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Investigator® Quantiplex® Pro Handbook for Applied Biosystems 7500 Real-Time PCR Systems

For quantification of human and male DNA in forensic samples

Sample to Insight

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Kit Contents

Investigator® Quantiplex® Pro Kit (200) Catalog no. Number of 20 µL reactions	387216 200
Quantiplex Pro Reaction Mix	1 x 1.9 mL
Quantiplex Pro Primer Mix	1 x 1.9 mL
Male Control DNA M1 (50 ng/µL)	0.2 mL
QuantiTect® Nucleic Acid Dilution Buffer	1 vial
Quick-Start Protocol	1

Shipping and Storage

Kit reagents should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer. After first use, store the kit components at 2–8°C. Avoid freezing the kit components. The QuantiTect Nucleic Acid Dilution Buffer may also be stored at –30 to –15°C, if desired. Quantiplex Pro Primer Mix must be stored protected from the light. DNA samples should be stored separately from PCR reagents. Under these conditions, the components are stable until the expiration date indicated on the kit.

Intended Use

The Investigator Quantiplex Pro Kit is intended for molecular biology applications in forensic, human identity, and paternity testing. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend that all users of QIAGEN® products adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of Investigator Quantiplex Pro Kits is tested against predetermined specifications to ensure consistent product quality. Investigator Quantiplex Pro Kit meets ISO 18385 requirements.

Introduction

Human identification is commonly based on the analysis of short tandem repeats (STRs), single nucleotide polymorphisms (SNPs), or deletion insertion polymorphisms (DIPs), depending on the demands of an examination or on the sample quality. These multiplex assays used for human identification are complex systems that require a defined range of template input.

The Investigator Quantiplex Pro Kit provides quantification of human genomic DNA, male DNA and the integrity of DNA in a sample using quantitative real-time PCR. The kit is designed to confirm whether a sample contains sufficient DNA to enable DNA fingerprinting analysis (such as STR, DIP, or SNP analysis). Furthermore, the kit may help in establishing if a sample contains inhibitors that may interfere with such applications, thus necessitating further sample purification. In addition the DNA degradation system allows for a more precise assessment of the degradation status of the DNA.

The Investigator Quantiplex Pro Kit uses a hot-start DNA polymerase enzyme and QuantiNova Guard additive. These unique components further improve the stringency of the antibody-mediated hot-start.

The kit also features a built-in control for visual identification of correct pipetting and Q-Bond®, an additive in the buffer that enables short cycling steps without loss of PCR sensitivity and efficiency.

Principle and Procedure

The Investigator Quantiplex Pro Kit is a ready-to-use system for the detection of human and male DNA and parallel assessment of DNA degradation using quantitative real-time PCR. The kit provides fast and accurate quantification of human DNA in forensic database and casework samples.

Detection of amplification is performed using TaqMan® probes and a fast PCR chemistry. The dual-labeled probes contain a fluorescent reporter and a quencher at their 5' and 3' ends, respectively. During the extension phase of PCR, the 5' and 3' exonuclease activity of the DNA polymerase cleaves the fluorophore from the quencher. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

Target regions

The target region for human DNA quantification (4NS1C®) is a 91 bp proprietary region present on several autosomes of the human genome. It was selected to give high sensitivity and is detected using the FAM™ dye channel on Applied Biosystems instruments.

Furthermore, the kit detects a longer autosomal amplification product (353 bp) targeting the same locus as the 91 bp 4NS1C autosomal target. Due to the differently sized autosomal targets, the longer autosomal target is more susceptible to DNA degradation, allowing for a precise assessment of the degradation status of the DNA. The larger 353 bp autosomal quantification target region is detected using the ATTO 550 dye channel on Applied Biosystems instruments.

The target region for male DNA quantification was selected in order to give high sensitivity in the presence of mixed female/male DNA samples. It is detected as an 81 bp fragment using the ATTO 647N dye channel on Applied Biosystems instruments.

Internal Control

In addition, the Investigator Quantiplex Pro Kit contains a balanced internal amplification control that is used to test successful amplification and identify the presence of PCR inhibitors. This heterologous amplification system is detected as a 434 bp internal control (IC) in the JOE™ dye channel on Applied Biosystems instruments. The IC is designed to be more sensitive to inhibitors than the human and the male quantification targets. The comparison of the C_T values of the IC system for DNA standards with the C_T values of the IC system for unknown samples may provide an indication of potential inhibition of the reaction in the unknown samples. Therefore, even if the IC system reports the presence of inhibitors in the sample, the DNA quantification will typically provide a reliable result. The presence of inhibitors in the sample may affect the downstream application and must be considered.

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

Table 1. Targets, amplicon lengths, and channels for Investigator Quantiplex Pro Kit

Target	Amplicon length	Channel	Ploidy	Copy number
Human target, small autosomal (Human)	91 bp	FAM	Diploid	Multi-copy
Human target, large autosomal (Degradation)	353 bp	ATTO550/TAMRA	Diploid	Multi-copy
Human male target (Male)	81 bp	ATTO647N/CY5	Diploid	Multi-copy
Internal PCR control	434 bp	JOE	N.A.	Synthetic fragment

Quantiplex Pro Reaction Mix

The Quantiplex Pro Reaction Mix contains a hot start DNA polymerase and Quantiplex Pro reaction buffer. The DNA polymerase is provided in an inactive state and has no enzymatic activity at ambient temperatures. The antibody-mediated hot-start mechanism prevents the formation and extension of nonspecific PCR products and primer–dimers during reaction setup and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and accurate quantification. At low temperatures, the DNA polymerase is kept in an inactive state by the antibody and QuantiNova Guard, which stabilize the complex and improve the stringency of the hot start. After raising the temperature for 2 minutes to 95°C, the antibody and QuantiNova Guard are denatured and the DNA polymerase is activated, enabling PCR amplification. The hot start enables rapid and convenient room-temperature setup.

Furthermore, the Quantiplex Reaction Mix contains the additive Q-Bond, which allows short cycling times on standard cyclers and on fast cyclers with rapid ramping rates. Q-Bond increases the affinity of the DNA polymerase for short, single-stranded DNA, reducing the time required for primer/probe annealing to a few seconds. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times that result in an overall PCR run time of approximately 60 minutes.

The Quantiplex Pro Reaction Mix is also based on the unique QIAGEN PCR buffer system. The buffer contains a balanced combination of KCl and NH_4Cl, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity.

Male Control DNA M1 and standard curve

DNA quantification standards are critical for accurate analysis. We strongly recommend a 27-fold dilution series with 4 concentration points in the standard curve for each assay. The Control DNA contains pooled male DNA at a concentration of 50 ng/µL. To ensure pipetting

accuracy, the minimum input volume of DNA for dilutions should be 5 µL. The standard curve is designed to be easily set up using a convenient 1:27 dilution series. If using QuantiTect Nucleic Acid Dilution Buffer to dilute the Control DNA, the dilutions are stable for at least 1 week at 2–8°C.

Important: Male Control DNA M1 is optimized for use with the Investigator Quantiplex Kits only.

Templates for routine work

In order to streamline the instrument setup and the analysis of the results on the Applied Biosystems 7500 Real-Time PCR System for Human Identification and Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems, QIAGEN has developed a set of template files. Download the template files from the product resources page at **www.qiagen.com/QPpro-template-files**.

Description of protocols

Protocols for 2 different cyclers are provided in this handbook.

- Applied Biosystems 7500 Real-Time PCR System for Human Identification
- Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Equipment

- Cooling device or ice
- Real-time thermal cycler
  - Applied Bioystems 7500 Real-Time PCR System for Human Identification, or
  - Applied Biosystems 7500 or 7500 Fast Real-Time PCR Systems

Material

- Pipettes and pipette tips
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your thermal cycler)

Reagents

- Nuclease-free (RNase/DNase-free) consumables: special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of human DNA

Investigator Quantiplex Pro Calibration Kit (cat. no. 387416)

- Calibration Standard FAM, Calibration Standard JOE, Calibration Standard ATTO 550, Calibration Standard ROX, Calibration Standard ATTO 647N, Quantiplex Pro Calibration Buffer

Important Notes

Selecting kits and protocols

This handbook contains protocols and recommendations for DNA quantification using the instruments listed in Table 2. Real-time cyclers other than those listed here or in HB-3355 Investigator Quantiplex Pro Handbook for QuantStudio 5 Real-Time PCR Systems have not been validated by QIAGEN for DNA quantification using the Investigator Quantiplex Pro Kit.

Table 2. Protocols for the Investigator Quantiplex Pro Kit with different real-time thermal cyclers

Real-time thermal cycler	Protocol
Applied Biosystems 7500 Real-Time PCR System for Human Identification	Page 14
Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems	Page 55

Contamination risks

Do not remove the seal on the reaction plates once the amplification is complete. Removing the plate seal increases the risk of contaminating subsequent reactions with amplified product.

All reaction mixtures should be set up in an area separate from that used for DNA isolation and PCR product analysis (post-PCR) in order to minimize the potential for cross-contamination. In addition, use disposable tips containing hydrophobic filters to minimize cross-contamination.

Controls

No-template control (NTC)

Replicates of NTC reactions should be included in each quantification run in order to detect contamination. NTCs should contain all the components of the reaction, except for the

template. Quantification using the Investigator Quantiplex Pro Kit is highly sensitive; despite the fact that the reagents contained in the Quantiplex Kit undergo strict quality controls to assess that they are free of human DNA contamination, background DNA may be detected in rare cases due to the high assay sensitivity. Take great care to avoid contamination when pipetting the NTC.

We recommend performing NTC reactions at least in duplicate.

Internal positive control

An internal, positive control (detected using a TaqMan probe) is used to test for successful amplification and for the presence of PCR inhibitors. Primers, TaqMan probe and template for the internal control are all contained in the Quantiplex Pro Primer Mix.

Calibration of Applied Biosystems 7500 Real-Time PCR Systems

When using the Applied Biosystems 7500 Real-Time PCR System with Investigator Quantiplex Pro, custom dye calibration for FAM, JOE, ATTO 550, ROX, and ATTO 647N is needed. To achieve optimal performance, calibrate with the Investigator Quantiplex Pro Calibration Kit (cat. no. 387416). However, standard system dye calibrations for FAM, JOE, TAMRA, ROX, and Cy®5 might be used. In this situation, use TAMRA for the Degradation target and Cy5 for the Male target. Refer to the instrument user manual for additional information on correct setup.

Protocol: Cycler Calibration using the Investigator Quantiplex Pro Calibration Kit and Applied Biosystems 7500 Real-Time PCR System for Human Identification

This protocol is optimized for use of the Investigator Quantiplex Pro Calibration Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification using HID Real-Time PCR Analysis Software v1.1 or 1.2.

For general instructions on instrument calibration, refer to the *Applied Biosystems 7500 Real-Time PCR System for Human Identification User Manual*.

Important points before starting

- Set up calibration plate in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use Applied Biosystems 7500 Real-Time PCR System-related consumables (optical 96-well reaction plates and optical adhesive films).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- Repeat dispenser-pipettes can be used to dispense 20 µL into all 96 wells of a plate assigned for one dye.
- For repeat dispensers use disposable, sterile, separately wrapped repeat dispenser tips.
- Always wear a suitable lab coat, disposable gloves, and protective goggles. Take care to minimize touching plate wells, optical adhesive films, and bottoms of the plates.
- We strongly recommend performing the Regions of Interest (ROI) calibration, background calibration and optical calibration before you perform the custom dye calibration with the Investigator Quantiplex Pro Calibration Kit. Further details on how to perform ROI

calibration, background calibration, and optical calibration can be found in the Applied Biosystems 7500 Real-Time PCR Systems *Installation and Maintenance Guide*.

Procedure A: Calibration plate setup

1. Thaw kit components if required. Mix all kit components before use.
2. Vortex each calibration standard for at least 5 s before removing an aliquot.
3. Dilute each Investigator Quantiplex Pro Calibration Kit standard in a separate tube (e.g., a 5 mL reaction tube), as described in Table 3.

Table 3. Dilution scheme for Investigator Quantiplex Pro Calibration Kit standards

Component	Volume (µL)				
Calibration Standard FAM	23	–	–	–	–
Calibration Standard JOE	–	23	–	–	–
Calibration Standard ATTO 550	–	–	23	–	–
Calibration Standard ROX	–	–	–	23	–
Calibration Standard ATTO 647N	–	–	–	–	23
Quantiplex Pro Calibration Buffer	2277	2277	2277	2277	2277
Total volume	2300	2300	2300	2300	2300

4. Mix each calibration standard by vortexing for at least 5 s.
5. Assign and label one separate optical 96-well reaction plate for each Investigator Quantiplex Pro Calibration Kit standard (5 in total).
6. Dispense 20 µL of diluted Investigator Quantiplex Pro Calibration Kit standard FAM into the assigned dye-specific optical 96-well reaction plate.
7. Close the plate with an optical adhesive film.
8. Repeat these steps for each of the other remaining Investigator Quantiplex Pro Calibration Kit standards (JOE, ATTO 550, ROX, and ATTO 647N).
9. Centrifuge plates briefly. Always protect plates from light.

10. After calibration, store the calibration plates at –30 to –15°C in a constant-temperature freezer, protected from light. Calibration plates can be stored and reused for up to 3 months.

Procedure B: Calibration Protocol for Applied Biosystems 7500 Real-Time PCR Systems for Human Identification

1. Open the HID Real-Time PCR Analysis Software v1.1 or 1.2 and select the **Instrument Maintenance Manager** from the instrument menu (Figure 1).

