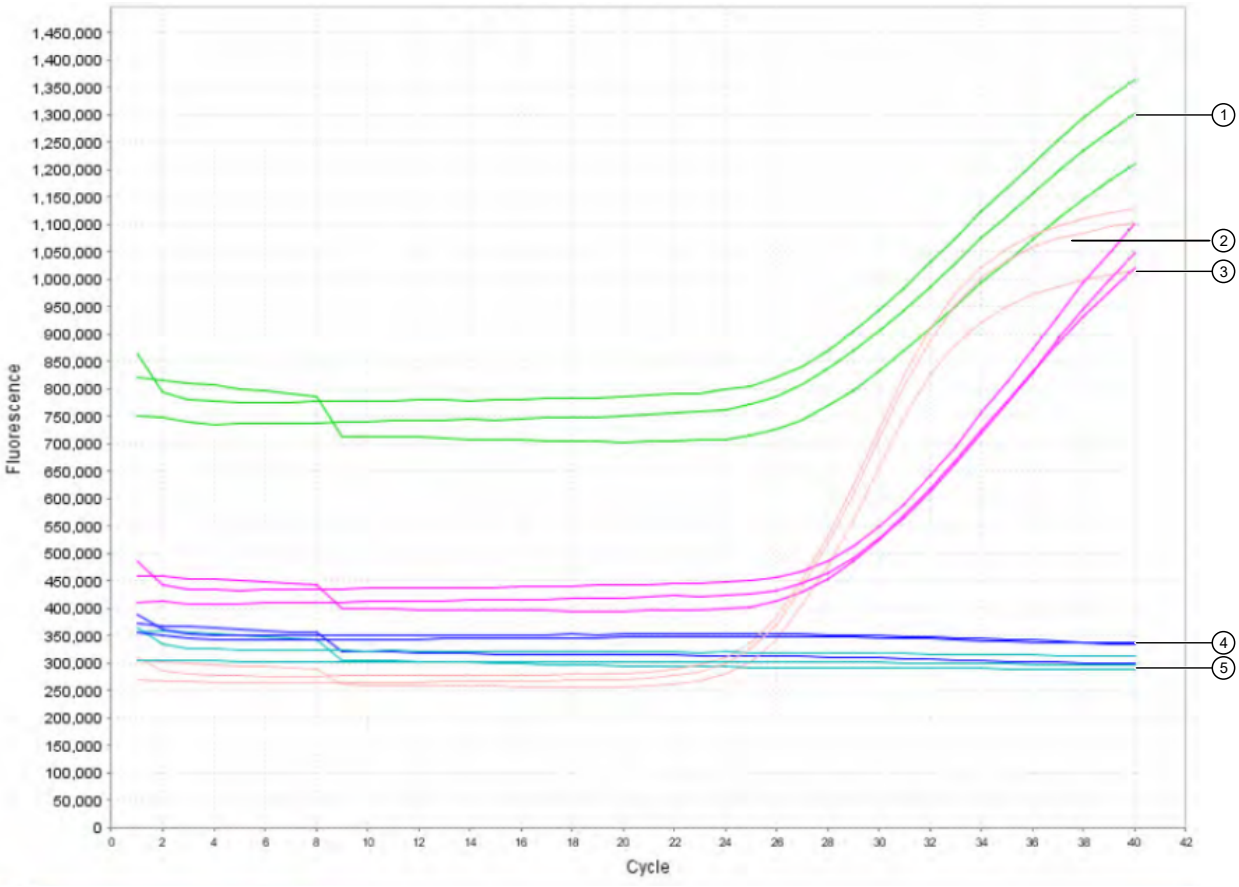


Figure 22 Multicomponent plot for F-C example data

① VIC™ dye
② ABY™ dye
③ JUN™ dye
④ FAM™ dye
⑤ MUSTANG PURPLE™ (MP) dye

If an inaccurate quantification result (caused by fluorescence noise) is used to determine DNA input volume for STR amplification, STR results may be inaccurate.

The sample concentration is ~0.88 ng/µL in Figure 22. If the third replicate was not identified as an outlier, approximately 2.2 µL of DNA might be added to an STR reaction to target an input of 1 ng, resulting in almost double the intended target being added to the reaction (1.94 ng).

Examine the standard curve

Examine the standard curve results to evaluate the quality of the results from the quantification standard reactions.

About standard curve results

The standard curve is a graph of the C_T of quantification standard reactions plotted against the starting quantity of the standards. The software calculates the regression line by calculating the best fit with the quantification standard data points. The regression line formula has the form:

$C_T = m\ [\log (Qty)] + b$

where `m` is the slope, `b` is the y-intercept, and `Qty` is the starting DNA quantity. The values associated with the regression analysis can be interpreted as follows:

- **R^2 value**—Measure of the closeness of fit between the standard curve regression line and the individual C_T data points of quantification standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.
- Regression coefficients:
  - **Slope**—Indicates the PCR amplification efficiency for the assay. A slope of –3.3 indicates 100% amplification efficiency.
  - **Y-intercept**—Indicates the expected C_T value for a sample with Qty = 1 (for example, 1 ng/µL).

Linearity

The standard curve for the Quantifiler™ HP and Trio Kits is linear from 5 pg/µL to 100 ng/µL.

The kits can detect DNA concentrations lower than <5 pg/µL, however, the CV (Coefficient of Variation) values may be higher than those observed for the 5 pg/µL to 100 ng/µL range.

R^2 value

An R^2 value ≥0.99 indicates a close fit between the standard curve regression line and the individual C_T data points of quantification standard reaction

If the R^2 value is ≤0.98 check the following:

- Quantity values entered for quantification standards in the Plate Setup - Assign Targets to the Selected Wells during experiment setup
- Making of serial dilutions of quantification standards
- Loading of reactions for quantification standards
- Failure of reactions containing quantification standards

Slope

A slope close to –3.3 indicates optimal, 100% PCR amplification efficiency.

The slope values listed in Table 6 represent the typical range of slope values observed during the development and validation of the Quantifiler™ HP and Trio Kits. Some deviations from this range may be observed due to instrument performance. If the slope varies beyond the typical range indicated in Table 6, check the following:

- Assay setup
- Software setup
- Reagents
- Instrument

Table 6 Range and average of standard-curve slope values

Quantifiler™ HP and Trio targets	Typical slope (range)	Average slope
Small Autosomal (SA)	–3.0 to –3.6	–3.3
Large Autosomal (LA)	–3.1 to –3.7	–3.4
Y Target (Y)	–3.0 to –3.6	–3.3

Y-intercept

You may observe some variation in the Y-intercept value, therefore we cannot provide a meaningful Y-intercept specification that will apply to all laboratories over time. We suggest that your lab monitor Y-intercept over time. In addition to variations that can be caused by pipetting of standards or minor lot-to-lot variations in the kits, Y-intercept can also be affected by:

- **Target-to-target variation**—The Y-intercept for the large autosomal target is typically lower than the Y-intercept for the small autosomal target or the Y target. This is because of the higher copy number of the large autosomal target relative to the copy number of the small autosomal and Y targets.
- **Instrument-to-instrument variation**—Differences between instruments result in small differences in Y-intercept values for each of the targets. Minor differences do not affect assay performance or quantification accuracy.

IPC C_T

To assess C_T values for the Internal PCR Control (IPC), view the JUN™ dye signal in the amplification plots for the quantification standards. Typical reactions are expected to show relatively consistent IPC amplification for standards with concentrations ≤5 ng/µL. With higher concentrations of human genomic DNA, competition between the human and/or male-specific and IPC PCR reactions may suppress IPC amplification. We have observed IPC C_T values begin to increase at concentrations >5 ng/µL, and a greater magnitude of increase at concentrations >50 ng/µL. Figure 23 below displays an example of how the IPC C_T values may deflect upwards with increasing DNA concentrations.

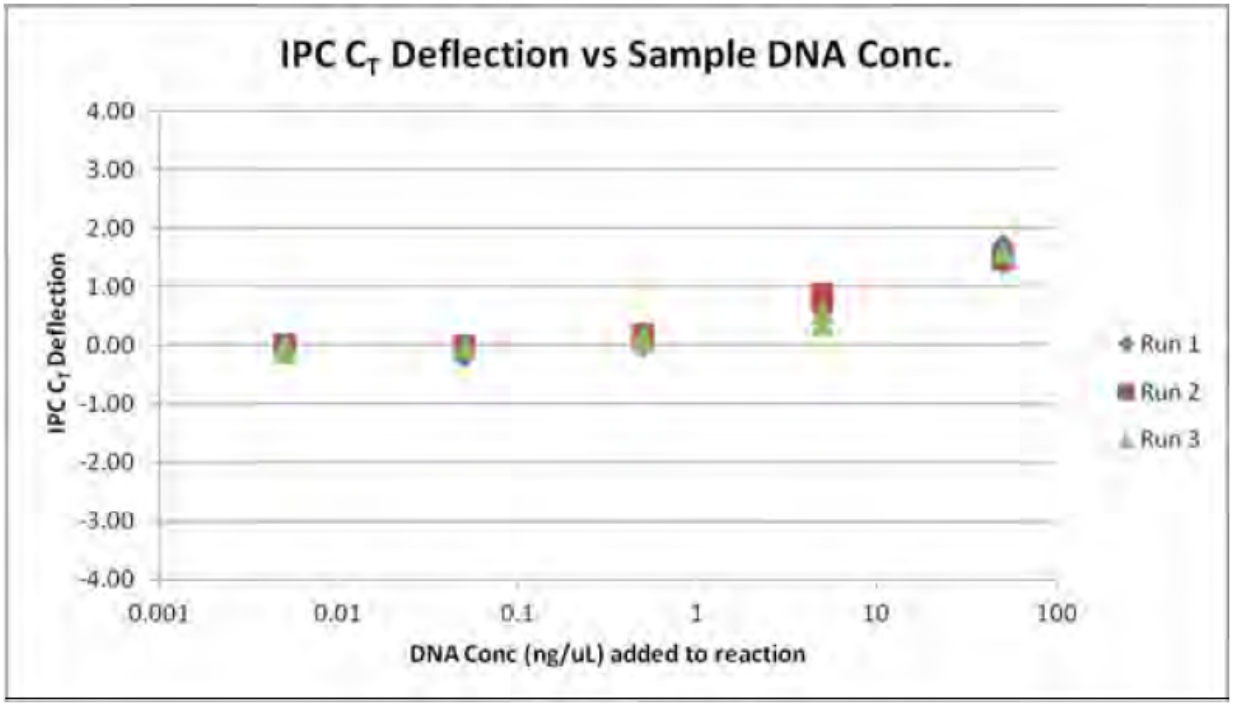


Figure 23 Example of impact of increasing sample DNA concentration on IPC C_T deflection

Note: This is only an example and the magnitude of deflection may vary and laboratory to laboratory this effect may differ in magnitude.

Troubleshoot the standard curve

The following table provides common errors that can result from incorrect quantities or tasks not being set properly.

Table 7 Troubleshooting the standard curve

Observation	Possible cause	Recommended action
Slope for the standard curve is outside the typical range *or* R^2 value is significantly less than 0.98	When defining quantification values for the standards, an incorrect quantity was applied.	1. From the analysis section, move the cursor over the well and verify that the quantity is correct. 2. Update with the correct values and reanalyze, if necessary.
At each concentration, the standard curves for all targets are not shown	For the standard curve samples, the task was set to unknown for one of the targets in the assay.	1. From the analysis section, move the cursor over the well and verify that the task is set to “S” for all of the standard curve samples. 2. Update and reanalyze, if necessary.