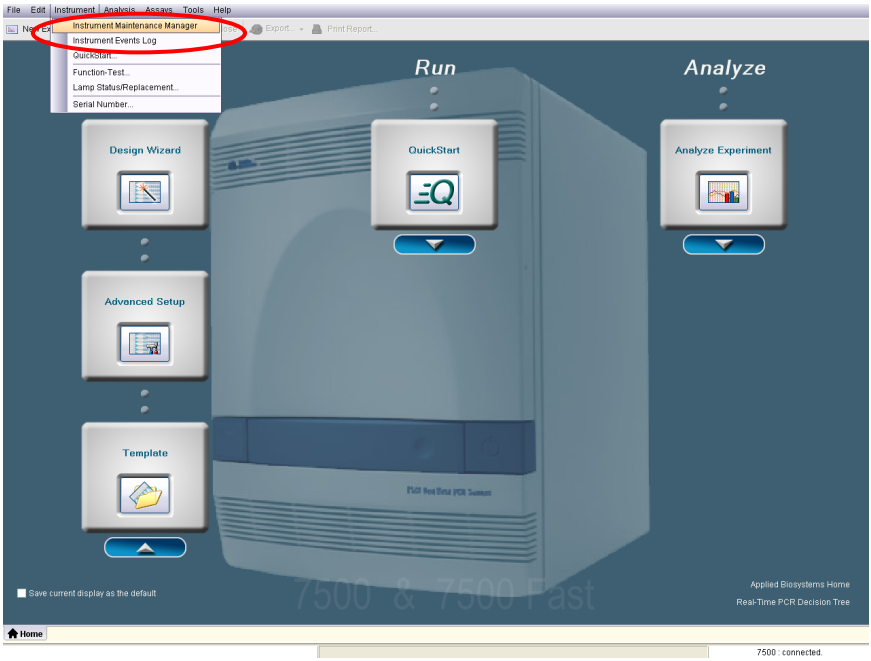


Figure 1. Selecting the Instrument Maintenance Manager.

2. Click the **Dye** section on the left panel (Figure 2).

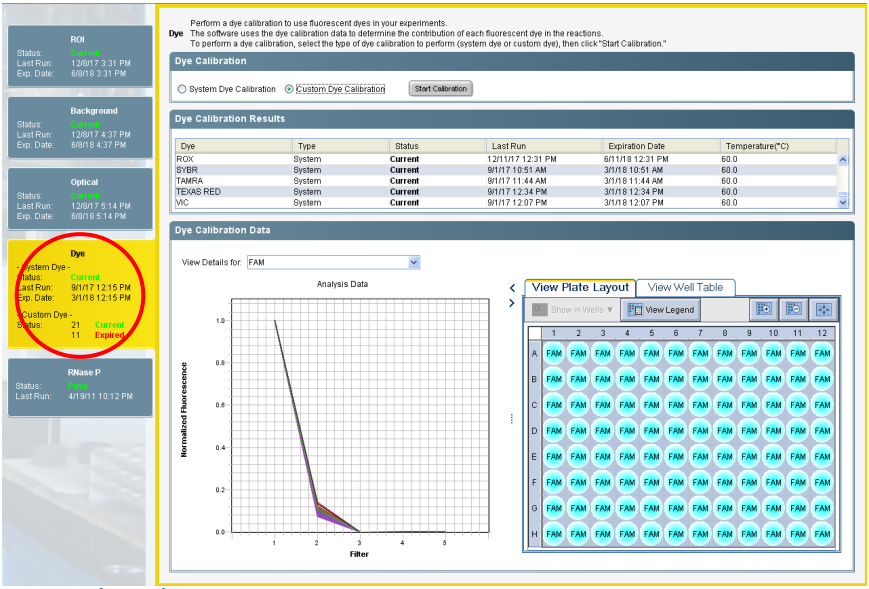


Figure 2. Selecting the Dye section.

3. Select **Custom Dye Calibration** and then click **Start Calibration** (Figure 3).

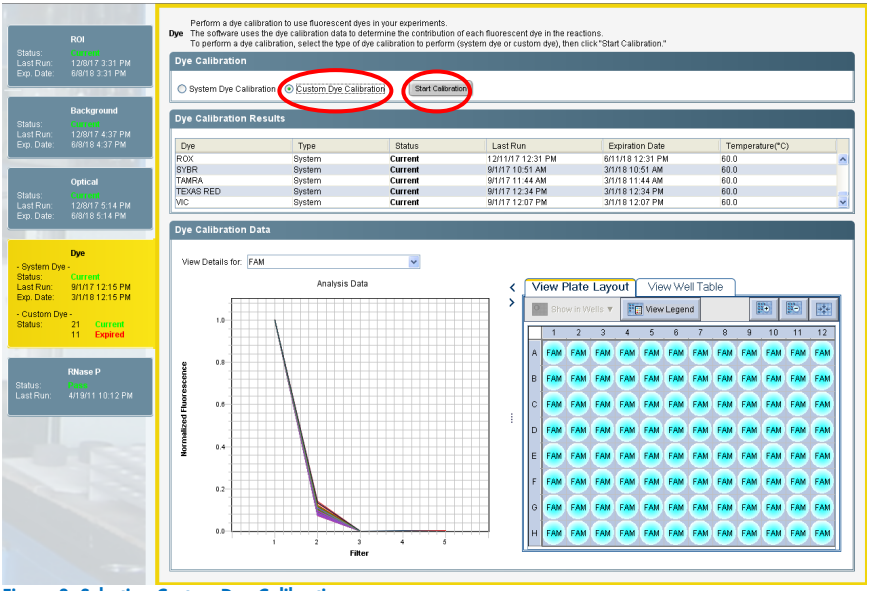


Figure 3. Selecting Custom Dye Calibration.

4. Select **New Dye** in the **Dye Calibration** window (Figure 4).

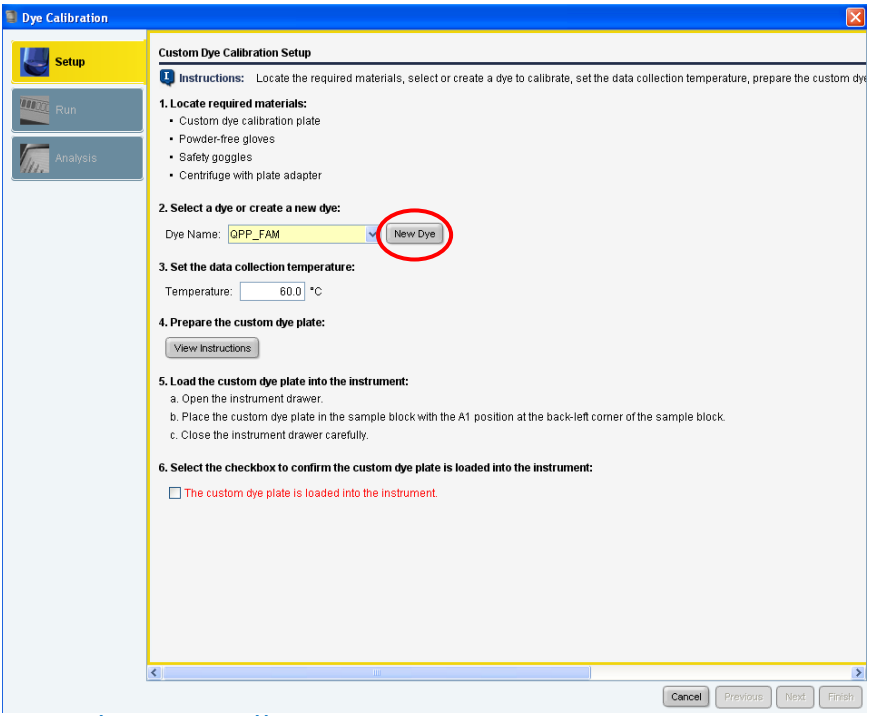


Figure 4. Selecting New Dye calibration.

5. In the **Dye Library** window click **New** (Figure 5).

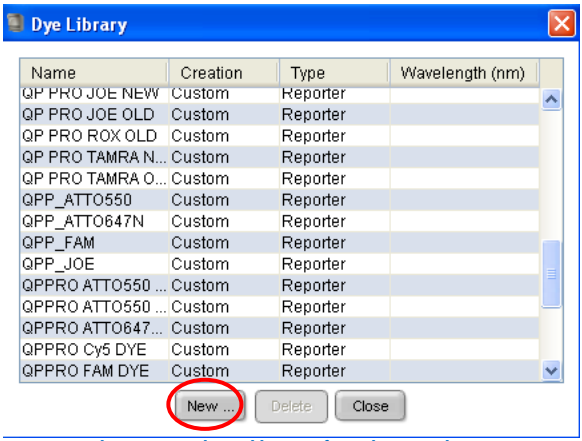


Figure 5. Selecting new dye calibration from the Dye Library.

6. Set names for the new custom dyes. To differentiate the new dyes from standard dyes, we recommend using the prefix QPP (for Quantiplex Pro). Enter **QPP_FAM** as a new dye name, select **Reporter** and click **OK** (Figure 6).

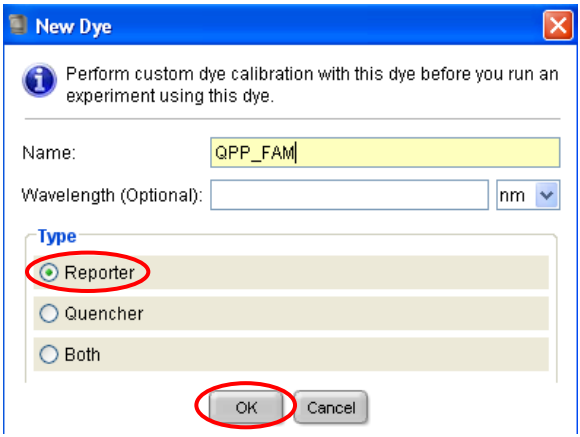


Figure 6. Dye naming.

7. Repeat step 6 to set new dye names for:
   - QPP_JOE
   - QPP_ATTO550
   - QPP_ATTO647N

8. Select **QPP_FAM** in the **Custom Dye Calibration Setup** window. Load the QPP_FAM plate prepared in Procedure A into the instrument. Confirm by checking the box for **The custom dye plate is loaded into the instrument** (Figure 7).

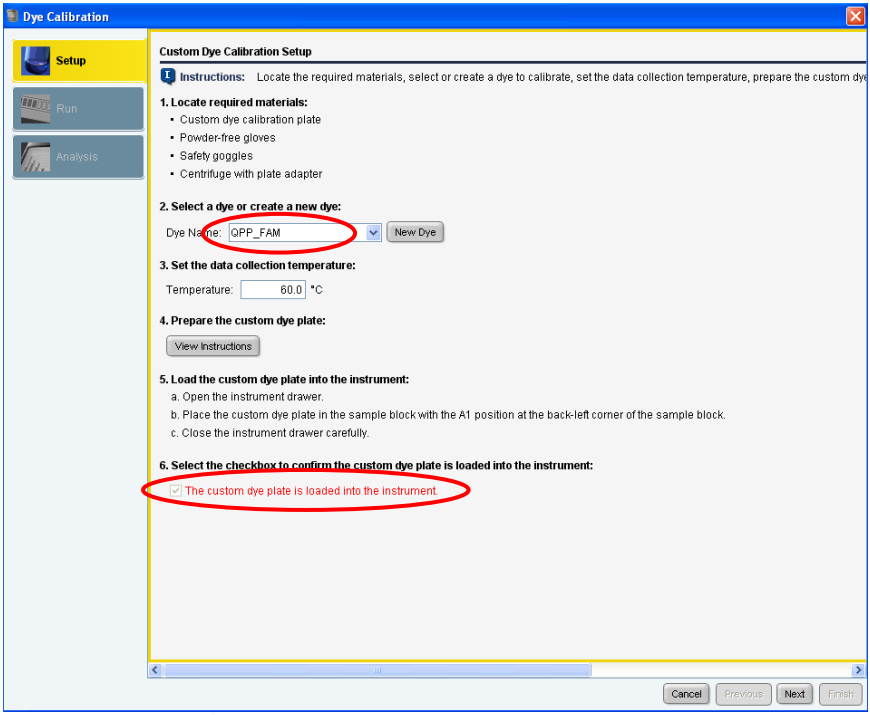


Figure 7. Selecting a dye from the drop down menu in the Custom Dye Calibration Setup.

9. Click **Next** and then **Start Run** (Figure 8).

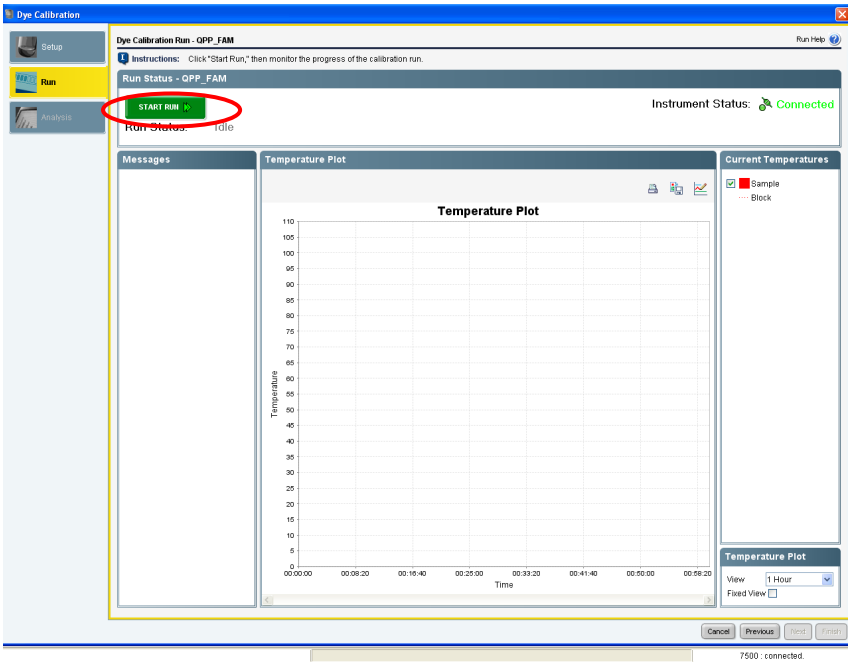


Figure 8. Starting calibration run.

10. After the calibration run has finished, click **Next** (Figure 9).

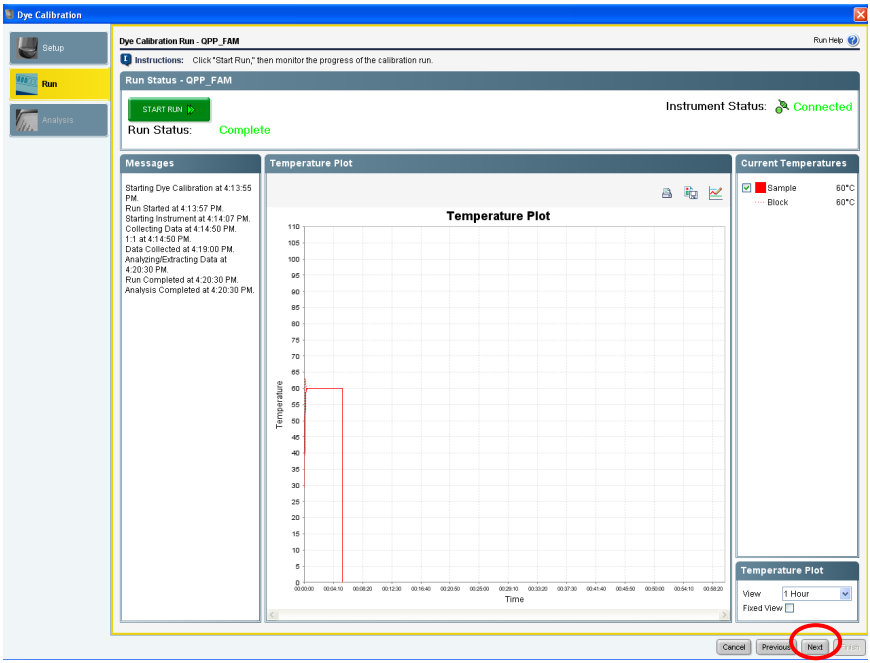


Figure 9. Completed calibration run.

11. The spectra and the status (e.g., passed) will be displayed (Figure 10). Click **Finish**.

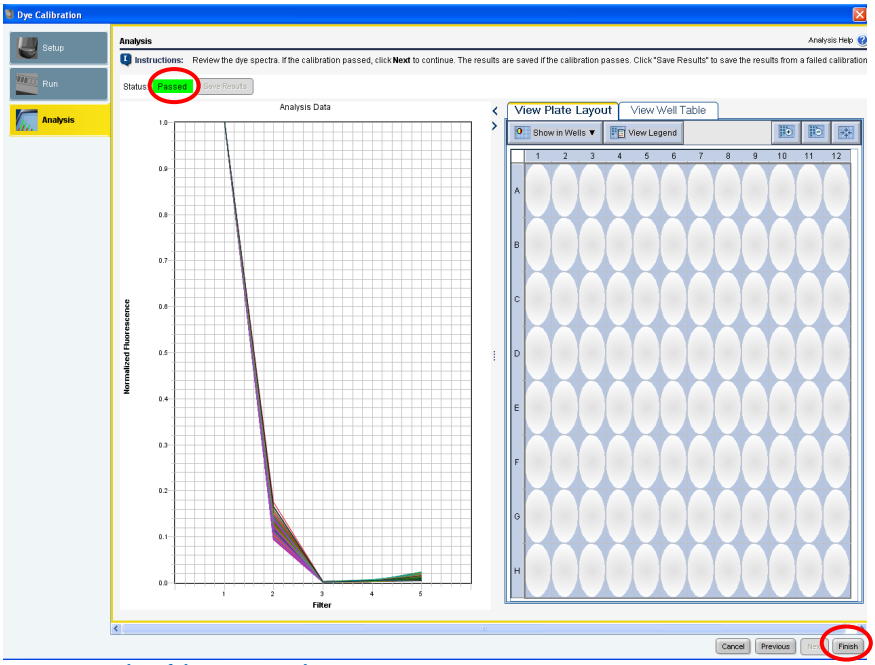


Figure 10. Display of dye spectra and status.

12. Repeat steps 8 to 11 to calibrate for the following dyes:

- QPP_JOE
- QPP_ATTO550
- QPP_ATTO647N

13. To calibrate ROX, open the **Dye** section and select **System Dye Calibration**. Click the **Start Calibration** button (Figure 11).

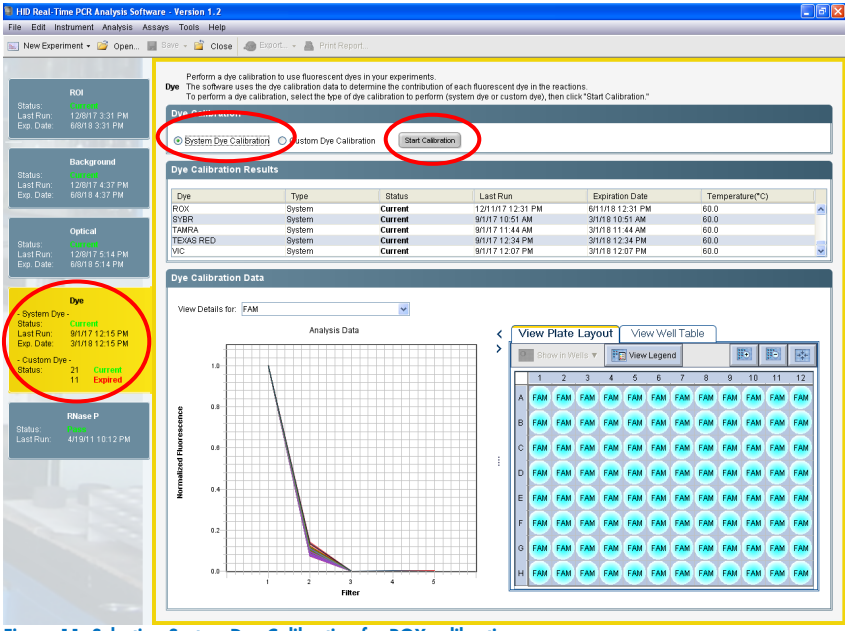


Figure 11. Selecting System Dye Calibration for ROX calibration.

14. In the **Overview** window click **Next**.

15. In the **Materials Required** window select only **ROX** and click **Next** (Figure 12).

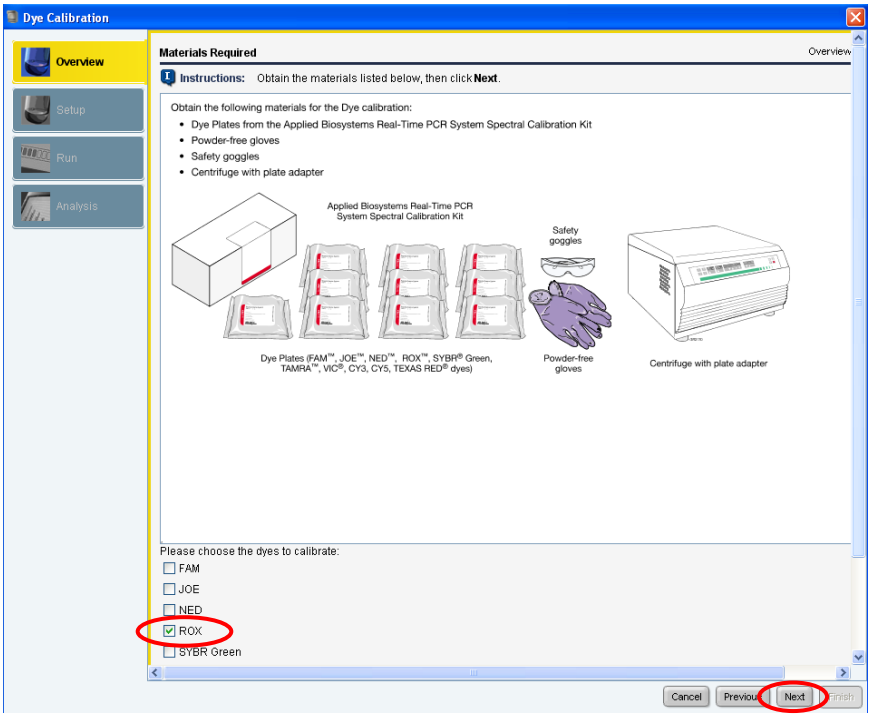


Figure 12. Selecting ROX dye to calibrate.

16. In the **Preparing the Plate** window, click **Next**.
17. The **Loading the Plate** window will open. Click **Next** and load the plate for Calibration Standard ROX into the instrument.
18. Click **Start Run**.
19. After the calibration run has finished, click the **Next** button in the right corner. The spectrum and status of ROX (e.g., passed) will be displayed.
20. Click **Next** and then click **Finish Calibrating ROX**.

21. Confirm that the calibrated dyes provide the highest signals in the filter as shown in Table 4.

Table 4. Calibrated Dye Filter Signal

Custom dye	Highest signal in filter
QPP_FAM	1
QPP_JOE	2
QPP_ATTO550	3
ROX	4
QPP_ATTO647N	5

Protocol: Cycler Calibration using the Investigator Quantiplex Pro Calibration Kit and Applied Biosystems 7500 Real-Time PCR System with 7500 Software v2.0.6

This protocol is optimized for use of the Investigator Quantiplex Pro Calibration Kit on the Applied Biosystems 7500 Real-Time PCR System with 7500 Software v2.0.6.

For general instructions on instrument calibration, refer to the *Applied Biosystems 7500 Real-Time PCR System for Human Identification User Manual*.

Important points before starting

- Set up calibration plate in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use Applied Biosystems 7500 Real-Time PCR System-related consumables (optical 96-well reaction plates and optical adhesive films).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- Repeat dispenser-pipettes can be used to dispense 20 µL into all 96 wells of a plate assigned for one dye.
- For repeat dispensers use disposable, sterile, separately wrapped repeat dispenser tips.
- Always wear a suitable lab coat, disposable gloves and protective goggles. Take care to minimize touching plate wells, optical adhesive films and bottoms of the plates.
- We strongly recommend performing the Regions of Interest (ROI) calibration, background calibration and optical calibration before you perform the custom dye calibration with the Investigator Quantiplex Pro Calibration Kit. Further details on how to perform ROI

calibration, background calibration and optical calibration can be found in the Applied Biosystems 7500 Real-Time PCR Systems *Installation and Maintenance Guide*.

Procedure A: Calibration plate setup

1. Thaw kit components if required. Mix all kit components before use.
2. Vortex each calibration standard for at least 5 s before removing an aliquot.
3. Dilute each Investigator Quantiplex Pro Calibration Kit standard in a separate tube (e.g., a 5 mL reaction tube), as described in Table 5.

Table 5. Dilution scheme for Investigator Quantiplex Pro Calibration Kit standards

Component	Volume (µL)				
Calibration Standard FAM	23	–	–	–	–
Calibration Standard JOE	–	23	–	–	–
Calibration Standard ATTO 550	–	–	23	–	–
Calibration Standard ROX	–	–	–	23	–
Calibration Standard ATTO 647N	–	–	–	–	23
Quantiplex Pro Calibration Buffer	2277	2277	2277	2277	2277
Total volume	2300	2300	2300	2300	2300

4. Mix each calibration standard by vortexing for at least 5 s.
5. Assign and label one separate optical 96-well reaction plate for each Investigator Quantiplex Pro Calibration Kit standard (5 in total).
6. Dispense 20 µL of diluted Investigator Quantiplex Pro Calibration Kit standard FAM into the assigned dye-specific optical 96-well reaction plate.
7. Close the plate with an optical adhesive film.
8. Repeat these steps for each of the other remaining Investigator Quantiplex Pro Calibration Kit standards (JOE, ATTO 550, ROX, and ATTO 647N).
9. Centrifuge plates briefly. Always protect plates from light.

10. After calibration, store the calibration plates at –30 to –15°C in a constant-temperature freezer, protected from light. Calibration plates can be stored and reused for up to 3 months.

Procedure B: Calibration Protocol for Applied Biosystems 7500 Real-Time PCR Systems with 7500 Software v2.0.6.

1. Open the 7500 Software v2.0.6 and select the **Instrument Maintenance Manager** from the instrument menu (Figure 13).

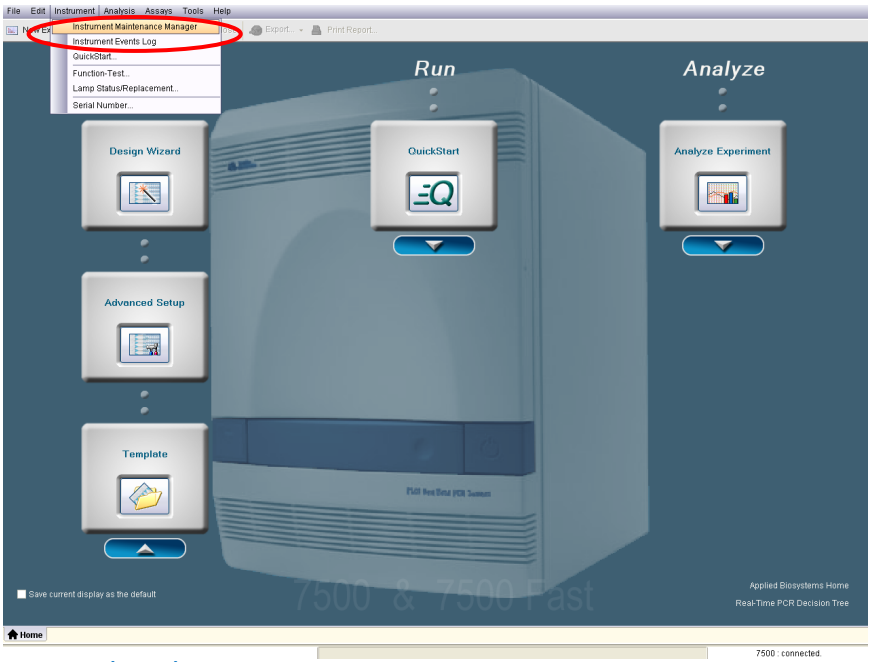


Figure 13. Selecting the Instrument Maintenance Manager.