Table 7 Troubleshooting the standard curve *(continued)*

Observation	Possible cause	Recommended action
Slope value for standard is outside the expected range (see "Slope" on page 54)	Standards have not been properly stored, or are older than 2 weeks.	Prepare fresh standards.
A failed standard is incorporated into the standard curve.	Standard DNA not loaded in well.	Exclude failed standard from standard curve analysis. (Select **Plate Setup** ▸ **Define Samples and Targets**, then change the Sample Type from Standard to Unknown), then reanalyze.

Use the internal PCR control system

Purpose

Use the Internal PCR Control (IPC) system to distinguish between true negative sample results and reactions affected by:

- The presence of PCR inhibitors
- Assay setup
- A chemistry or instrument failure

Note: The IPC in the Quantifiler™ HP and Trio DNA Quantification Kits have been developed with increased inhibitor tolerance to better correlate with our more recently introduced STR kits, such as NGM Detect™, VeriFiler™ Plus , NGM SElect™, GlobalFiler™, and GlobalFiler™ IQC PCR Amplification Kits. STR kits are For Forensic or Paternity Use Only.

Components

The following components of the IPC system are present in the Quantifiler™ Trio Primer Mix:

- Synthetic DNA template
- Primers that hybridize specifically to the synthetic DNA template
- Probe labeled with JUN™ dye

Interpret IPC results

Positive amplification occurs when the C_T value for the target is <40. Because samples contain unknown amounts of DNA and inhibitors, a large range of C_T values is possible. The IPC system template DNA is present at a consistent concentration across all reactions on a plate. Therefore, the IPC (JUN™ dye) C_T should be relatively constant in typical reactions. However, the presence of PCR inhibitors and/or higher concentrations of DNA can increase the IPC C_T relative to the average IPC C_T of the quantification standards on the same plate.

In the amplification plot window of the HID Real-Time PCR Analysis Software, observe amplification of the assay targets, then interpret the IPC results. For guidance about how to understand IPC results, see Table 9.

IMPORTANT! Perform validation studies to determine the IPC interpretation guidelines appropriate for your sample types, sample concentrations, and protocols.

Table 8 Quantifiler™ Trio Automated Kit targets

Target	Dye/quencher
Human Target, small autosomal	VIC™ dye with MGB quencher
Human Target, large autosomal	ABY™ dye with QSY™ quencher
Human Male Target	FAM™ dye with MGB quencher
Internal PCR Control	JUN™ dye with QSY™ quencher

Table 9 Interpret IPC amplification results

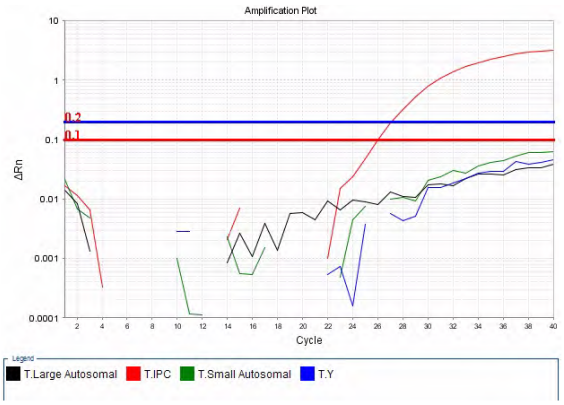
Quantifiler™ Trio Automated Human (VIC™ and ABY™ dyes) and Male (FAM™ Dye)	Quantifiler™ Trio Automated IPC (JUN™ Dye)	Interpretation
No amplification 	Amplification	Negative result - no human DNA detected

Table 9 Interpret IPC amplification results *(continued)*

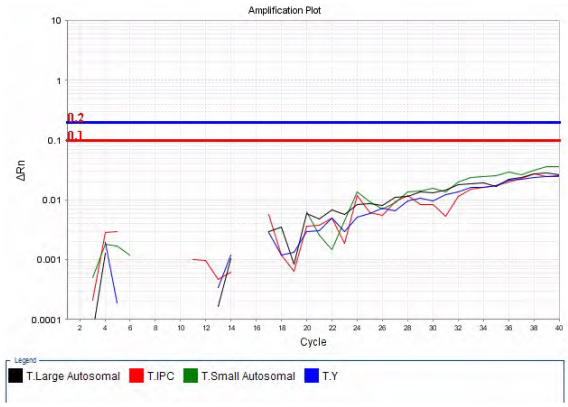
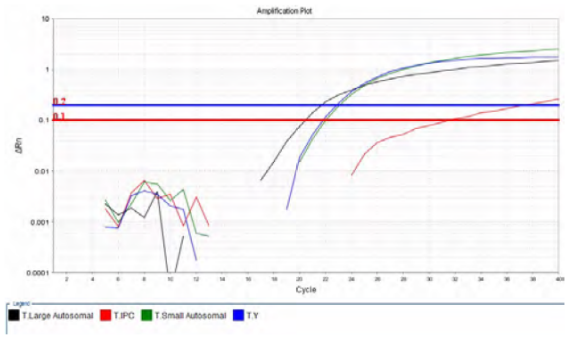
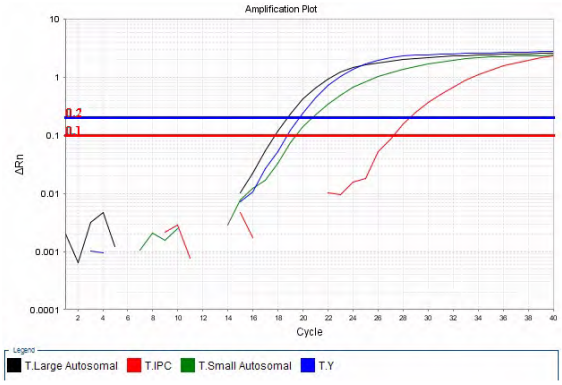
Quantifiler™ Trio Automated Human (VIC™ and ABY™ dyes) and Male (FAM™ Dye)	Quantifiler™ Trio Automated IPC (JUN™ Dye)	Interpretation
No amplification 	No amplification	Invalid result, perhaps caused by severe PCR inhibition, improper formulation of reagents, or failure of critical assay components
Amplification  **Note:** Suppressed amplification (high C_T value and low ΔR_n value) of the human and/or male-specific targets can also occur due to PCR inhibition. This is often more pronounced in the large autosomal target that is more susceptible to inhibitory effects.	No amplification or amplification appears significantly reduced relative to the average IPC C_T value for quantification standards.	Possible Inhibitor present

Table 9 Interpret IPC amplification results *(continued)*

Quantifiler™ Trio Automated Human (VIC™ and ABY™ dyes) and Male (FAM™ Dye)	Quantifiler™ Trio Automated IPC (JUN™ Dye)	Interpretation
Amplification, Quantity >5 ng/µL The example below is a sample free of PCR inhibitors with 100 ng/µL DNA. It illustrates that it is possible to see increased IPC C_T with no inhibition. **Note:** The IPC C_T shown below is 1.11 higher than the average IPC C_T value for the quantification standards from 50 ng/µL to 0.005 ng/µL (5 standards, 2 replicates each). For additional information, see also Figure 23. 	Amplification appears reduced relative to the average IPC C_T value for the quantification standards.	High sample concentration may contribute to suppression of IPC amplification. This may occur independently or in combination with the effect of PCR inhibitors, yielding inconclusive IPC results.

Negative results

No human DNA is detected when:

- No signal for the Small Autosomal, Large Autosomal and Y targets (VIC™, ABY™ and FAM™ dyes, respectively) is detected, indicating that the human and/or male-specific targets did not amplify.
- The IPC target (JUN™ dye) amplifies and amplification does not appear reduced relative to the average IPC C_T value for quantification standards.

Complete amplification failure

Undetected results for all assay targets, including human and male-specific targets and the IPC target, indicates a complete failure of PCR amplification for the reaction. This could be caused by conditions such as incorrect thermal cycling or incorrect formulation of PCR reagent mix (which would affect multiple reactions or possibly the entire plate), or by severe PCR inhibition affecting individual samples. This type of result is invalid, and the samples should be prepared again to confirm the result or new samples should be extracted.

PCR inhibition

No amplification or weak amplification of the IPC may indicate PCR inhibition (partial or complete) in the sample. In addition, suppressed amplification (high C_T value and low ΔR_n value) of the human and/or male-specific targets can occur due to PCR inhibition. This is typically more pronounced in the large autosomal target than the small autosomal target since the large autosomal target is more susceptible to inhibitory effects.

IPC results inconclusive

With increasing concentrations of human genomic DNA (>5 ng/µL), competition between the human and/or male-specific and IPC PCR reactions may suppress IPC amplification for that sample. This can occur independently or in combination with the effect of PCR inhibitors, yielding inconclusive results. However, samples with high DNA concentration will be diluted during STR reaction setup to meet the optimal target input amount of DNA in the STR reaction. Therefore, the effect of most inhibitors, if present in the sample, on next generation STR kit performance is likely to be minimized.

Evaluate IPC amplification

If the IPC amplification for certain samples appears reduced relative to IPC amplification for quantification standards or is completely suppressed, it may be caused by:

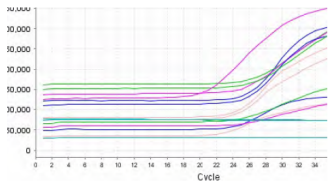
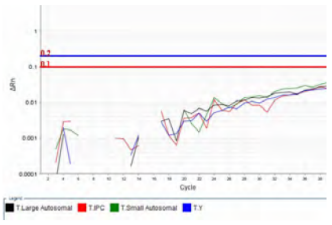
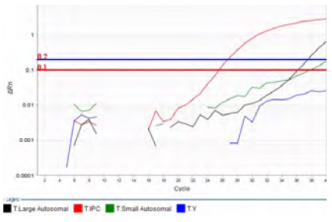
- Presence of PCR inhibitors
- Higher concentrations of DNA (for example, >5 ng/uL)

The IPC results can help you determine the next step:

- Proceed directly to an STR analysis of the sample
- Dilute the sample before adding it to the STR reaction
- Perform additional cleanup of the sample to remove potential inhibitors and requantify the sample if necessary
- Select a next generation STR kit for improved performance with inhibited samples

Troubleshoot amplification plots

Table 10 Troubleshooting amplification plots

Observation	Possible cause	Recommended action
ΔR_n and C_T values inconsistent with replicates 	Incorrect volume of Quantifiler™ THP PCR Reaction Mix added to some reactions.	**1.** Select the multicomponent plot. Wells with incorrect volume of Quantifiler™ THP PCR Reaction Mix should generate significantly less fluorescence compared to unaffected wells. **2.** Verify that the correct volume of reaction mix was added to the plate by comparing the volume of the affected wells to the surrounding wells.
High C_T value and low ΔR_n value 	High levels of PCR inhibition resulting in no amplification of the human and male targets.	Consider diluting the sample before adding to STR reaction. If inhibition is still present, repurify the sample and rerun.
Unpredictable pattern of positive/undetected results from assay targets, with very high C_T values (for example, >37) 	Stochastic effects with very low-concentration samples may cause wide variations in C_T results among replicates, or result in unpredictable patterns of positive/undetected results with assay targets.	Perform validation studies to determine analysis guidelines for samples with extremely low concentrations of DNA that are close to or below the detection threshold for standard STR assays.

Assess quantity

Purpose

After viewing the results and assessing the quality of the results, determine whether sufficient DNA is present to proceed with a short tandem repeat (STR) assay.

Note: The primary quantification value is from the small autosomal target. Use this value for determination of STR input amount.