2. Click the **Dye** section in the left panel (Figure 14).

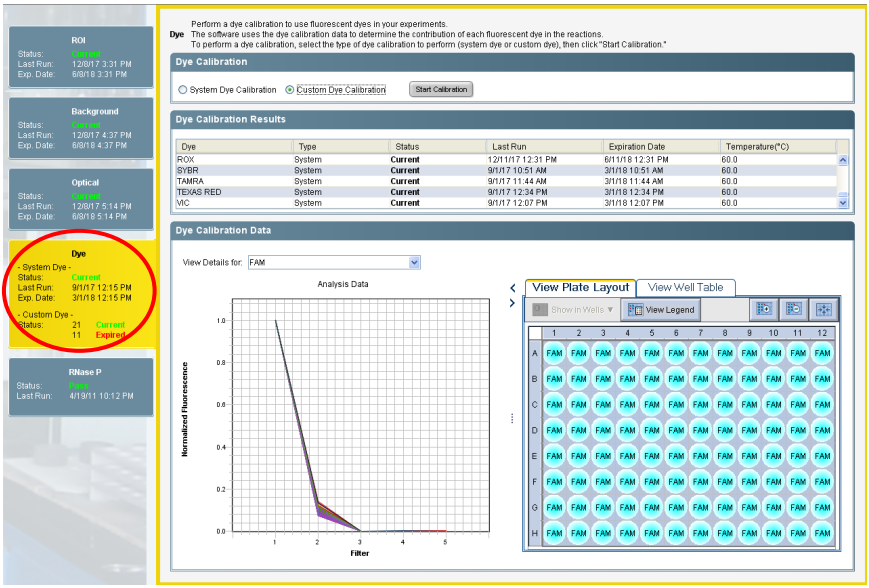


Figure 14. Selecting the Dye section.

3. Select **Custom Dye Calibration** and then click **Start Calibration** (Figure 15).

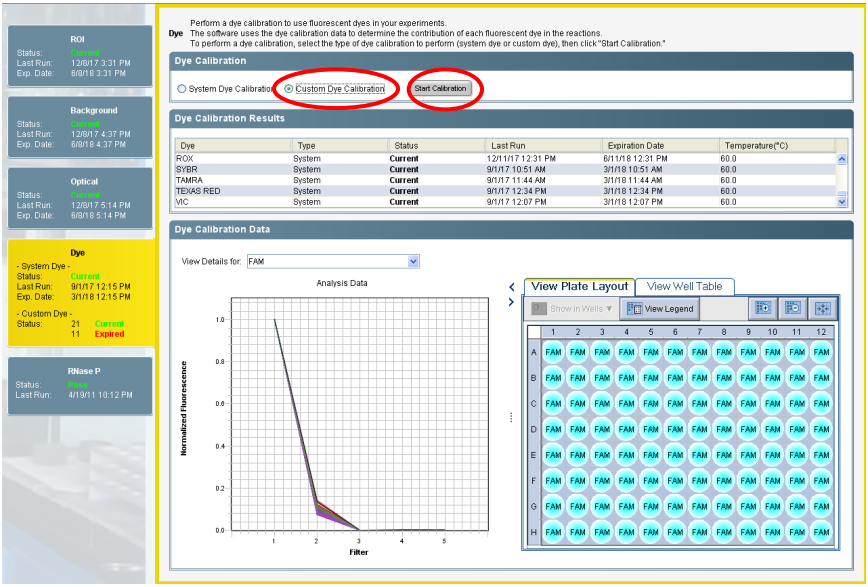


Figure 15. Selecting Custom Dye Calibration.

4. Select **New Dye** in the **Dye Calibration** window (Figure 16).

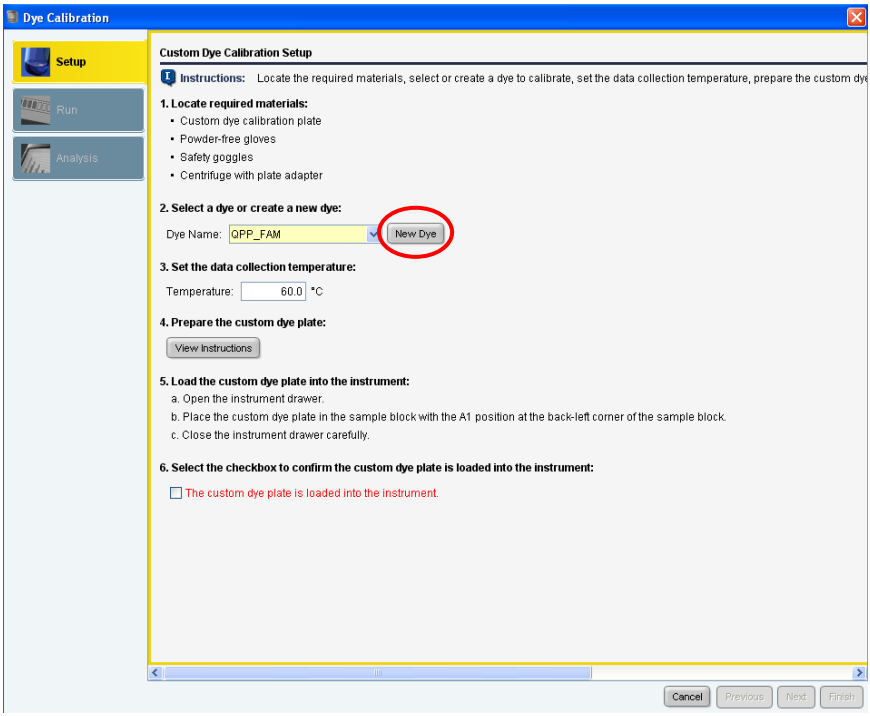


Figure 16. Selecting New Dye calibration.

5. In the **Dye Library** window click **New** (Figure 17).

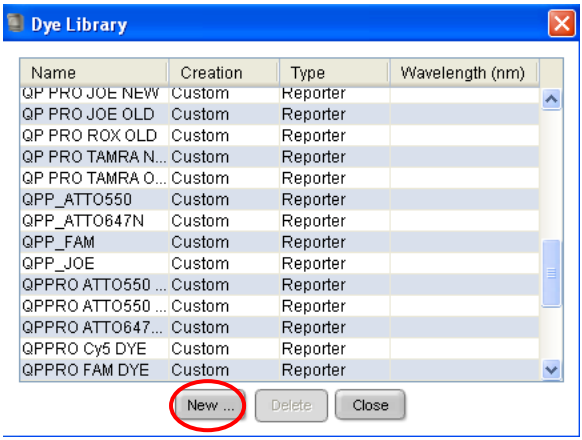


Figure 17. Selecting new dye calibration from the Dye Library.

6. Set names for the new custom dyes. To differentiate the new dyes from standard dyes, we recommend using the prefix QPP (for Quantiplex Pro). Enter **QPP_FAM** as a new dye name, select **Reporter,** and click **OK** (Figure 18).

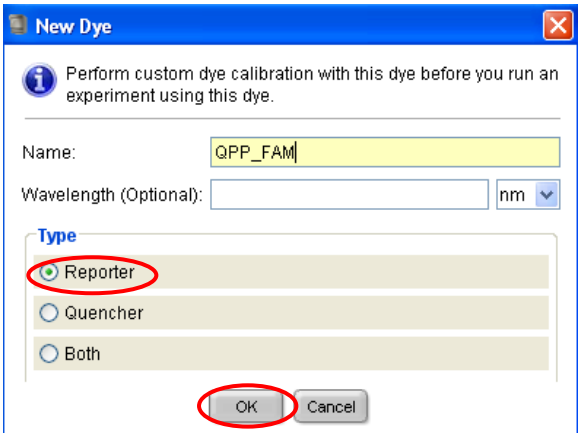


Figure 18. Dye naming.

7. Repeat step 6 to set new dye names for:
   - QPP_JOE
   - QPP_ATTO550
   - QPP_ROX
   - QPP_ATTO647N

8. Select **QPP_FAM** in the **Custom Dye Calibration Setup** window. Load the QPP_FAM plate prepared in Procedure A into the instrument. Confirm by checking the box for **The custom dye plate is loaded into the instrument** (Figure 19).

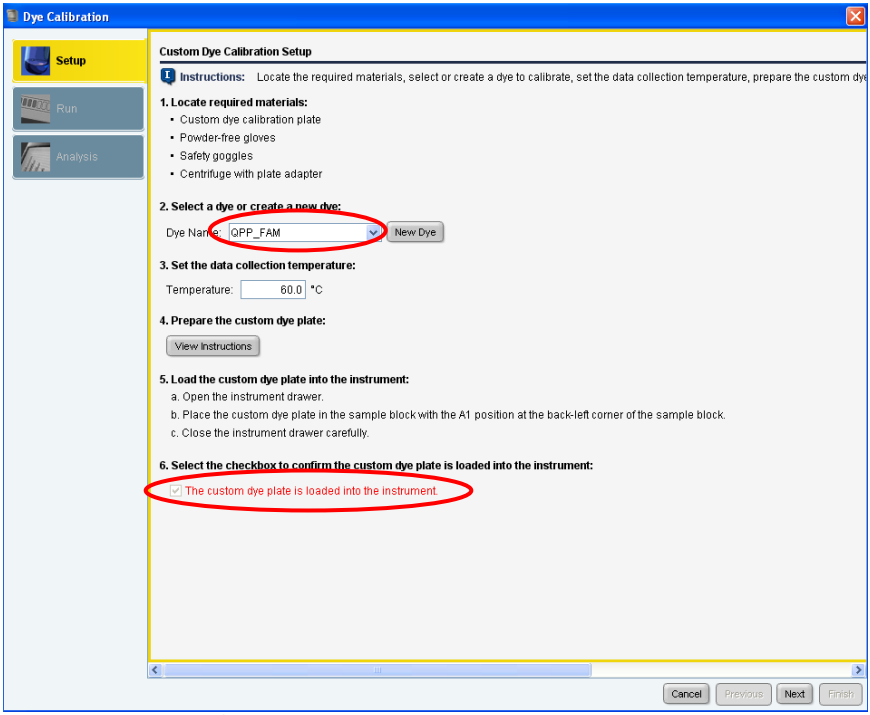


Figure 19. Selecting a dye from the drop down menu in the Custom Dye Calibration Setup.

9. Click **Next** and then **Start Run** (Figure 20).

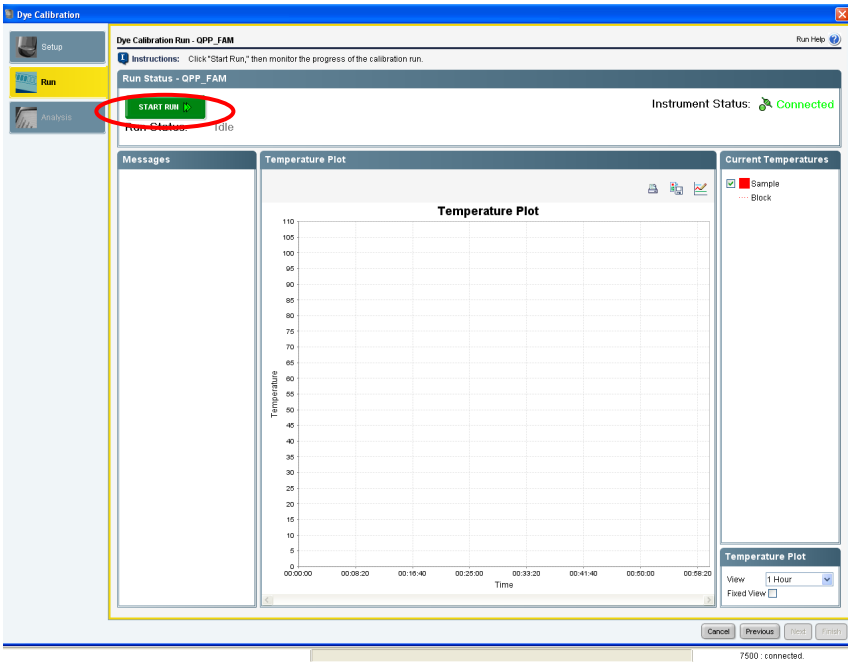


Figure 20. Starting calibration run.

10. After the calibration run has finished, click **Next** (Figure 21).

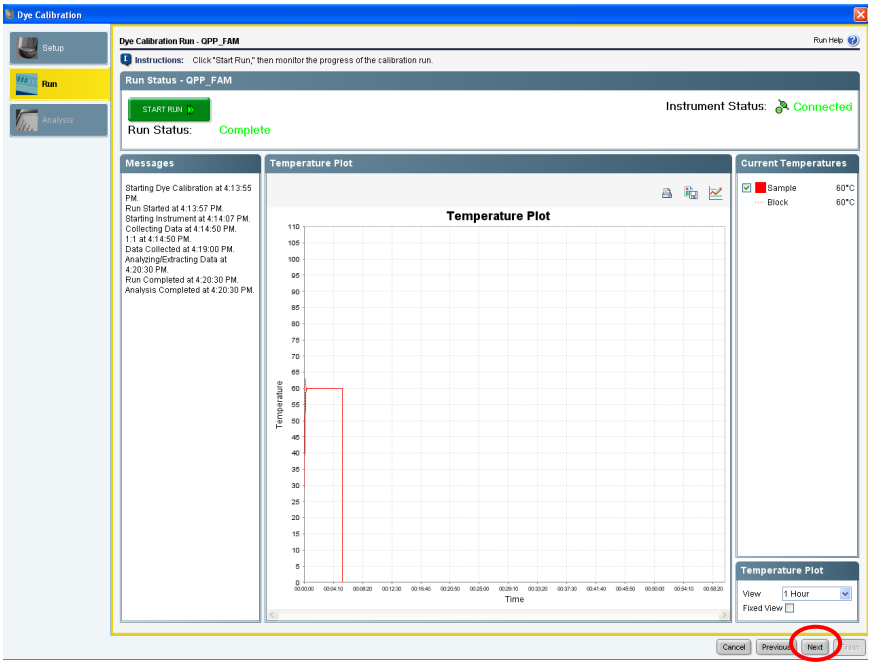


Figure 21. Completed calibration run.

11. The spectra and the status (e.g., passed) will be displayed (Figure 22). Click **Finish**.

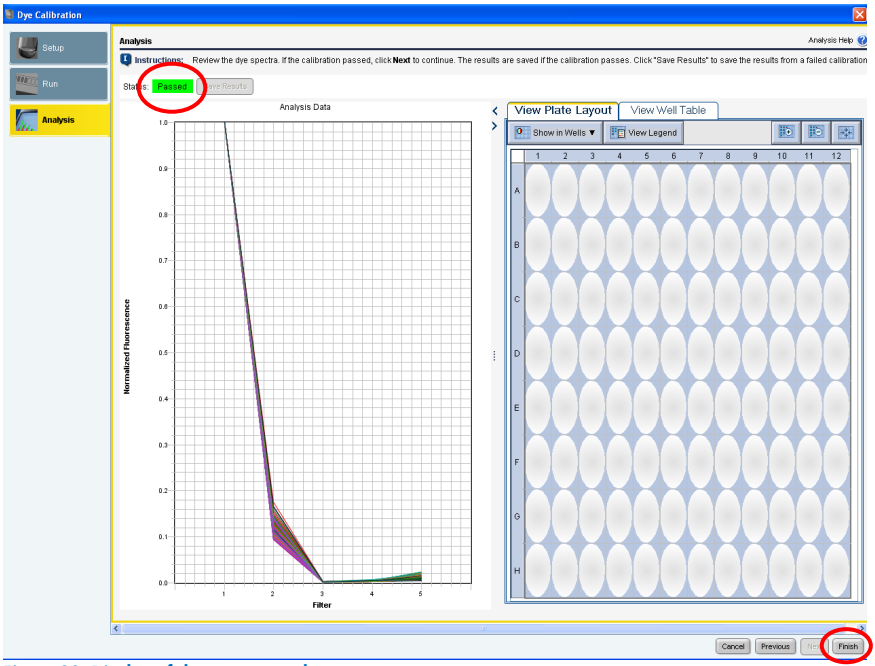


Figure 22. Display of dye spectra and status.

12. Repeat steps 8 to 11 to calibrate for the following dyes:

- QPP_JOE
- QPP_ATTO550
- QPP_ROX
- QPP_ATTO647N

13. Confirm that the calibrated dyes provide the highest signals in the filter as shown in Table 6.

Table 6. Calibrated Dye Filter Signal

Custom dye	Highest signal in filter
QPP_FAM	1
QPP_JOE	2
QPP_ATTO550	3
QPP_ROX	4
QPP_ATTO647N	5

Protocol: Quantification of DNA Using the Applied Biosystems 7500 Real-Time PCR System for Human Identification

This protocol is optimized for use of the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System for Human Identification using HID Real-Time PCR Analysis Software v1.1 or v1.2 or 7500 Software v2.0.6.

For general instructions on instrument setup and other software versions, refer to the *Applied Biosystems 7500 Real-Time PCR System for Human Identification User Manual*.

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- When using the Applied Biosystems 7500 Real-Time PCR System for Human Identification with Investigator Quantiplex Pro, custom dye calibration for FAM, JOE, ATTO 550, ROX, and ATTO 647N is strongly recommended. To achieve optimal performance, calibrate with the Investigator Quantiplex Pro Calibration Kit (cat. no. 387416).
- Use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Use the template volume specified in the protocol. The reaction is optimized for use with 2 µL template DNA. Do not use more or less than 2 µL per 20 µL reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored between 2°C and 8°C for at least 1 week.

- Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure A: PCR

1. Mix all solutions thoroughly before use to avoid localized concentrations of salt.
2. Prepare fresh serial dilutions of the Male Control DNA M1 according to Table 7. Vortex for at least 5 s, and centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipette tip for each dilution. Make sure not to introduce cross-contamination.

Table 7. Serial dilutions of Male Control DNA M1

Serial dilution of Control DNA (ng/µL)	Control DNA (µL)	QuantiTect Nucleic Acid Dilution Buffer (µL)
50	Undiluted DNA	–
1.8519	5	130
0.0686	5	130
0.0025	5	130

Note: Alternative standard curves are listed in "Appendix: Alternative Standard Curves", page 84.

3. Thaw the template nucleic acids.

   Mix all solutions thoroughly before use to avoid localized concentrations of salt.

4. Prepare a master mix according to Table 8 (next page).

   The master mix contains all of the components needed for PCR except the template (sample) DNA and nuclease-free water.

   Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. This should include positive and negative control reactions.

Reaction setup can usually be done at room temperature (15–25°C). However, we recommend keeping the reagents, samples, and controls on ice or in a cooling device.

Table 8. Master mix for DNA quantification

Component	Volume per 20 µL reaction	Final concentration
Quantiplex Pro Reaction Mix	9 µL	1x
Quantiplex Pro Primer Mix	9 µL	1x
Total volume of master mix	18 µL	–

5. Mix the master mix thoroughly, and dispense 18 µL into the wells of a PCR plate.
6. Add 2 µL QuantiTect Nucleic Acid Dilution Buffer to the NTC wells.

   Make sure that the NTC wells do not come in contact with human DNA.
7. Add 2 µL control DNA dilutions or 2 µL unknown sample DNA to the individual wells and mix thoroughly. Close the plate.

   Mix carefully in order to avoid localized concentrations of salt.

   Table 9 (next page) shows a possible plate setup. Make sure that the master mix and template are thoroughly mixed.

   It is required to run duplicates of the control DNA dilutions for each assay and on each reaction plate.

Table 9. Possible plate setup of reactions on the Applied Biosystems 7500 Real-Time PCR System for Human Identification

Well contents												
	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	1.8519	1.8519	0.0686	0.0686	0.0025	0.0025	NTC	NTC	UNK	UNK
B	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
F	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
G	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

All amounts in ng/µL. **UNK**: Unknown sample.

8. Open the HID Real-Time PCR Analysis Software v1.1 or v1.2 in the Custom Assays mode.

9. If you are using a template file, then select **New Experiment/From Template** (Figure 23, next page) and proceed to step 13, page 47, to assign the Targets to the Plate Layout. Then proceed to step 17, page 49, to save and start the run.
   If you are not using a template file, proceed with step 10.

   The template file loads all of the settings needed to start an Investigator Quantiplex Pro run, including the standard curve settings, the cycling profile, and the targets needed for fluorescence acquisition. Download the template files from the product resources page at **www.qiagen.com/QPpro-template-files**.

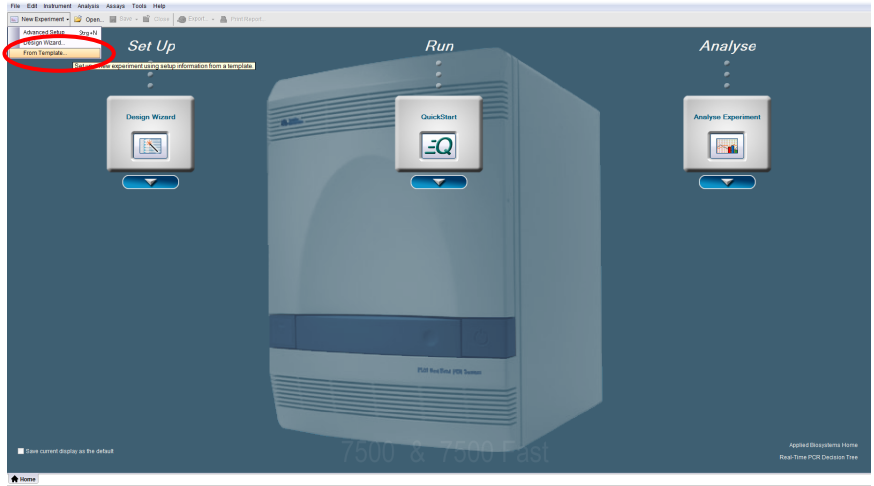


Figure 23. Starting a new experiment from a template.

10. If you are not using a template file, select **Advanced Setup** by clicking the arrow below **Design Wizard** (Figures 24 and 25).

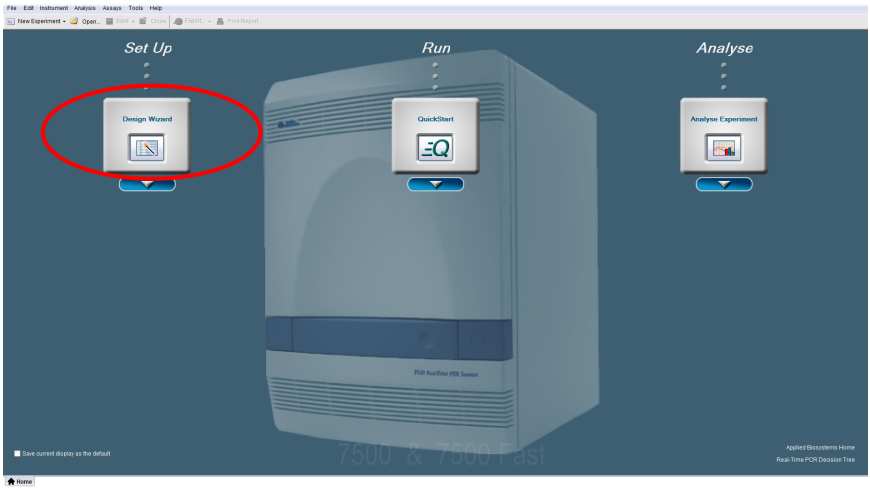


Figure 24. Starting a new experiment.

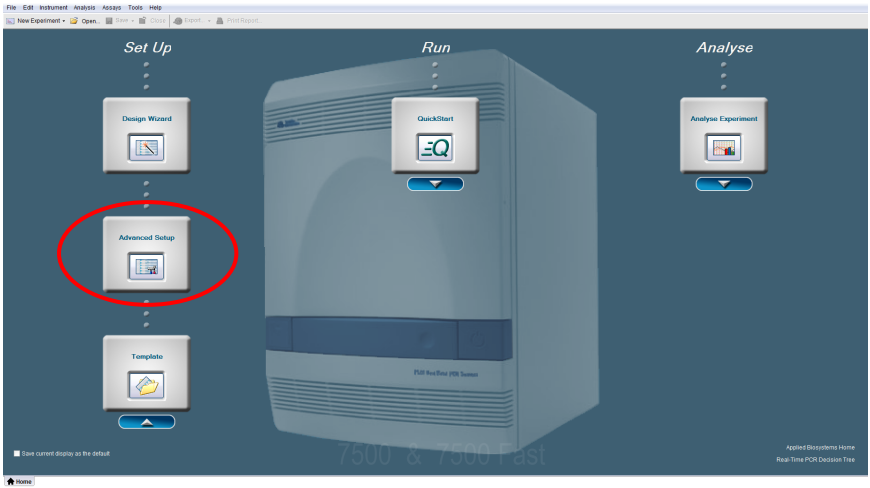


Figure 25. Starting a new experiment using Advanced Setup.

11. Once the new window opens, enter a new **Experiment Name** in the appropriate field. Select the following settings (Figure 26):

- Instrument: 7500 (96 Wells)
- Experiment Type: Quantitation – Standard curve
- Reagents: TaqMan Reagents
- Ramp Speed: Standard
- Unselect the **Include Melt Curve** option

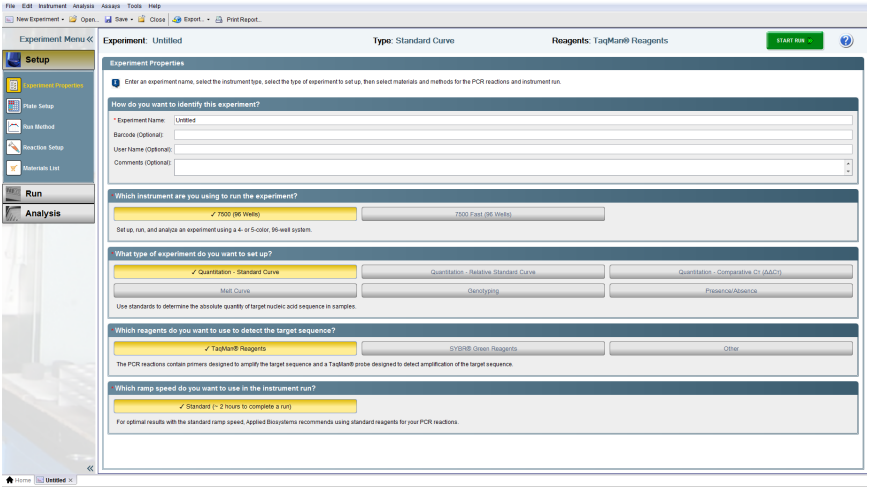


Figure 26. Selecting the correct Experiment Properties settings.

12. Click **Plate Setup** and define four Targets by clicking twice on **Add New Target**. Select the following settings (Figure 27).

- **Human**, Reporter: **QPP_FAM**, Quencher: **None**
- **IC**, Reporter: **QPP_JOE**, Quencher: **None**
- **Degradation**, Reporter: **QPP_ATTO550**, Quencher: **None**
- **Male**, Reporter: **QPP_ATTO647N**, Quencher: **None**

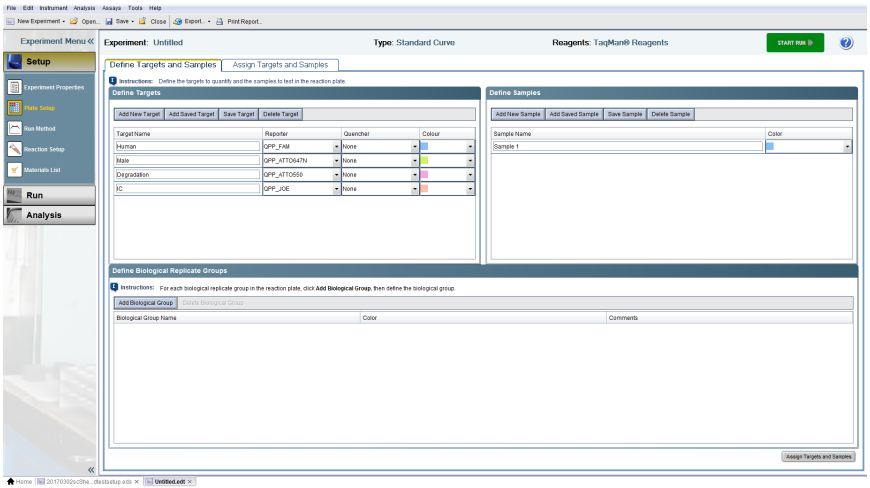


Figure 27. Selecting the correct Target settings.