Assay sensitivity

The Quantifiler™ Trio Automated Kit can reproducibly quantify 5 pg/µL of human genomic DNA in a sample. When 2.0 µL of a sample at the lowest concentration standard (5 pg/µL) is loaded in a reaction, the well contains approximately 1.5 diploid human genome equivalents.

Stochastic effects

The Quantifiler™ Trio Automated Kit can detect DNA concentrations <5 pg/µL; however, at concentrations <5 pg/µL, stochastic effects, or the statistical effect of random sampling of alleles present at a very low copy number, can produce significant variability in assay results. When using samples containing DNA in this concentration range, you can perform replicate analysis to confirm true absence of DNA.

If insufficient DNA is present

If the results from Quantifiler™ Trio Automated Kit reactions indicate that insufficient DNA is present to perform an STR assay, some options available to improve STR kit performance are:

- Re-extract the DNA, then repeat the test with the Quantifiler™ Trio Automated Kit before performing STR analysis.
- Concentrate the sample, then repeat the test with the Quantifiler™ Trio Automated Kit before performing STR analysis.
- Use an STR assay that allows for higher volume of DNA input, for example, the GlobalFiler™ PCR Amplification Kit.

Calculate male:female DNA ratio

Forensic DNA samples may contain mixtures of DNA from multiple individuals. In DNA mixtures of male and female individuals, it may be useful to calculate the ratio of total autosomal DNA to the male-specific Y-chromosome DNA.

Note: The ratio is automatically calculated in the HID Real-Time PCR Analysis Software.

The Quantifiler™ Trio Automated Kit assesses the quantity of human and male DNA in biological samples. The quantity of human DNA in this calculation is based on the quantity value for the small autosomal target. From these values, one can calculate the ratio of male and female DNA using the following equation:

Male DNA:Female DNA Ratio = Quantity of Male DNA/Quantity of Male DNA : (Quantity of Human DNA - Quantity of Male DNA)/Quantity Male DNA

All quantities in the above equation are ng/µL.

For example, assuming:

Male DNA concentration = 2 ng/µL

Human DNA concentration = 8 ng/µL then the Male DNA:Female DNA ratio is:

2/2: (8-2)/2 = 1:3

This ratio helps determine the extent of the mixture and is useful in determining whether to proceed with autosomal STR or Y-STR analysis.

As the ratio of female DNA increases relative to male DNA, the ability to detect the minor male component may be limited with autosomal STR analysis. In these instances Y-STR analysis may be considered. Based on each laboratory's protocols, detection instrumentation and analysis thresholds, internal validation studies should be performed to determine M:F ratio thresholds to indicate when Y-STR analysis should be considered. In house experiments have shown that the Quantifiler™ Trio Automated assay can accurately quantify 20 pg/µL male DNA in >1,000-fold excess female DNA.

Determine quality index

Quality index

You can use two results from the HID Real-Time PCR Analysis Software to determine the Quality Index for a sample:

- Degradation Index
- $IPCC_T$ flag

Degradation index

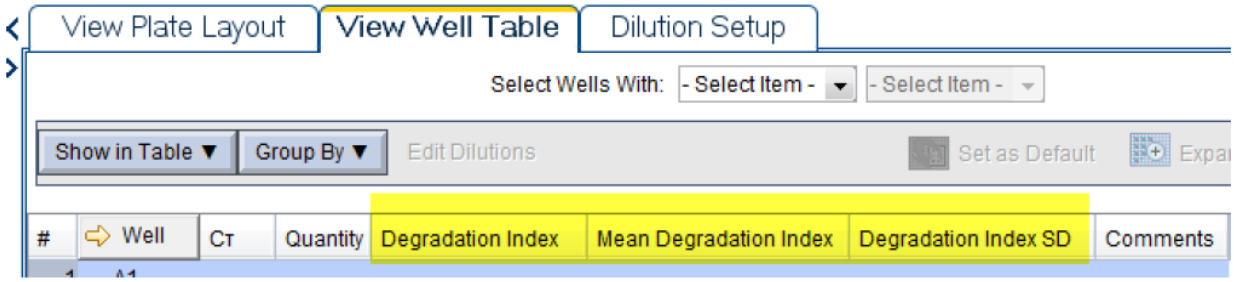
"Degradation Index" refers to the data observed when a sample displays a decrease in measured amount for large DNA fragments compared to small DNA fragments. The Degradation Index is for use as a general indicator of whether large DNA fragments may perform more poorly relative to small DNA fragment in STR reactions.

The Degradation Index is automatically calculated by the HID Real-Time PCR Analysis Software using the following formula:

$$\frac{\text{Small autosomal target DNA conc. (ng/µL)}}{\text{Large autosomal target DNA conc. (ng/µL)}}$$

The Degradation Index value is displayed in the Well Table view in any of the analysis screens (you may have to scroll to the right to display it.) The mean and standard deviation for replicates are also displayed in the Well Table view.

Note: When the quantity for the small or large autosomal target is undetermined, the Degradation Index is not calculated and the Degradation Index field in the Well Table will be empty. When the large autosomal target is undetermined, this can be an indication of significant degradation and/or inhibition affecting the sample. See "Determining the quality index" on page 66 for more information.



The Degradation Index can be affected by:

- Degree of degradation of the large autosomal target DNA
- Presence of PCR inhibitors

  PCR inhibitors (particularly target-specific inhibitors) act in many ways to disrupt amplification. PCR inhibitors that negatively affect the large autosomal target in comparison to the small autosomal target cause less efficient amplification and higher C_T values for the large autosomal target. Evaluate Degradation Index in conjunction with the IPC C_T as described below.

IPC C_T flag

The IPC C_T flag is triggered for an unknown sample that has an IPC C_T of:

- Undetermined
- Greater than the average of the IPC C_T values for all the standards plus the threshold you set in the software HID Settings

For example if you set the IPCT C_T Variance to 2.0 and the average IPC C_T for the standards is 29, the IPC C_T flag is triggered for samples with a C_T ≥31.

The IPC C_T flag is displayed in Analysis QC Summary screen and the Plate View or Well Table view in any of the analysis screens.

When the IPC C_T flag is triggered this typically indicates the presence of PCR inhibitors in sufficient concentration to significantly impact downstream performance with next generation STR kits. See the "Interpret IPC results" on page 57 for more information.

IMPORTANT! Perform validation studies to determine an IPC C_T threshold appropriate for your laboratory's sample types and protocols.

Determining the quality index

To determine the Quality Index, evaluate the Degradation Index in conjunction with the IPC C_T to assess the potential presence of PCR inhibitors and degradation that may have an impact on downstream sample processing.

IPCCT flag triggered?	Degradation Index	Quality Index interpretation[1]
No	<1	Typically indicates that DNA is not degraded or inhibited.
	1 to 10	Typically indicates that DNA is slightly to moderately degraded. PCR inhibition is also possible, however, not enough to significantly suppress IPC amplification.
	>10 or blank (no value)	Typically indicates that DNA is significantly degraded. PCR inhibition is also possible, however, not enough to significantly suppress IPC amplification. Highly degraded samples that cannot be recovered by STR can be analyzed with HID-Ion AmpliSeq™ Panels and the Ion Personal Genome Machine™ (PGM™) (see Appendix D, "Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel").
Yes	<1	Although theoretically possible, this result is unlikely because PCR inhibitors in sufficient concentration to trigger the IPCCT flag typically would affect the large autosomal target as well.
	>1 or blank (no value)	Typically indicates that the DNA is affected by degradation and/or PCR inhibition.

[1] These are general guidelines that may not apply to all samples depending on the inhibitors present, the varying quantity of contributor DNA in mixed samples and the STR kit used. (STR kits are For Forensic or Paternity Use Only.)

IMPORTANT! Perform validation studies to determine interpretation guidelines for the Quality Index for your laboratory.

The Quality Index results can help you determine next steps, including:

- Proceed directly to an STR analysis of the sample
- Dilute the sample before adding to the STR reaction
- Perform additional cleanup of the sample to remove potential inhibitors and requantify the sample if necessary
- Use one of the next generation STR kits for improved performance with inhibited samples
- Use an STR assay that includes a high number of miniSTR loci, such as the GlobalFiler™ and MiniFiler™ PCR Amplification Kits (or a combination of those kits), for increased data recovery from degraded samples
- Use an HID-Ion AmpliSeq™ Panels and the Ion Personal Genome Machine™ (PGM™) for samples that cannot be recovered by STR (see Appendix D, "Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel").

Assess sensitivity and results

About assay sensitivity

Real-time PCR assays are extremely sensitive, and detection of C_T values >35 may indicate the presence of exceedingly low quantities of DNA. It is possible to detect C_T values <40 for extraction blank and negative control samples while performing a real-time PCR reaction with the Quantifiler™ Trio Automated Kit.

Detection of such a low quantity of DNA can vary from amplification to amplification based on stochastic effects. Such levels may be considered background signal and may vary from laboratory to laboratory, and may not produce detectable product when the STR Kits are used. (STR kits are For Forensic or Paternity Use Only.)

The Quantifiler™ Trio Automated Kit reagents undergo rigorous quality control to help ensure that the reagents are free of extraneous DNA. However, due to the extreme sensitivity of the test, background DNA from the environment can be detected on rare occasions.

Each laboratory should take standard precautions to minimize contamination in its own facility. Each laboratory should also establish a C_T value above which a positive result represents background signal only.

Evaluating the strengths and limitations of any test is common practice in forensic laboratories. We recommend applying a similar approach when validating the Quantifiler™ Trio Automated Kit.

Negative control samples, DNA contamination, and spectral artifacts

Due to the extremely high sensitivity of the Quantifiler™ Trio Automated Kit assays, you may occasionally observe amplification in:

- Negative Control (no template control or NTC) samples caused by contamination of assay reagents or consumables
- Case samples containing minute amounts of DNA below the detection limit for the assay

It is possible to obtain sporadic signal in any of the genomic targets. However, detection of signal may be more likely for the large autosomal target. In these samples, amplification is most likely caused by the high copy number of the large autosomal target (which leads to a higher probability of amplification). Samples with a C_T >38 for the large autosomal target and no amplification for the small autosomal and Y targets typically contain extremely small quantities (a fraction of 1 genome equivalent) of DNA. Amplification of only the large autosomal target may not indicate the presence of DNA quantity sufficient for STR analysis.

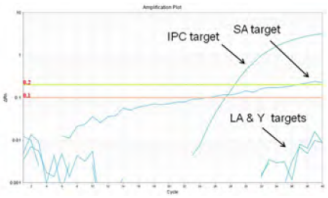
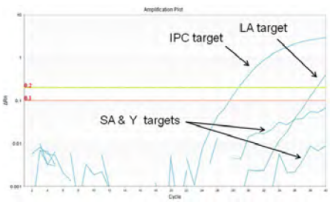
IMPORTANT! Perform validation studies to determine the minimum C_T value for each of the assay targets that correlate to a DNA quantity that will yield an interpretable STR result.

See Table 11 for information to help you distinguish between a real DNA signal due to the contamination of assay reagents or consumables and an apparent positive result due to spectral artifacts that can (very rarely) generate a fluorescence signal that crosses the ΔR_n threshold. Such artifacts may be the result of anomalous baseline signals, and can often be eliminated by changing the baseline window setting.

Note: The HID Real-Time PCR Analysis Software uses a specialized multicomponenting algorithm that provides precise deconvolution of multiple dye signals in each well. This algorithm helps ensure minimal crosstalk when using multiple fluorophores for multiplex assays. However, a residual spectral overlap may be observed if the instrument is in need of calibration.

Assess apparent positive results in negative control samples

Table 11 Troubleshooting apparent positive results in negative control samples

Observation	Possible cause	Recommended action
Amplification plot for the SA target (VIC™ dye channel) shows a very gradual increase in ΔR_n with abnormal appearance (compared to IPC amplification plot), eventually crossing the 0.2 threshold to register as a positive. 	If the HID Real-Time PCR Analysis Software Analysis Settings are set to automatic baseline, spurious fluorescence signals in early cycles may cause an artifact that falsely elevates the ΔR_n signal.	If the Analysis Settings are set to use automatic baseline, change them to manual baseline: 1. In the HID Real-Time PCR Analysis Software, select **Analysis** ▸ **Analysis Settings**. 2. Click the **C_T Settings** tab. 3. Select **Use Default Settings** to apply the Manual Baseline method: Manual C_T = 0.2, Baseline Start Cycle = 3, and Baseline End Cycle = 15. 4. Click **Apply Analysis Settings**. 5. In the main **Analysis** window, click **Analyze**.
C_T value <40 is observed for one or more genomic targets in an NTC reaction, normally expected to be negative for all genomic detectors. 	Contamination of reagents or consumables (assay plate, pipette tips, and so on) with human genomic DNA or amplified PCR products.	Ensure that stringent contamination controls and laboratory cleanliness protocols are in place. Always wear clean disposable gloves when handling assay consumables and ensure that reagent tubes and consumable boxes are opened using appropriate safeguards.

Prevent PCR contamination

Laboratory practices to minimize false positives

PCR assays require special laboratory practices to avoid false positive amplifications, as detailed in Table 11. The high sensitivity of these assays may result in the amplification of a single DNA molecule.

To minimize false positives due to the presence of amplified material in your work area, follow these recommended laboratory practices:

- When possible, maintain separate work areas, dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Wear a clean lab coat (not previously worn while handling amplified PCR products or during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect they are contaminated and before entering or leaving the work area.
- Establish procedures for handling new, unopened and partially used packages of sample tubes and reaction plates to prevent interaction between clean and used packaging.
- Use positive-displacement pipettes or aerosol-resistant pipette tips.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully.
- Try not to splash or spray PCR samples.
- When pipetting from a kit component tube, hold the cap of the tube in your gloved hand, or be sure to set it down on a clean, decontaminated surface.
- Keep reactions and components sealed when possible.
- Do not open sealed reaction tubes or plates after amplification.
- Clean work areas periodically with freshly diluted 10% bleach or other cleaning solution known to destroy DNA. If using bleach, rinse the areas with DI water to help ensure the work areas do not contain residual bleach after cleaning.

A Supplemental information

Assay overview

The Quantifiler™ Trio Automated assay combines four 5’ nuclease assays:

- Two separate target-specific human assays; one with a short PCR amplicon and one with a long PCR amplicon
- A target-specific human male DNA assay
- An internal PCR control (IPC) assay

Target-specific assay components

Each target consists of PCR primers and dye-labeled TaqMan™ probes (with non-fluorescent quenchers) for the amplification of multicopy genomic loci. For information about the targets of PCR amplification in the Quantifiler™ Trio Automated DNA Quantification Kit, see “Target-specific assay components” on page 70.

Table 12 Quantifiler™ Trio Automated DNA Quantification Kit targets

Target	Amplicon length	Ploidy	Copy Number	Dye/Quencher
Human Target, small autosomal	80 bases	Diploid	multicopy	VIC™ dye with MGB quencher
Human Target, large autosomal	214 bases	Diploid	multicopy	ABY™ dye with QSY™ quencher
Human Male Target	75 bases	Haploid	multicopy	FAM™ dye with MGB quencher
Internal PCR Control	130 bases	NA	Synthetic IPC template is included in the primer mix	JUN™ dye with QSY™ quencher

The Quantifiler™ Trio Automated assay targets serve the following functions in the multiplex system:

- **Small Autosomal (SA) Target:** The SA target is the primary quantification target for total human genomic DNA. Its smaller amplicon size (80 bp) is aligned with the sizes of typical "mini" STR loci and makes it better able to detect degraded DNA samples.
- **Large Autosomal (LA) Target:** The LA target is used mainly as an indicator of DNA degradation, by comparing the ratio of its quantification result with that of the SA target.
- **Y chromosome Target:** The Y target allows the quantification of a sample's human male genomic DNA component, and is particularly useful in assessing mixture samples of male and female genomic DNAs.

Internal PCR control system components

The internal PCR control (IPC) system consists of:

- IPC template DNA (a synthetic sequence not found in nature)
- Primers for amplifying the 130 base IPC template DNA
- TaqMan™ probe dye-quencher—JUN™ dye with QSY™ quencher

The IPC present in each sample contains a synthetic DNA template, and provides positive confirmation that all assay components are functioning as expected. This internal control is particularly useful to confirm the validity of negative results. It is also useful to identify samples that contain PCR inhibitors.

About the probes

The TaqMan™ MGB probes contain:

- A reporter dye (FAM™ or VIC™ dye) linked to the 5' end of the probe
- A minor groove binder (MGB) at the 3' end of the probe
  This modification increases the melting temperature (T_m) without increasing probe length (Afonina et al., 1997; Kutyavin et al., 1997), to allow for the design of shorter probes.
- A nonfluorescent quencher (NFQ) at the 3' end of the probe

The TaqMan™ QSY™ probes contain:

- A reporter dye (ABY™ or JUN™ dye) linked to the 5' end of the probe
- A nonfluorescent quencher (QSY™) at the 3' end of the probe

5' nuclease assay process

The 5' nuclease assay process (Figure 24 through Figure 28) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

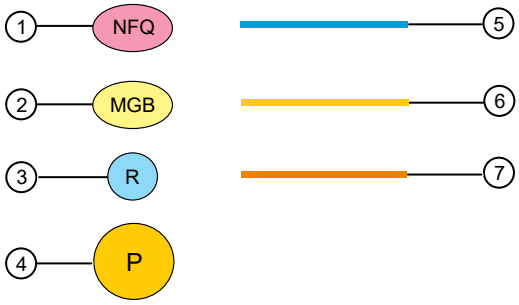


Figure 24 Legend for 5′ nuclease assay process figures

1. Non-fluorescent quencher
2. Minor groove binder
3. Reporter
4. Hot start DNA polymerase
5. TaqMan™ probe
6. Forward primer
7. Reverse primer

During PCR, the TaqMan™ MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 25).

When the probe is intact (Figure 25 and Figure 26), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förstertype energy transfer.

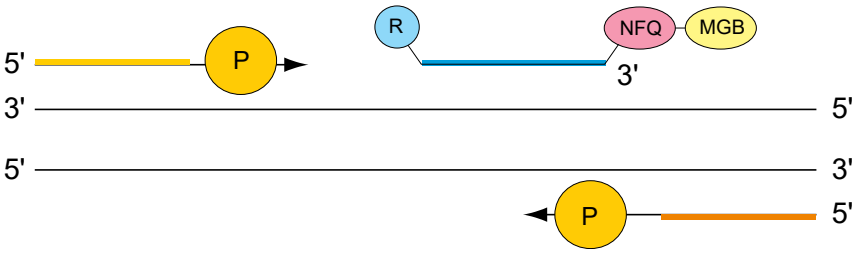


Figure 25 Polymerization

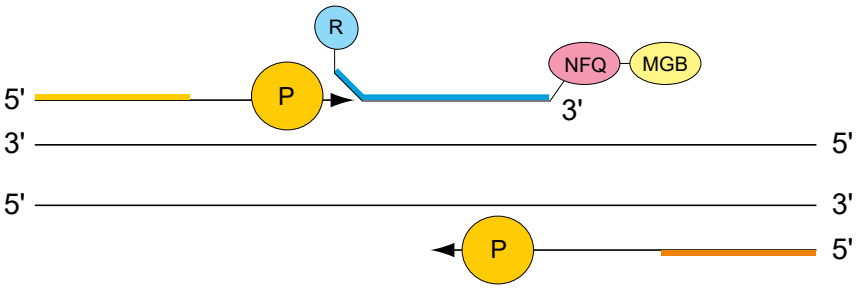


Figure 26 Strand displacement

The DNA polymerase cleaves only probes that are hybridized to the target (Figure 27). Cleavage separates the reporter dye from the quencher dye, resulting in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

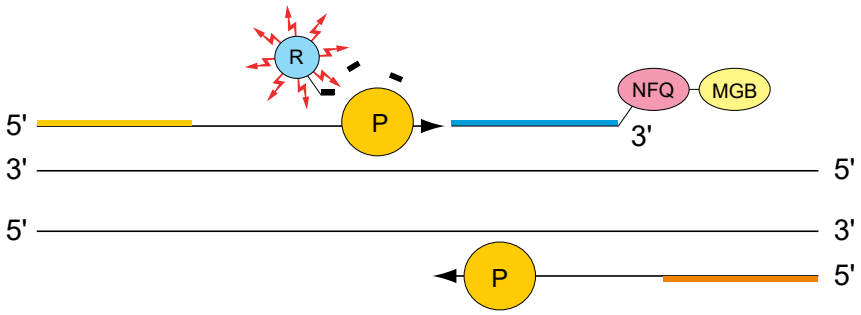


Figure 27 Cleavage

Polymerization of the strand continues, but because the 3' end of the probe is blocked, there is no extension of the probe during PCR (Figure 28).

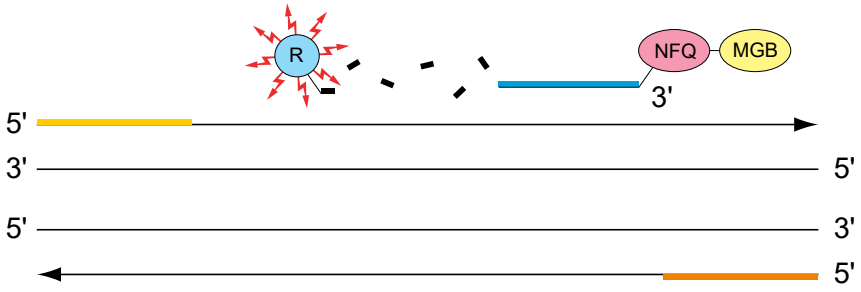


Figure 28 Completion of polymerization

Normalization of reporter signals

During a run, the software displays cycle-by-cycle changes in normalized reporter signal (ΔR_n). The software normalizes each reporter signal by dividing it by the fluorescent signal of the passive reference dye. Because the passive reference is one component of the PCR master mix, it is present at the same concentration in all wells of the reaction plate. By normalizing the reporter signal using the passive reference, the software can account for minor variations in signal caused by pipetting inaccuracies and make better well-to-well comparisons of the reporter signal.

Human DNA standard

The human DNA used to generate the DNA quantification standards dilution series consists of pooled human male genomic DNA. As such, the performance of the Quantifiler™ Trio Automated assay is optimized for use with this DNA standard. The use of an alternate DNA standard may result in the reporting of different concentration values for the unknown samples. Use of an alternate DNA standard is not recommended.

B

7500 Real-Time PCR System for Human Identification

7500 Real-Time PCR System for Human Identification

Instrument overview

For more information about the 7500 Real-Time PCR System, see the *Applied Biosystems™ 7500/7500 Fast Real-Time PCR System: Maintenance Guide* (Pub. No. 4387777).

The 7500 Real-Time PCR System for Human Identification provides an advanced, validated solution for casework, databasing, and paternity applications.