13. Define the Sample names and also the names for the Standards (e.g., Standard 1 or Std1, Standard 2 or Std2, etc.) using the **Define Samples** tool on the right panel.

    Naming of Standards is required for proper subsequent analysis with the QIAGEN Quantification Assay Data Handling Tool.

    Important: If replicates are needed they should be assigned before you proceed to the next step. Define replicates by using the same sample name or in the **Define Biological Replicate Groups** panel.

    If you are using a template file, proceed to step 17, page 49, to save and start the run.

14. Switch to the tab **Assign Targets and Samples**. In the **Plate Layout**, select the wells in use and assign all four Targets by checking the boxes (Figure 28).

    Important: Do not highlight the wells that are not in use (i.e., those without reaction mix). Including unused wells will significantly impact the scale of the X and Y axes when viewing the data.

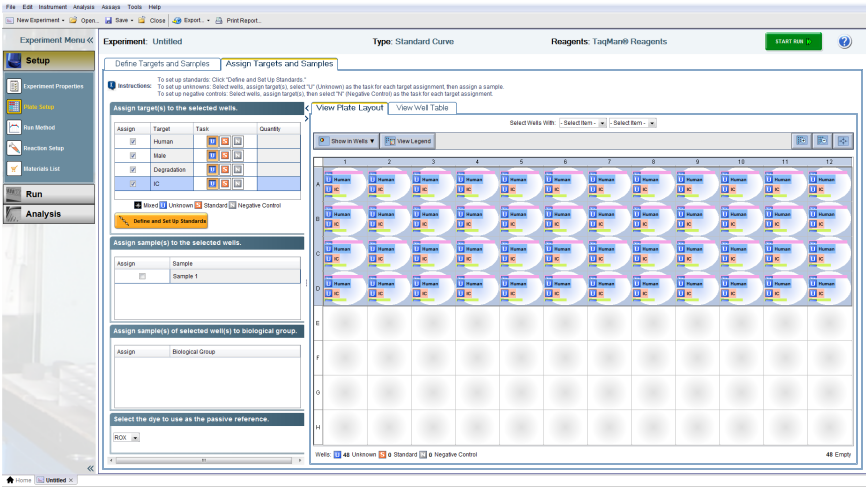


Figure 28. Assigning the Targets.

15. Select the wells for the no-template controls (NTC) and flag them as **Negative Control** using the gray **N** button.

    Note: Leave the IC (QPP_JOE) Task for NTC reactions set to **Unknown**. Enter the sample name.

16. Select the wells for the standard curve and flag them as **Standard** using the orange **S** button. Select **Quantity** for the appropriate detector and enter the quantity of DNA in the well according to Table 7, page 41.

    Important: Although units are not entered for **Quantity**, a common unit must be used for all standard quantities (e.g., ng/µL). The units used for standard quantities define the quantification units for analysis of results.

Note: Leave the IC (QPP_JOE) Task for standard reactions set to **Unknown**.

17. Assign the samples to the plate layout by clicking on the wells and checking the appropriate box on the left panel (Figure 29).

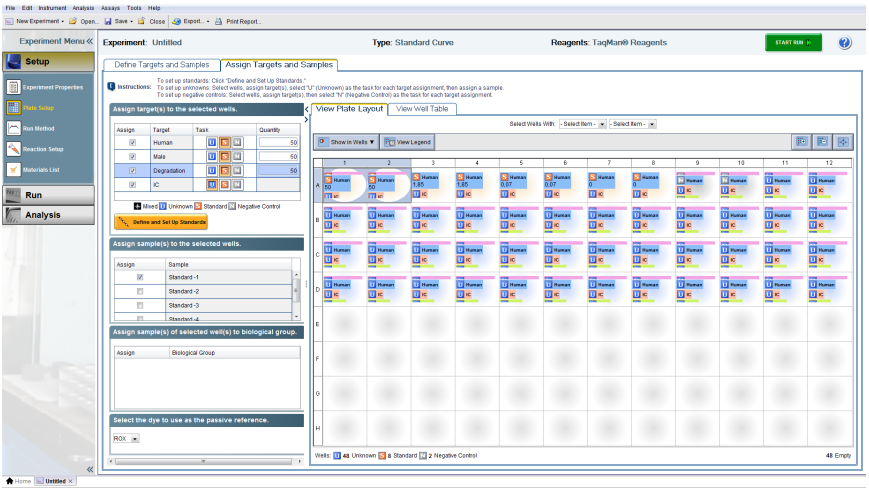


Figure 29. Entering the sample name and assigning the samples.

18. Click **Run Method**. Program the cycler according to Table 10 (next page).

Table 10. Cycling protocol using an Applied Biosystems 7500 Real Time PCR System for Human Identification

Step	Temperature	Time	Number of cycles	Comment
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C to activate the DNA polymerase
Denaturation	95°C	5 s	40	
Combined annealing/extension	60°C	35 s		Perform fluorescence data collection

19. On the thermal profile, change the holding times to those in Table 10. Change the Sample volume to **20 µL** (Figure 30).

    Data acquisition should be performed during the combined annealing/extension step.

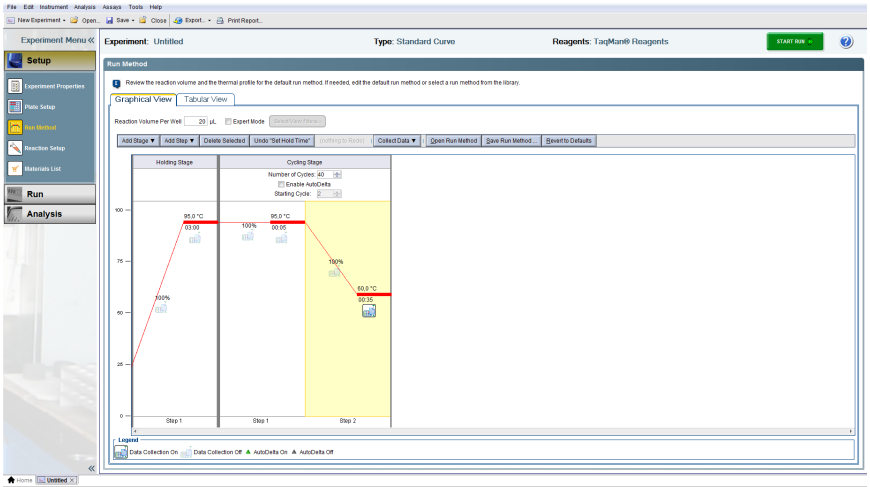


Figure 30. Adjusting the thermal profile (HID Real-Time PCR Analysis Software v1.2).

20. Before running the reaction plate, save the plate document as an EDS Document (***.eds**) file. Click **File** and then **Save**. Enter a name for the plate document, then click **Save** again.

21. Load the plate into the instrument.

    Ensure that position A1 on the plate is on the top-left side of the tray.

22. Start the reaction by clicking **Start**.

Procedure B: Data analysis

Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

1. Open the run file using the HID Real-Time PCR Analysis Software v1.1 or v1.2. First you must open the software in the Custom Assays Mode. Then go to **Open** and then **Browse** to locate the saved file.
2. Standards must first be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 4, below.
3. Go to **Setup** and select **Plate Setup**. Define the wells that contain DNA standards as explained in step 14, page 48.

   Important: Although units are not entered for **Quantity**, a common unit must be used for all standard quantities (e.g., ng/µL). The units used for standard quantities define the quantification units for the analysis of results.

   Note: Leave the IC (QPP_JOE) Task for standard reactions set to **Unknown**. Enter the sample name (e.g., Standard 50 ng/µL).
4. In the **Amplification Plot** tab (found in the **Analysis** tab), select the appropriate samples in the table below the amplification plot. Choose **Human**, set threshold to **0.2,** deselect **Auto Baseline** and then press **Analyze**. Repeat this for Male, Degradation, and IC with the following thresholds listed (Figure 31, next page):

   Note: Alternatively, click the **Analysis Settings** button in the upper right corner to set all analysis settings.

- Male: **0.2**
- Degradation: **0.2**
- IC: **0.05**
- Manual Baseline for all targets: Baseline Start Cycle: **3**, Baseline End Cycle: **15**

Note: Verify that options for **Auto Threshold** and **Auto Baseline** are deselected for all Targets. Setting the appropriate threshold value may require further internal validation at your facility.

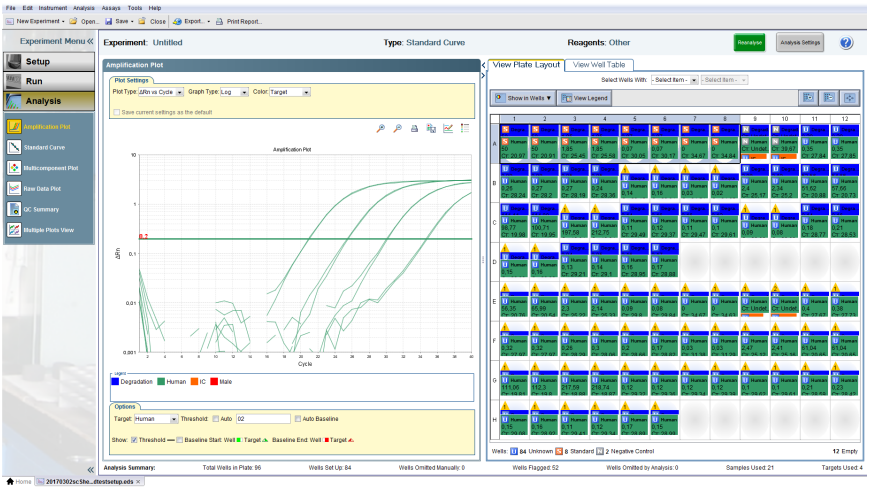


Figure 31. Sample analysis for the QPP_FAM, QPP_JOE, QPP_ATTO550 and QPP_ATTO647N channels.

5. To view the standard curve, select the **Standard Curve** tab (found in the **Results** tab). View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values (Figure 32).

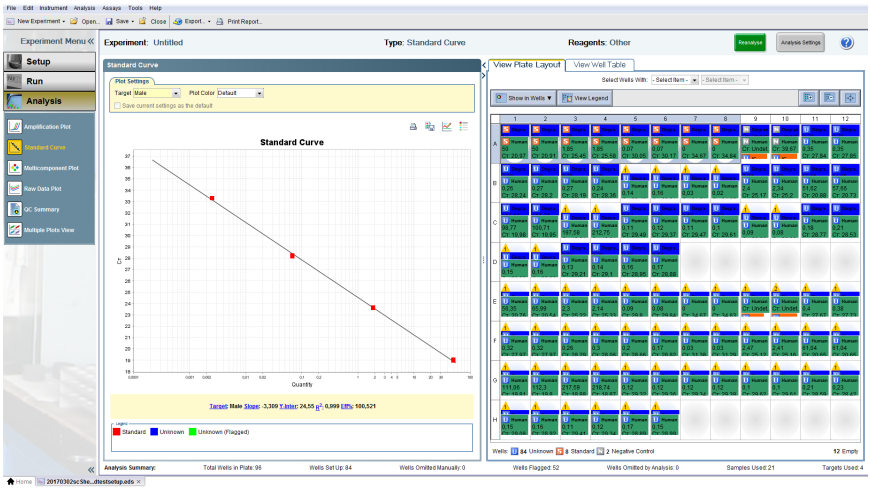


Figure 32. Standard curve.

6. View the concentrations of the unknown samples (Figure 33, next page).

   The **Well Table** displays data for the selected wells and summarizes the quantity of DNA present in the unknown samples. The **Human** Target shows the quantity of DNA present with the same units as used for the standards (i.e., if ng/µL was used for the definition of the standards, then the quantities for the unknowns will be reported in ng/µL). The **Male** and **Degradation** Targets show the quantity of male and human DNA present, with the same units as used for the standards.

   The **IC** Target shows the C_T value for the internal control.

   Important: If replicates are needed they have to be assigned before you proceed to the next step and export your data for further analysis with the Data Handling Tool. Mean quantification values should be displayed if replicates have been assigned.

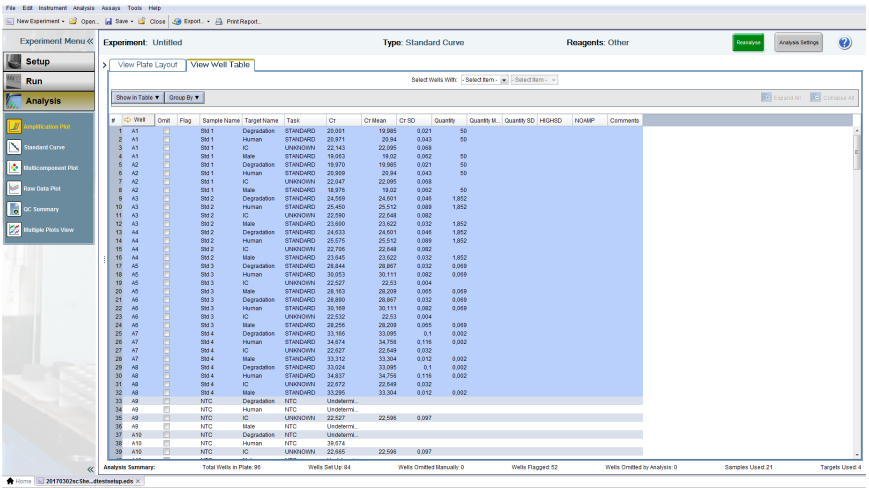


Figure 33. Unknown sample concentrations.

7. To export and save the results report, go to **File**, followed by **Export** and then **Results**. The analysis settings must be saved first, then the results may be saved in the format **Results Export Files *.csv**.

8. To interpret the results, see "Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool", page 70.

Protocol: Quantification of DNA Using the Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems

This protocol is optimized for use of the Investigator Quantiplex Pro Kit on the Applied Biosystems 7500 Real-Time PCR System, using SDS software version 1.4.

For general instructions on instrument setup, and other software versions, refer to the *Applied Biosystems 7500 Real-Time PCR System User Manual.*

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- When using the Applied Biosystems 7500 Real-Time PCR System with Investigator Quantiplex Pro, custom dye calibration for FAM, JOE, ATTO 550, ROX, and ATTO 647N is strongly recommended. To achieve optimal performance, calibrate with the Investigator Quantiplex Pro Calibration Kit (cat. no. 387416). Refer to the instrument user manual for correct setup.
- Use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Use the template volume specified in the protocol. The reaction is optimized for use with 2 µL template DNA. Do not use more or less than 2 µL per 20 µL reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored between 2°C and 8°C for at least 1 week.

- Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure A: PCR

1. Mix all solutions thoroughly before use to avoid localized concentrations of salt.
2. Prepare fresh serial dilutions of the Male Control DNA M1 according to Table 11. Serial dilutions of Male Control DNA M1. Vortex for at least 5 s and centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipette tip for each dilution.
   Make sure not to introduce cross-contamination.

Table 11. Serial dilutions of Male Control DNA M1

Serial dilution of Control DNA (ng/µL)	Control DNA (µL)	QuantiTect Nucleic Acid Dilution Buffer (µL)
50	Undiluted DNA	–
1.8519	5	130
0.0686	5	130
0.0025	5	130

Note: Alternative standard curves are listed in "Appendix: Alternative Standard Curves", page 84.

3. Thaw the template nucleic acids.
   Mix all solutions thoroughly before use to avoid localized concentrations of salt.
4. Prepare a master mix according to Table 12 (next page).
   The master mix contains all of the components needed for PCR except the template (sample) DNA and nuclease-free water.
   Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. This should include positive and negative control reactions.

Reaction setup can usually be done at room temperature (15–25°C). However, we recommend keeping the reagents, samples and controls on ice or in a cooling device.

Table 12. Master mix for DNA quantification

Component	Volume per 20 µL reaction	Final concentration
Quantiplex Pro Reaction Mix	9 µL	1x
Quantiplex Pro Primer Mix	9 µL	1x
Total volume of master mix	18 µL	–

5. Mix the master mix thoroughly, and dispense 18 µL into the wells of a PCR plate.
6. Add 2 µL QuantiTect Nucleic Acid Dilution Buffer to the NTC wells.

   Make sure that the NTC wells do not come in contact with human DNA.
7. Add 2 µL control DNA dilutions or 2 µL unknown sample DNA to the individual wells and mix thoroughly. Close the plate.

   Mix carefully in order to avoid localized concentrations of salt and DNA.

   Table 13 (next page) shows a possible plate setup. Make sure that the master mix and template are thoroughly mixed. It is required to run duplicates of the control DNA dilutions for each assay and on each reaction plate.

Table 13. Possible plate setup of reactions on the Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems

Well contents												
	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	1.8519	1.8519	0.0686	0.0686	0.0025	0.0025	NTC	NTC	UNK	UNK
B	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
F	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
G	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

All amounts in ng/µL. **UNK**: Unknown sample.

8. Open the Sequence Detection Software and select **Create a New Document**. Click **Next** to continue.

   If you are not using a template file from the Investigator Quantiplex Pro product page, then select the settings in step 8a in the **New Document Wizard** window and proceed with step 9.

   If you are using a template file, then select the settings in step 8b and proceed to step 13.

   Note: Download the template files from the product resources page at: **www.qiagen.com/QPpro-template-files**.

   8a. If you are not using a template file, select the following settings (Figure 34, next page) and proceed with step 9:

   - Assay: **Standard Curve (Absolute Quantification)**
   - Container: **96-Well Clear**
   - Template: **Blank Document**
   - Run Mode for the 7500 Real-Time PCR System: **Standard 7500**
   - Run Mode for the 7500 Fast Real-Time PCR System: **Standard 7500**

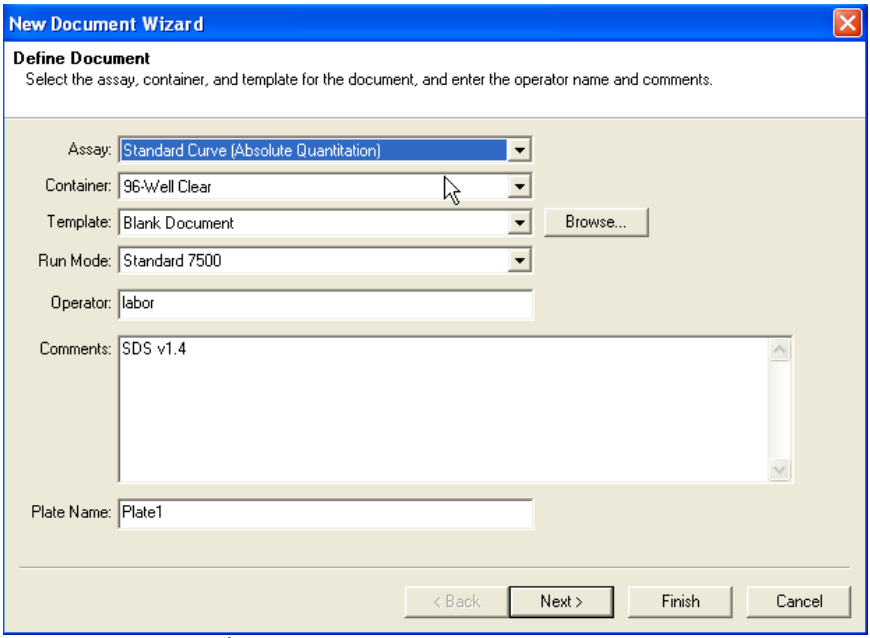


Figure 34. Creating a new document.

8b. If you are using a template file, then select the following settings and proceed to step 13.

- Assay: **Standard Curve (Absolute Quantification)**
- Container: **96-Well Clear**
- Template: **Investigator Quantiplex Pro Columns 7500 123.sdt** or **Investigator Quantiplex Pro Rows 7500 123.sdt**
- Run Mode for the 7500 Real-Time PCR System: **Standard 7500**
- Run Mode for the 7500 Fast Real-Time PCR System: **Standard 7500**

9. Add the detectors QPP_FAM, QPP_JOE, QPP_ATTO550, and QPP_ATTO647N to the document and set QPP_ROX as passive reference. Click **Next** to continue (Figure 35, next page).

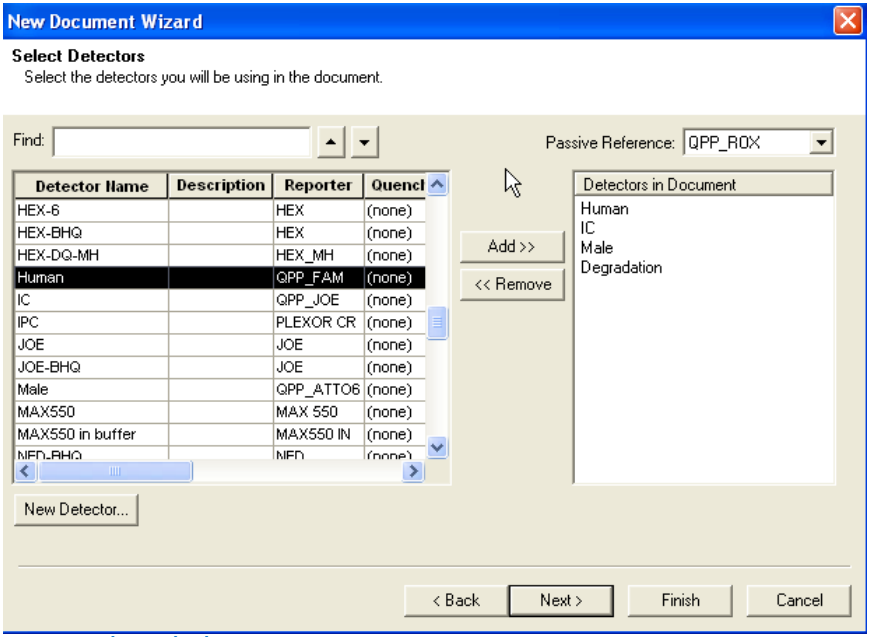


Figure 35. Selecting the detectors.

10. Select the wells in use and check the box for all 4 detectors.

    Important: Do not highlight the wells that are not in use (i.e., those without reaction mix). Including unused wells will significantly impact the scale of the X and Y axes when viewing the data.

11. Enter the concentrations of the standard curve to the wells containing the control reactions for the QPP_FAM, QPP_ATTO550 and the QPP_ATTO647N detectors.

12. Click **Unknown** in the Task column and then select **Standard** from the dropdown menu (Figure 36). Select **Quantity** for the appropriate detector and enter the quantity of DNA in the well according to Table 11, page 56.

Important: Although units are not entered for **Quantity**, a common unit must be used for all standard quantities (e.g., ng/µL). The units used for standard quantities define the quantification units for the analysis of results.

Note: Leave the QPP_JOE detector Task for standard reactions set to **Unknown**. Enter the sample name (e.g., Standard 50 ng/µL).

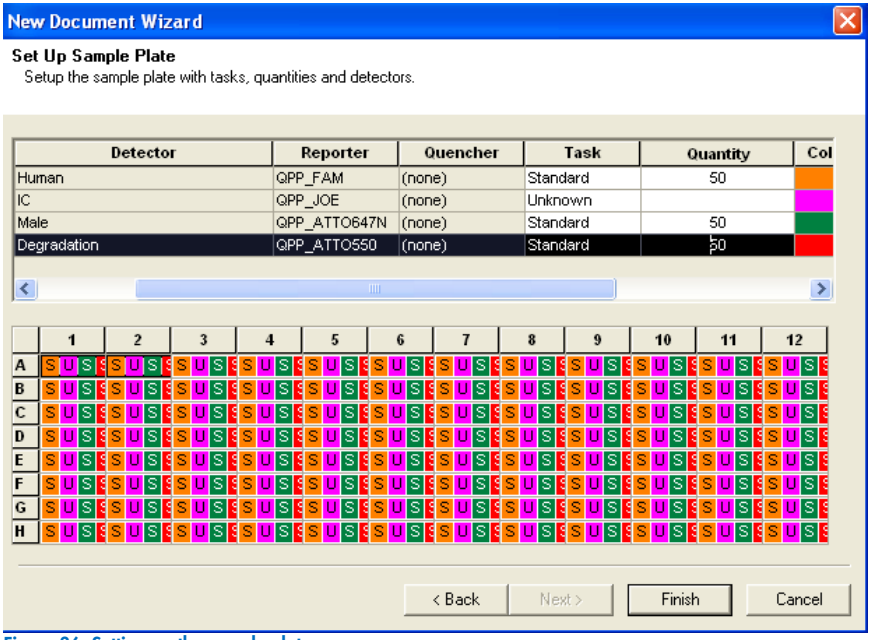


Figure 36. Setting up the sample plate.

13. Click **Finish**. To program the cycler according to Table 14 (next page), click on the **Instrument** tab.

Table 14. Cycling protocol using an Applied Biosystems 7500/7500 Fast Real-Time PCR System

Step	Temperature	Time	Number of cycles	Comment
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C to activate the DNA polymerase
Denaturation	95°C	5 s	40	
Combined annealing/extension	60°C	35 s		Perform fluorescence data collection

14. On the thermal profile, delete the first step by pressing **Shift** and clicking within the Stage 1 hold step (50°C for 2 min). After the hold step is selected, press the **Delete** key. Change the holding times to those in Table 13. Be sure to change the Sample volume to **20 µL** and select the correct run mode (**Standard 7500**) (Figure 37, next page).

    Data acquisition should be performed during the combined annealing/extension step. If using software version 1.2.3, be sure to uncheck the box **9600 Emulation**.

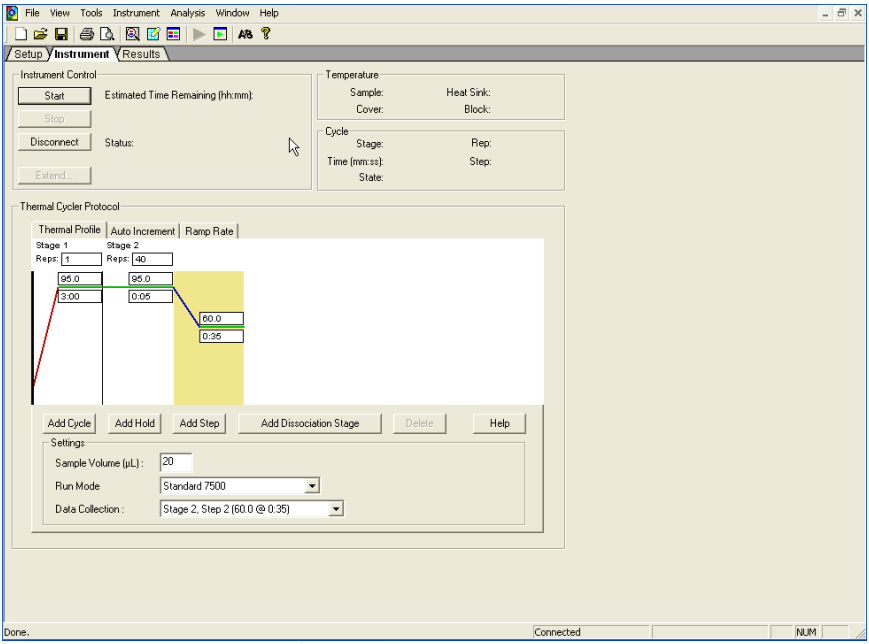


Figure 37. Adjusting the thermal profile (SDS Software Version 1.4).

15. Before running the reaction plate, save the plate document as an SDS Document (***.sds**) file. Click **File** and then **Save**. Enter a name for the plate document, and then click **Save** again.

16. Load the plate into the instrument.

    Ensure that position A1 on the plate is on the top-left side of the tray.

17. Start the reaction by clicking **Start**.

Procedure B: Data analysis

Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

1. Open the run file using the SDS software. Go to **File** followed by **Open** and then **Browse** to locate the saved file.
2. Standards must first be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 4, below.
3. Go to **View** and select **Well Inspector** from the menu (Figure 38, next page). In the **Task** column, define the wells that contain DNA standards for the QPP_FAM, QPP_ATTO550 and the QPP_ATTO647N channel as **Standard**. Name them as Standard (e.g., Standard 1 or Std1, Standard 2 or Std2, etc.), and enter the quantity of DNA in the well according to Table 11, page 56.