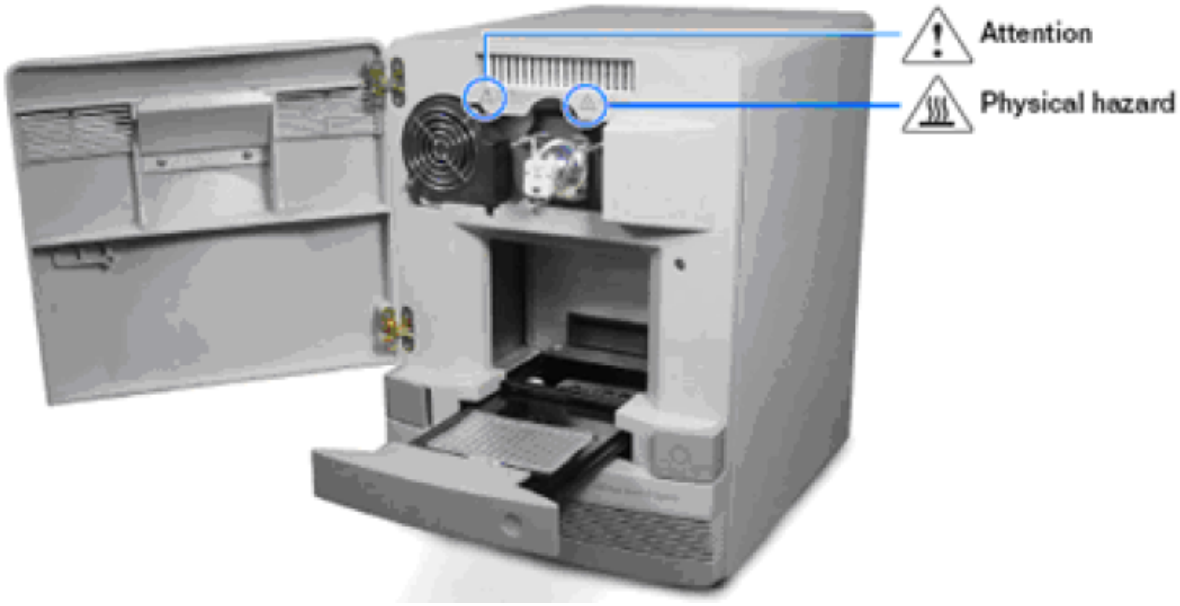
The 7500 Real-Time PCR System is controlled by the HID Real-Time PCR Analysis Software.

The 7500 instrument is calibrated with several dyes including: FAM™, SYBR™ Green, VIC™, ABY™, TAMRA™, NED™, Cy3™, ROX™, Texas Red™, Cy5™, JUN™, and MUSTANG PURPLE™ (MP).

The 7500 Real-Time PCR System uses the data obtained from the pure-dye calibration to distinguish the individual contribution of each dye in the collective fluorescence, as gathered by the instrument during a run. After each run, the instrument software receives raw spectra-signal data for each reading. To make sense of the raw data, the software determines the contribution of each fluorescent dye used in the sample by comparing the raw spectra data to a set of pure dye standards contained in the pure spectra file. When an experiment is saved after analysis, the software stores the pure spectra information with the collected fluorescent data for that experiment.

During a run

1. A tungsten-halogen lamp directs light to each well on the reaction plate. The light excites the fluorescent dyes in each well of the plate.
2. The CCD camera detects the fluorescence emission.
3. The software obtains the fluorescence emission data from the CCD camera and applies data analysis algorithms.



Real-time data analysis

The 7500 Real-Time PCR instrument can be used to determine the relative quantity of a target nucleic acid sequence in a sample by analyzing the cycle-to-cycle change in fluorescent signal as a result of amplification (Figure 29).

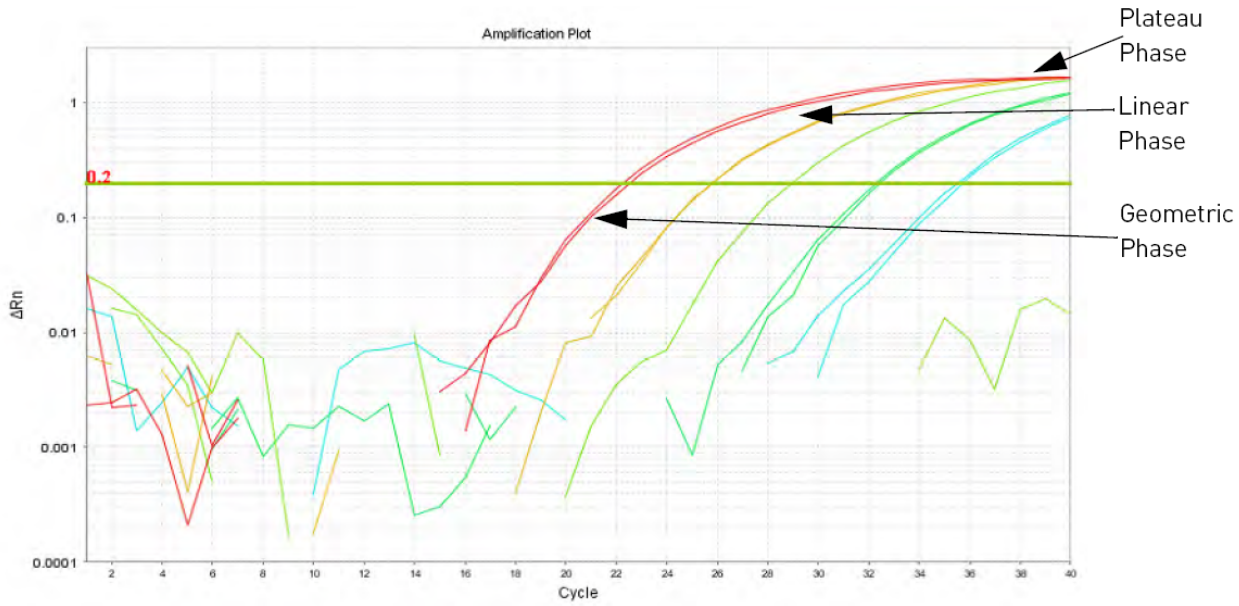


Figure 29 Phases of PCR amplification

Amplification plot example

When using TaqMan™ probes with the 7500 Real-Time PCR instrument, the fluorescence signal (or normalized reporter, R_n) increases as the amount of specific amplified product increases. Figure 29 shows the amplification of PCR product in a plot of R_n vs. cycle number during PCR. This amplification plot contains three distinct phases that characterize the progression of the PCR.

Phases of amplification

Phase 1: Geometric (exponential)

Upon detection, the signal increases in direct proportion to the increase of PCR product. As PCR product continues to increase, the ratio of Taq DNA polymerase enzyme to PCR product decreases.

During the geometric phase, amplification is characterized by a high and constant efficiency. Amplification occurs between the first detectable rise in fluorescence and the beginning of the linear phase. During the geometric phase, a plot of DNA concentration versus cycle number on a log scale should approximate a straight line with a slope. Typically, the real-time PCR system is sufficiently sensitive to detect at least 3 cycles in the geometric phase, assuming reasonably optimized PCR conditions.

Phase 2: Linear

During the linear phase, the slope of the amplification plot decreases steadily. At this point, one or more components of the PCR has decreased below a critical concentration, and the amplification efficiency begins to decrease. This phase is termed linear because amplification approximates an arithmetic progression, rather than a geometric increase. Because amplification efficiency is continually decreasing during the linear phase, the amplification curves exhibit low precision.

Phase 3: Plateau

The amplification plot achieves the plateau phase when the PCR stops, the R_n signal remains relatively constant, and the template concentration reaches a plateau at about 10-7 M (Martens and Naes, 1989).

Relationship of amplified PCR product to initial template concentration

Because of the progressive cleavage of TaqMan™ fluorescent probes during the PCR, as the concentration of amplified product increases in a sample, so does the R_n value. The following equation describes the relationship of amplified PCR product to initial template during the geometric phase:

$N_c = N(1 + E)^c$

where N_c is the concentration of amplified product at any cycle, N is the initial concentration of target template, E is the efficiency of the system, and c is the cycle number.

For example, with the dilutions of RNase P target in the TaqMan™ RNase P Instrument Verification Plate, the ratio of template concentration to detectable signal is preserved in the geometric phase for all dilutions (Figure 30). As the rate of amplification approaches a plateau, the amount of product is no longer proportional to the initial number of template copies.

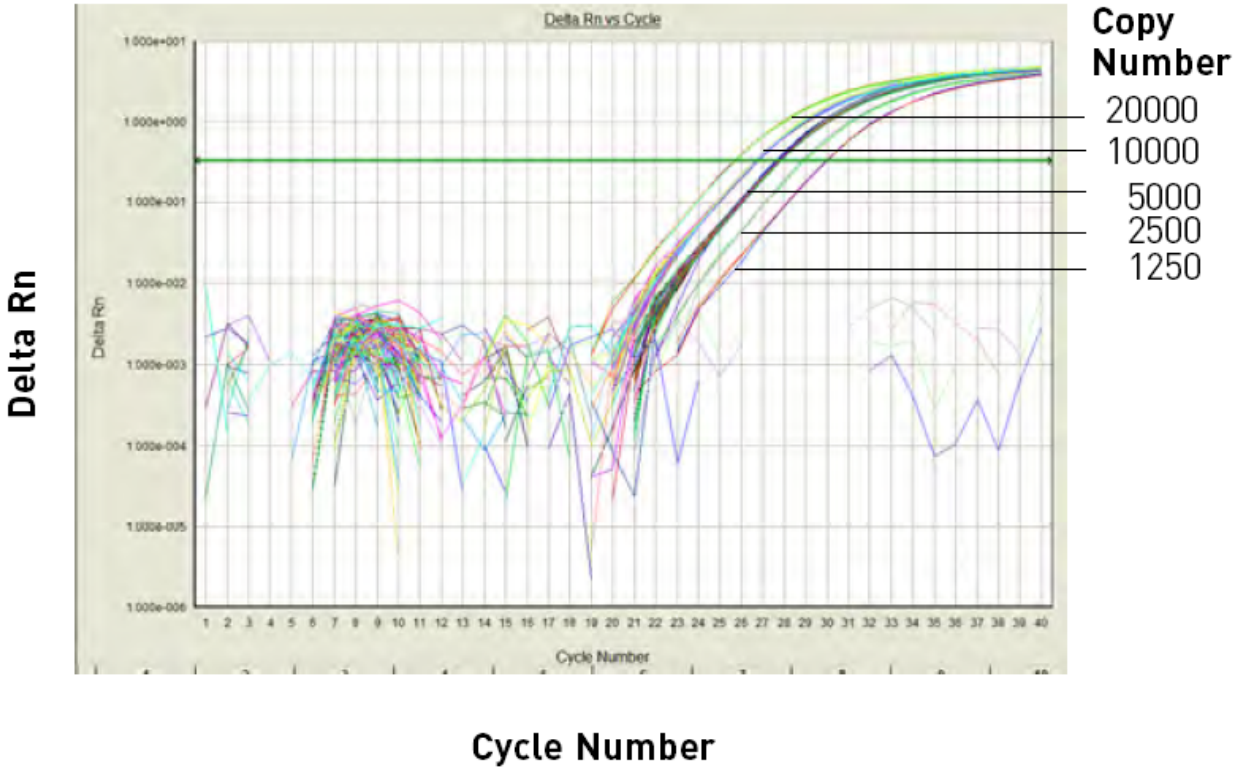


Figure 30 Amplification plot from a real-time run of an RNase P instrument verification plate

About the threshold

The HID Real-Time PCR Analysis Software uses a threshold setting to define the level of detectable fluorescence. Based on the number of cycles required to reach the threshold, the software can compare test samples quantitatively: A sample with a higher starting template copy number reaches the threshold earlier than a sample with a lower starting template copy number.

About the threshold cycle

The threshold cycle (C_T) for a specified amplification plot occurs when the fluorescent signal increases beyond the value of the threshold setting. The C_T value depends on:

- Starting template copy number
- Efficiency of DNA amplification by the PCR system

How C_T values are determined

To determine the C_T value, the HID Real-Time PCR Analysis Software uses the R_n values collected from a predefined range of PCR cycles called the baseline (the default baseline occurs between cycles 3 and 15 on the 7500 Real-Time PCR instrument):

1. The software generates a baseline-subtracted amplification plot of ΔR_n versus cycle number.
2. An algorithm defines the cycle where the ΔR_n value crosses the threshold setting as the threshold cycle (C_T).

Relationship of threshold cycles to initial template amount

The following equation describes the exponential amplification of the PCR:

$X_n = X_m(1 + E_X)^{n-m}$

where:

X_n = number of target molecules at cycle n (so that n > m)

X_m = number of target molecules at cycle m

E_X = efficiency of target amplification (between 0 and 1)

n – m = number of cycles elapsed between cycle m and cycle n

Our amplicons are designed and optimized to yield optimum amplification efficiencies. Therefore $E_X = 1$ so that:

$X_n = X_m(1 + 1)^{n-m}$

$= X_m (2)^{n-m}$

To define the significance in amplified product of one thermal cycle, set n – m = 1 so that:

$X_n = X_m(2)^1$

$= 2X_m$

Therefore, each cycle in the PCR reaction corresponds to a two-fold increase in product. Likewise, a difference in C_T values of 1 equates to a two-fold difference in initial template amount.

Calibrate the 7500 instrument

IMPORTANT! For system layout, electrical, power, safety, and other site requirements, see the *Applied Biosystems™ 7500/7500 Fast Site Preparation Guide* (Pub. No. 4412843).

If you	Perform
Installed new HID Real-Time PCR Analysis Software with a new instrument	Perform all calibrations and run the RNase P plate
Upgraded from an earlier version of the HID Real-Time PCR Analysis Software	After restoring the calibration files from the earlier HID software version (see the Release Notes), perform a custom dye calibration to calibrate ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes.
Replaced SDS Software v1.2.3	Perform all calibrations and run the RNase P plate

Required materials not supplied

Table 13 7500 Real-Time PCR Instrument—Materials required for calibration

If you	Material	Cat. No.
Replaced SDS Software v1.2.3	7500 Real-Time PCR Systems Spectral Calibration Kit I	4349180
	TaqMan™ RNase P Instrument Verification Plate	4350584
	96-Well Spectral Calibration Plate with ABY™ Dye	4461591
	96-Well Spectral Calibration Plate with JUN™ Dye	4461593
	96-Well Spectral Calibration Plate with MUSTANG PURPLE™ Dye	4461599
Upgraded from an earlier version of the HID Real-Time PCR Analysis Software	96-Well Spectral Calibration Plate with ABY™ Dye	4461591
	96-Well Spectral Calibration Plate with JUN™ Dye	4461593
	96-Well Spectral Calibration Plate with MUSTANG PURPLE™ Dye	4461599

Calibration procedures

The following is an outline of the calibration procedures for the 7500 Real-Time PCR Instrument. For complete instructions, see the *7500/7500 Fast Real-Time PCR Systems System Maintenance Guide* (Pub. No. 4387777).

- Regions of Interest (ROI) calibration
- Background calibration
- Optical calibration
- Dye calibration:
  - Perform dye calibration of the ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes. Follow the custom dye procedure.
  - Perform dye calibration of all system dyes for new instrument installations, or if replacing SDS Software v1.2.3.
  - Use 60°C as the default temperature for all dye calibrations.
- TaqMan™ RNase P Instrument Verification Plate run

New dye spectra for the 7500 Real-Time PCR Instrument

Figure 31 through Figure 33 show the calibration spectra for ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes.

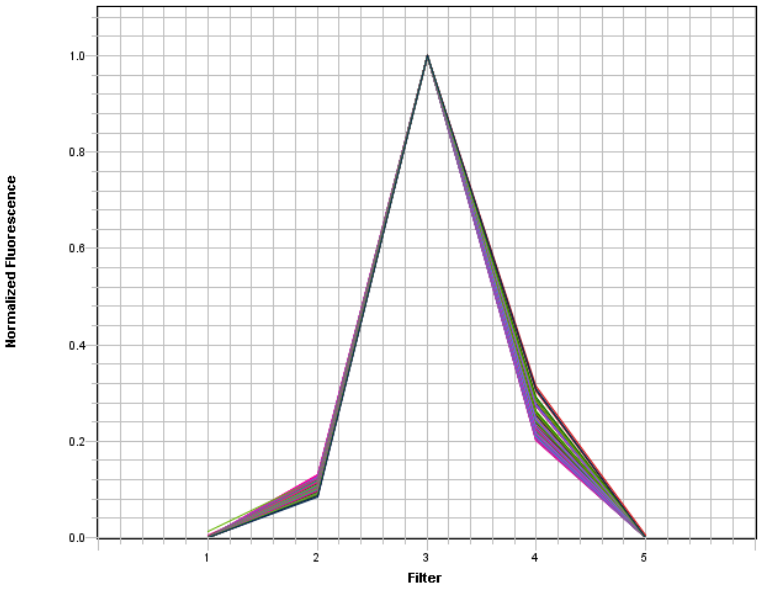


Figure 31 ABY™ dye spectra

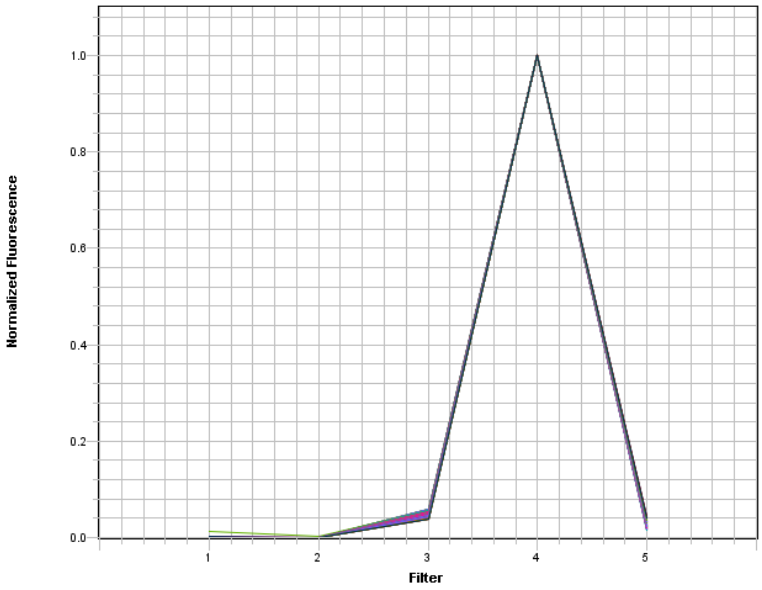


Figure 32 JUN™ dye spectra

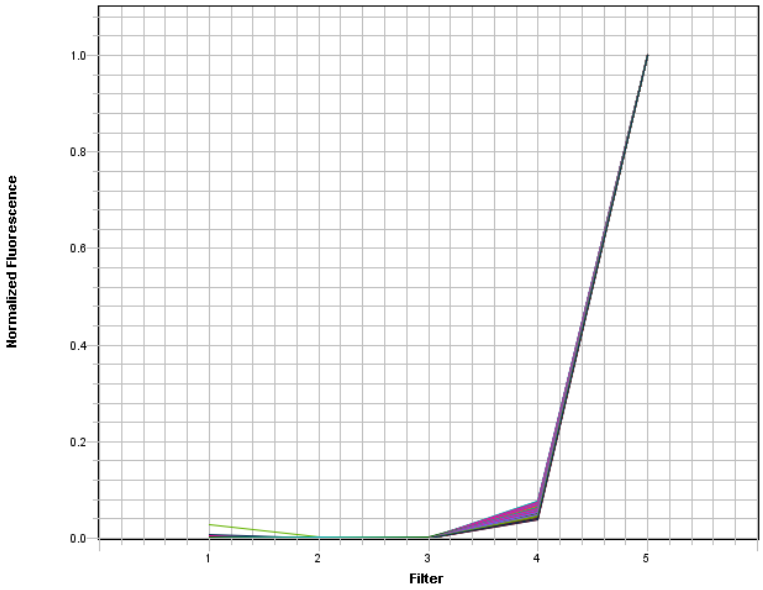


Figure 33 MUSTANG PURPLE™ (MP) dye spectra

C

QuantStudio™ 5 Real-Time PCR System for Human Identification

Instrument overview

For more information about the QuantStudio™ 5 Real-Time PCR Instrument, see the *QuantStudio™ 5 Real-Time PCR Instrument User Guide (for Human Identification)* (Pub. No. MAN0017162).

The QuantStudio™ 5 Real-Time PCR Instrument uses fluorescence-based polymerase chain reaction (PCR) reagents to perform:

- Quantitative detection of target nucleic acid sequences (targets).
- Qualitative detection of targets (endpoint analysis, genotyping, and presence/absence).

The instrument is configured with a 96-well 0.2-mL fixed block (6 color de-coupled).

To run experiments using HID assays, the instrument must be integrated with the HID Real-Time PCR Analysis Software v1.3 or later.

Calibrate the QuantStudio™ 5 Instrument

IMPORTANT! For system layout, electrical, power, safety, and other site requirements, see the *QuantStudio™ 5 Real-Time PCR Instrument Site Preparation Guide (for Human Identification)* (Pub. No. MAN0016701).

The QuantStudio™ 5 Real-Time PCR Instrument is calibrated during manufacturing; however, you *must* recalibrate the instrument for the dyes that are used for HID analysis before use. If you installed HID Real-Time PCR Analysis Software with a new instrument, perform custom dye calibrations for the ABY™ and JUN™ dyes.

Required materials not supplied

Table 14 QuantStudio™ 5 Real-Time PCR Instrument—Materials required for calibration

Material	Cat. no.
96-Well Spectral Calibration Plate with ABY™ Dye	4461591
96-Well Spectral Calibration Plate with JUN™ Dye	4461593
TaqMan™ RNase P Instrument Verification Plate, 96-Well 0.2-mL	4432382

Calibration procedures

The following is an outline of the calibration procedures for the QuantStudio™ 5 Real-Time PCR Instrument. For complete instructions, see the *QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide* (Pub. No. MAN0010407).

- Dye calibration:
  - Perform dye calibration of the ABY™ and JUN™ dyes. Follow the custom dye procedure.
  - Use 60°C as the default temperature for all dye calibrations.

IMPORTANT! You *must* calibrate the ABY™ dye as **ABY-HID** and the JUN™ dye as **JUN-HID**. Calibrating either dye without the "-HID" suffix (as **ABY** and **JUN**) overwrites the existing calibrations for the factory-calibrated system dyes. Doing so potentially creates confusion if the instrument is ever calibrated using the QuantStudio™ 3 and 5 Calibration Kit, which does not have the HID versions of the dyes.

- TaqMan™ RNase P Instrument Verification Plate run

New dye spectra for the QuantStudio™ 5 Real-Time PCR Instrument

Figure 34 through Figure 36 show the calibration spectra for ABY™, JUN™, and MUSTANG PURPLE™ (MP) dyes.

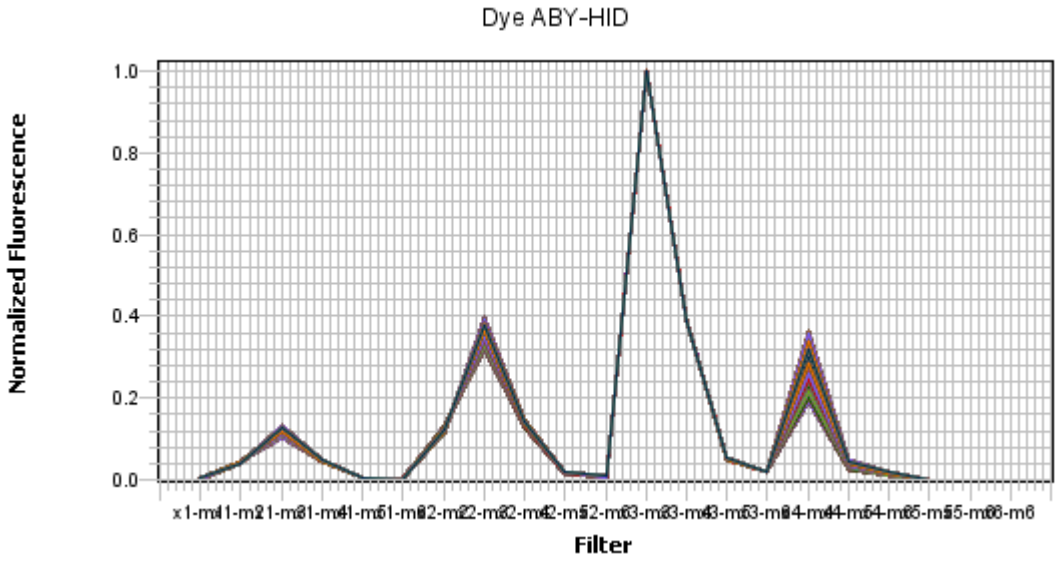


Figure 34 ABY™ dye spectra

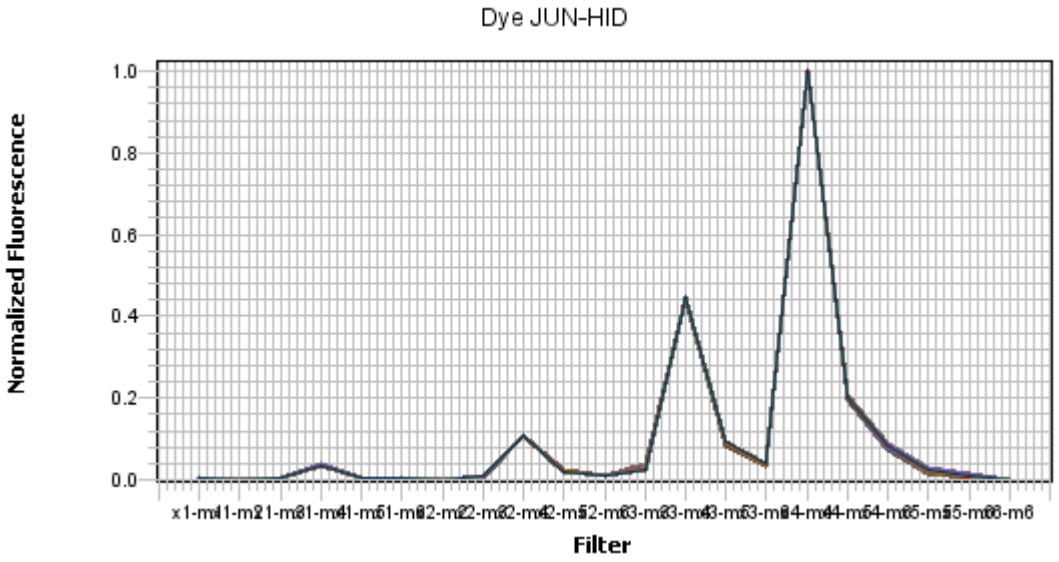


Figure 35 JUN™ dye spectra

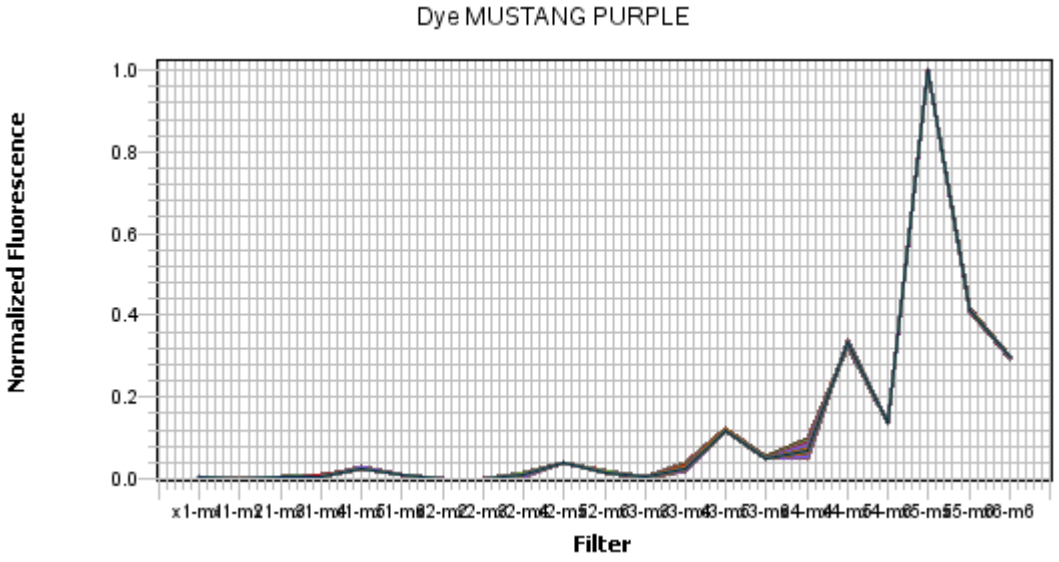


Figure 36 MUSTANG PURPLE™ (MP) dye spectra

Degraded sample studies: GlobalFiler™ STR Kit and HID-Ion AmpliSeq™ Identity and Ancestry Panel

This section provides examples of degraded samples with lower Degradation Indices as determined by the HID Real-Time PCR Analysis Software that yield incomplete STR profiles. It compares the results and Degradation Indices for sample data generated using the GlobalFiler™ STR Kit by capillary electrophoresis (CE) with data obtained with the HID-Ion AmpliSeq™ Identity Panel on the Ion Personal Genome Machine™ (PGM™). It also shows examples of the additional information provided by the HID-Ion AmpliSeq™ Identity and Ancestry Panel that complement CE STR results.

After performing internal validation studies to correlate Degradation Index with incomplete profile generation by STR, you may choose to analyze degraded samples with Degradation Indices below your laboratory-determined threshold with HID-Ion AmpliSeq™ Identity and Ancestry Panels.

Data in this section was produced using products that have been internally tested but that have not been validated under SWGDAM guidelines. Perform internal validation studies to determine the appropriate procedures for your laboratory.

Probability of identity in STR analysis of degraded samples

The relationship between degradation index and the Probability of Identity (PI) derived from the alleles identified in an artificially degraded Raji DNA sample is shown in the following figure (see “Probability of identity in STR analysis of degraded samples” on page 86). The sample was quantified with the Quantifiler™ Trio DNA Quantification Kit and analyzed with the GlobalFiler™ PCR Amplification Kit. PI values were obtained from the *GlobalFiler™ and GlobalFiler™ IQC PCR Amplification Kits User Guide* (Pub. No. 4477604).

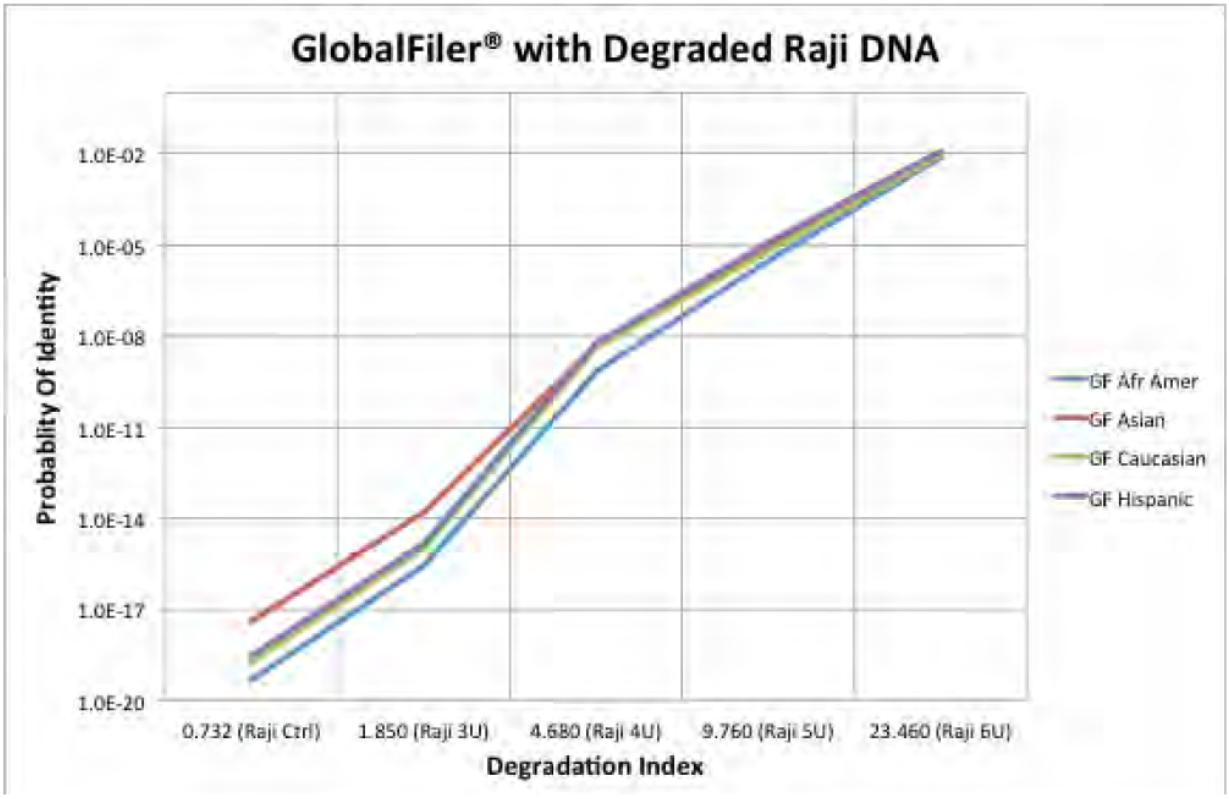


Figure 37 Probability of identity vs degradation index in STR analysis of one sample; results may differ with more samples

As the degradation index increases, the number of alleles identified decreases and may yield incomplete profiles.

Probability of identity in HID-Ion AmpliSeq™ Identity Panel analysis (autosomal SNPs) of degraded samples

The HID-Ion AmpliSeq™ Identity and Ancestry Panel is a high multiplex system consisting of 90 Autosomal and 34 upper Y-Clade SNPs chosen by Dr. Kenneth Kidd from Yale University and the SNPforID Consortium. This panel provides probabilities from 1 x 10^{-31} to 6 x 10^{-35}.

Note: Pakstis, A. J., Speed, W. C., Fang, R., Hyland, F. C., Furtado, M. R., Kidd, J. R., & Kidd, K. K. (2010). SNPs for a universal individual identification panel. Human Genetics, 127(3), 315-324.

Note: Phillips, C., Fang, R., Ballard, D., Fondevila, M., Harrison, C., Hyland, F., et al. (2007). Evaluation of the Genplex SNP typing system and a 49plex forensic marker panel. Forensic Science International: Genetics, 1(2), 180-185.

Note: Karafet, T. M., Mendez, F. L., Meilerman, M. B., Underhill, P. A., Zegura, S. L., & Hammer, M. F. (2008). New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. Genome Research, 18(5), 830-838.

Figure 38 shows the correlation of the Probability of Identity (PI) obtained with STR analysis and the PI of the same artificially degraded Raji DNA sample (quantified with the Quantifiler™ Trio DNA Quantification Kit and analyzed with the HID-Ion AmpliSeq™ Identity Panel; PI values obtained from 1000 Genomes **http://www.1000genomes.org/**) obtained with HID-Ion AmpliSeq™ Identity Panel analysis.

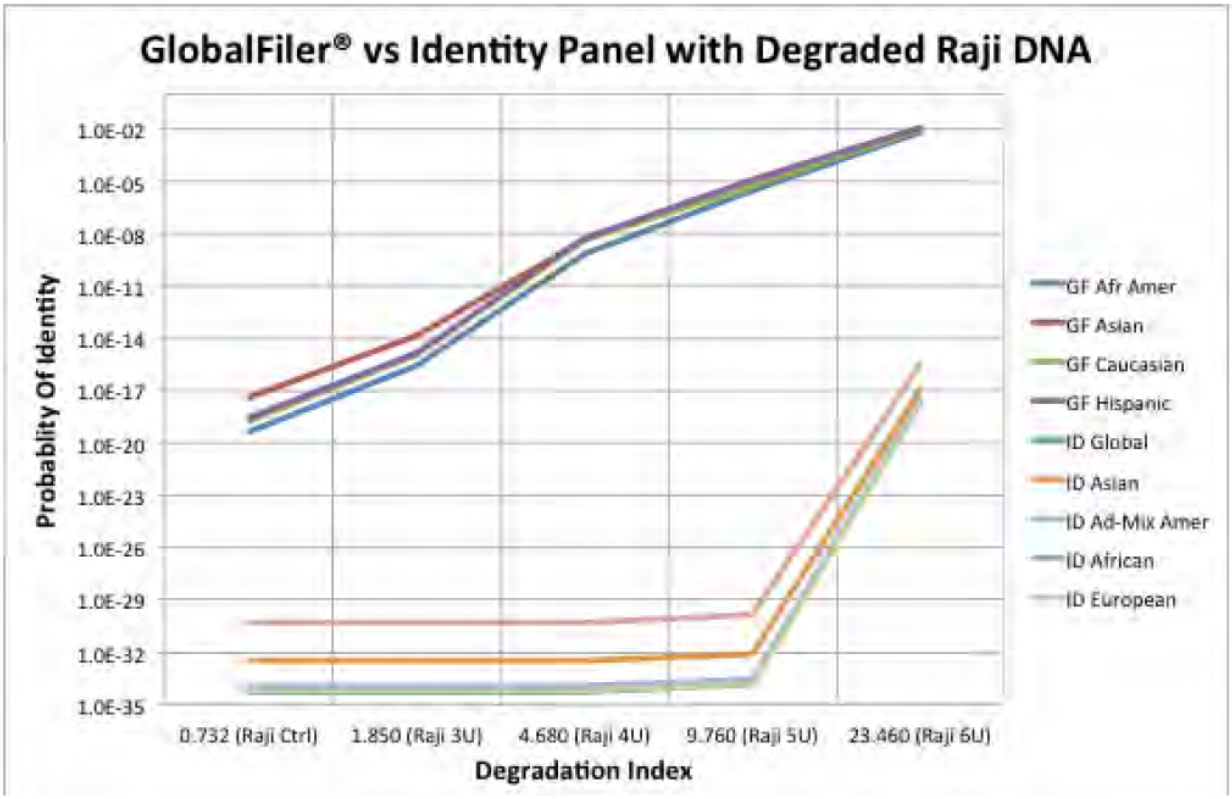


Figure 38 Probability of identity vs degradation index in HID-Ion AmpliSeq™ Identity Panel; results may differ depending on sample type and integrity

Additional Y SNP and ancestry information provided by HID-Ion AmpliSeq™ identity and ancestry panel analysis

Y haplo type information

Figure 39 shows Y haplogroup results for the same degraded Raji DNA.

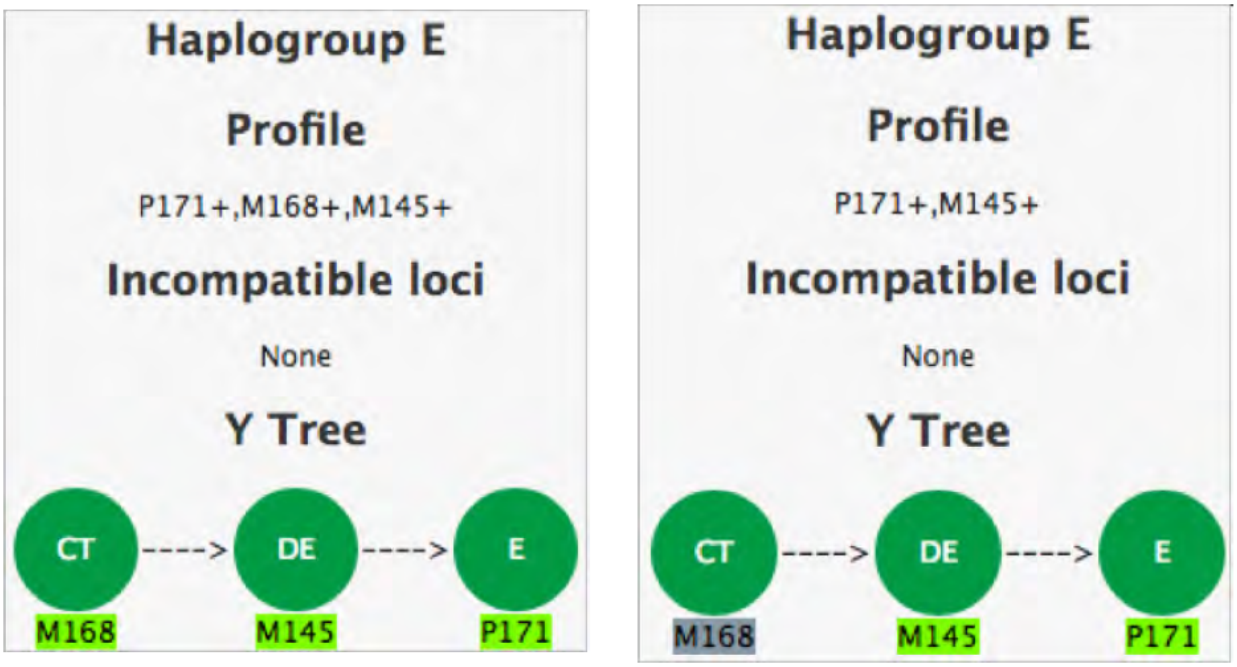


Figure 39 Y haplogroup of raji DNA 0 U no degradation (left) and 6 U degradation (right)

Results shown are for example purposes. Results will vary depending on sample.

Ancestry information

The HID-Ion AmpliSeq™ Ancestry Panel includes 165 autosomal markers chosen by Dr. Kenneth Kidd from Yale University and Michael Seldin from University of California, Davis. The panel provides additional information useful in analyzing degraded samples.

- Kidd et. al. Poster: Better SNPs for Better Forensics: Ancestry, Phenotype, and Family Identification. Shown at National Institute of Justice annual meeting, Arlington VA, June 2012.
- Kosoy R, Nassir R, Tian C, et al. (2009) Ancestry informative marker sets for determining continental origin and admixture proportions in common populations in America. Hum Mutat 30(1) 69-78.

The similarity of the ancestral profiles of the samples are shown in the following figures (see Figure 40 and Figure 41).

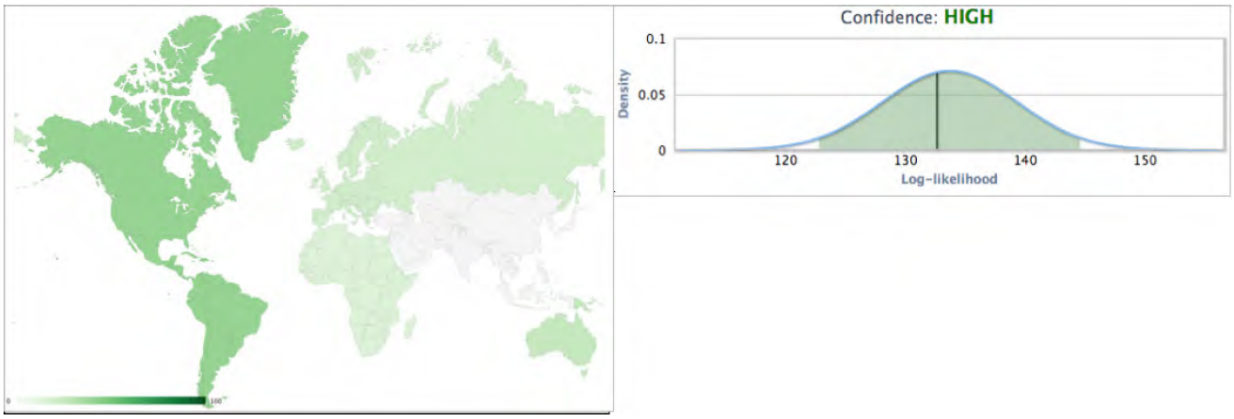


Figure 40 Biogeographical ancestry of PB001 Ctrl

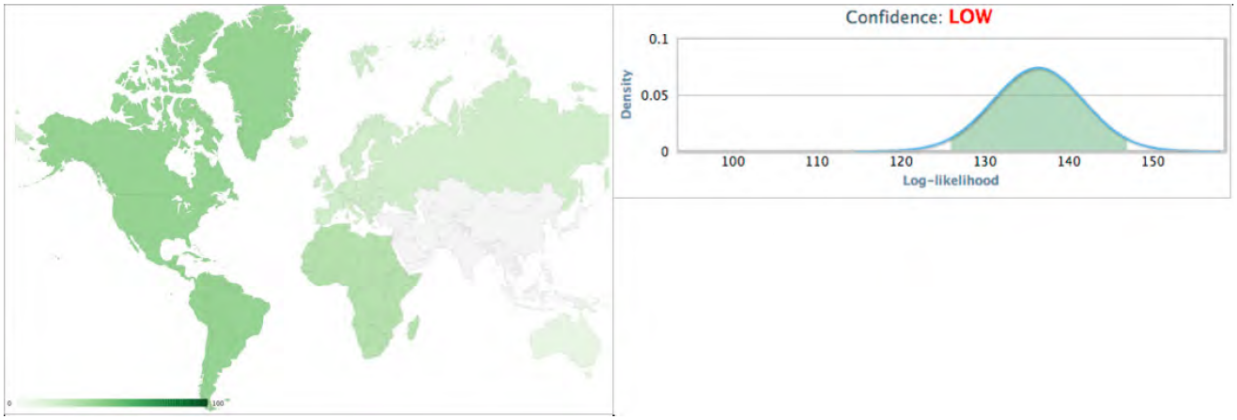


Figure 41 Biogeographical ancestry of PB001 high

Note: Because of the sensitivity and configuration of the panel (no redundant SNPs), the degradation of any critical SNPs may generate a Low Confidence result, even though the ancestry results are similar.

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Related documentation

Document	Publication number
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System: Maintenance Guide	4387777
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve	4347825
QuantStudio™ 5 Real-Time PCR Instrument User Guide (for Human Identification)	MAN0017162
HID Real-Time PCR Analysis Software User Guide	MAN0009819
Quantifiler™ Trio Automated DNA Quantification Kit Product Information Sheet	MAN1000067
Automated DNA Quantification, Normalization, and Amplification Setup User Bulletin	MAN1000064

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  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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