   Naming of Standards is required for proper subsequent analysis with the QIAGEN Quantification Assay Data Handling Tool.

   Important: Although units are not entered for **Quantity**, a common unit must be used for all standard quantities (e.g., ng/µL). The units used for standard quantities define the quantification units for the analysis of results.

   Note: Leave the QPP_JOE detector Task for standard reactions set to **Unknown**. Quantity values are not needed for the internal control. Enter the sample name (e.g., Standard 50 ng/µL). If replicates are needed they should be assigned before you proceed to data export. Define replicates by using the same sample name.

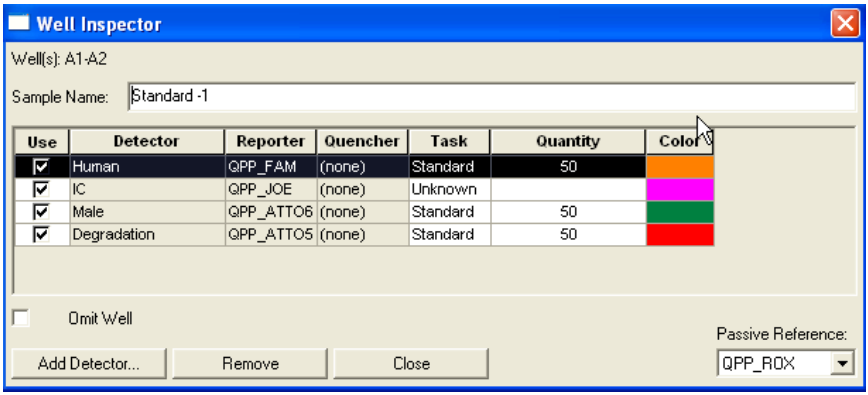


Figure 38. Well inspector.

4. In the **Amplification Plot** tab (found in the **Results** tab), select the appropriate samples in the table below the amplification plot. Choose **Human** and set Threshold to **0.2** then press **Analyze**. Repeat this for Male, Degradation and IC with the thresholds below (Figure 39, next page):

   - Male: **0.2**
   - Degradation: **0.2**
   - IC: **0.05**
   - Manual Baseline for all targets: Baseline Start Cycle: **3**, Baseline End Cycle: **15**

   Note: Verify that options for **Auto Ct** and **Auto Baseline** are deselected for all targets. Setting the appropriate threshold value may require further internal validation at your facility.

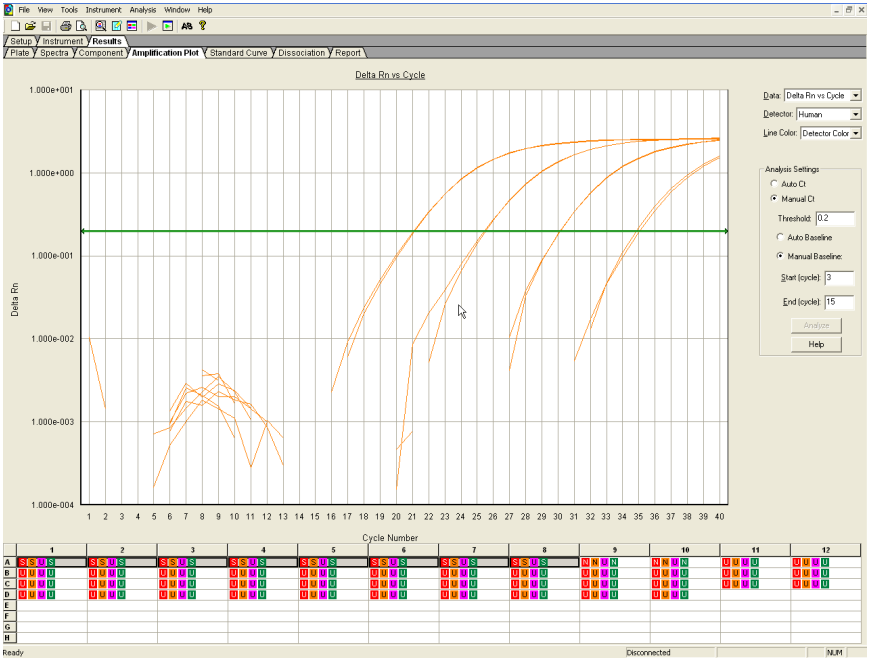


Figure 39. Amplification plot for Human.

5. To view the standard curve, select the **Standard Curve** tab (found in the **Results** tab). View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values for QPP_FAM, QPP_ATTO550, and the QPP_ATTO647N channels (Figure 40).

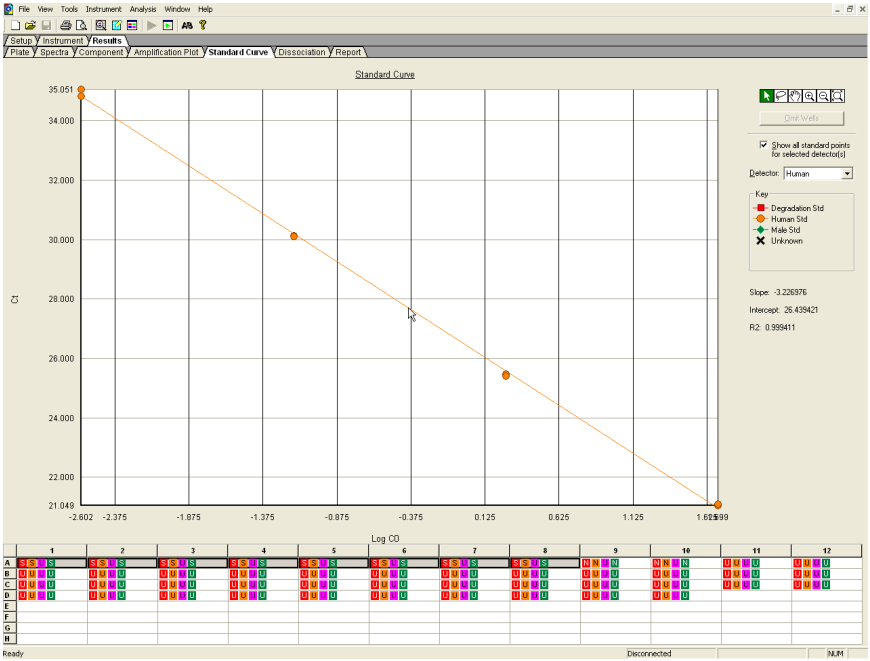


Figure 40. Standard curve.

6. View the concentration of the unknown samples (Figure 41).

   The report displays data for the selected wells and summarizes the quantity of DNA present in the unknown samples. The QPP_ATTO647N detector shows the quantity of total male DNA present, QPP_FAM detector shows quantity of total human DNA, while the QPP_ATTO550 detector shows the quantity of the large human system for DNA degradation assessment. The quantity is shown with the same unit as used for the standards (i.e., if ng/µL was used for the definition of the standards, then the quantities for the unknowns will be reported in ng/µL).

   The QPP_JOE-detector shows the C_T value for the internal control.

   Important: If replicates are needed, they have to be assigned before you proceed to the next step and export your data for further analysis with the Data Handling Tool. Mean quantification values should be displayed, if replicates have been assigned.

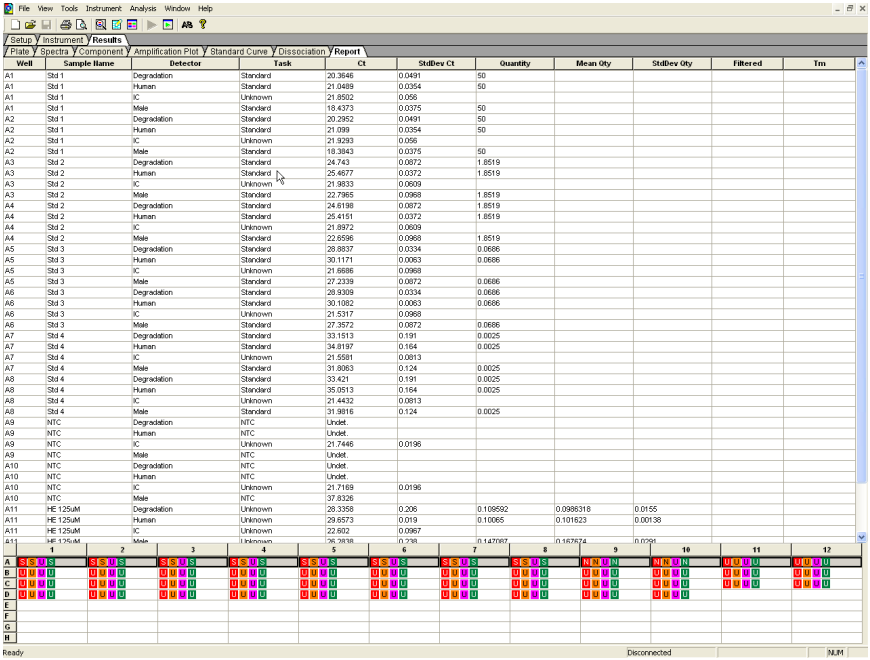


Figure 41. Unknown sample concentrations.

7. To export and save the results report, go to **File**, followed by **Export** and then **Results**. The analysis settings must be saved first, then the results may be saved in the format **Results Export Files *.csv** (Figure 42).

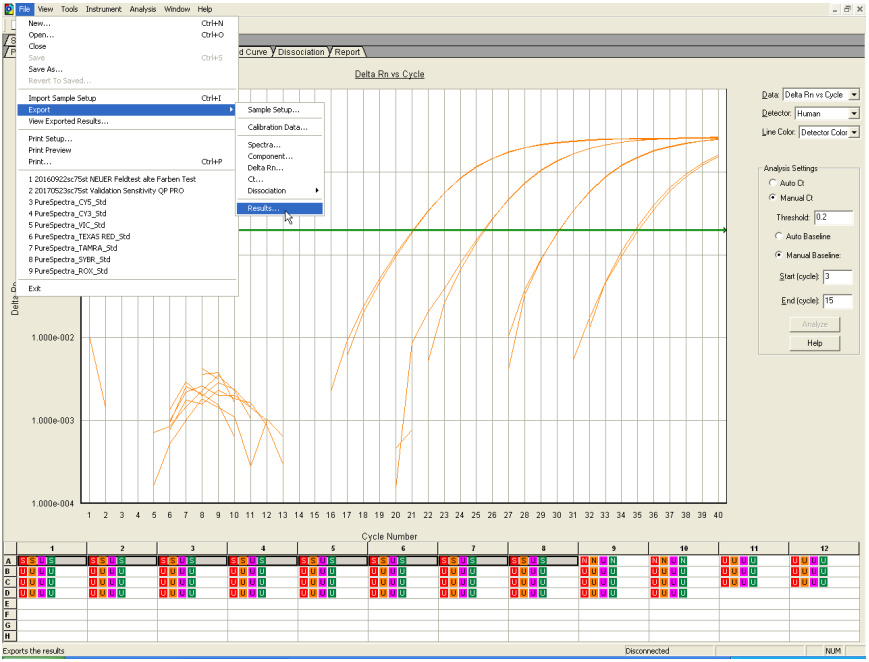


Figure 42. Exporting results.

8. To interpret the results, see “Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool”, page 70.

Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool

The QIAGEN Quantification Assay Data Handling Tool is designed for accurate quantification, data analysis, and interpretation. The Opening Page worksheet contains information on version number and software requirements/compatibility. On the Configuration worksheet, the root directories for data processing, result import options, and default values for analysis criteria and thresholds can be set. Each worksheet contains an instruction button, which, when pressed, provides detailed instructions on using the functions of the specific worksheet.

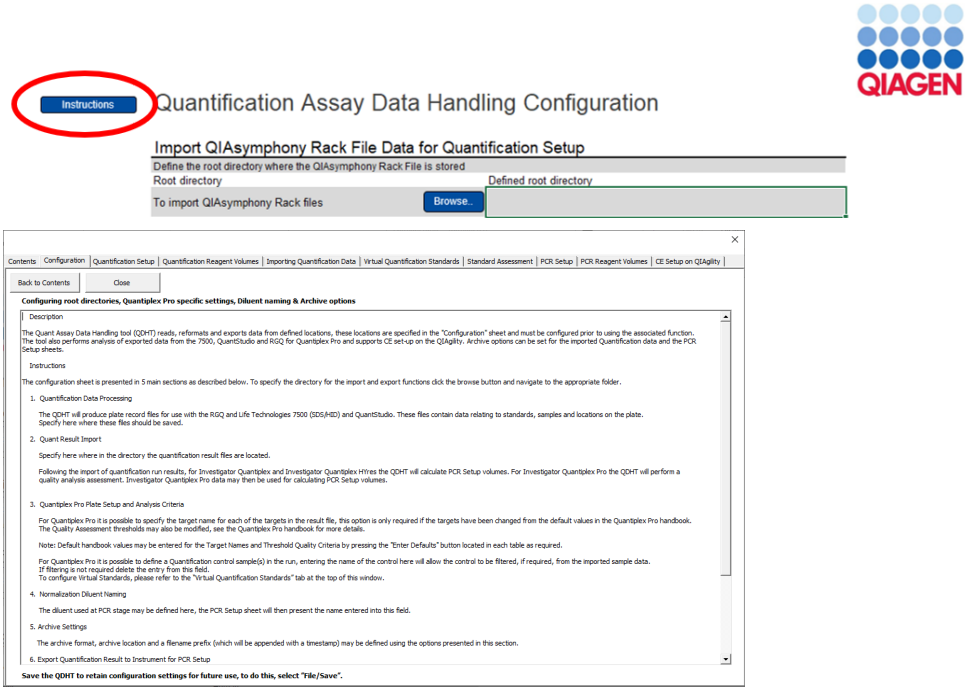


Figure 43. Instructions button. Each worksheet contains an instructions button. When pressed it will provide detailed instructions.

Procedure

1. Open QIAGEN Quantification Assay Data Handling Tool.
2. If the cycler has been calibrated with the Investigator Quantiplex Pro Calibration Kit and if the Data Handling Tool is being used for the first time on your computer, a one-time update has to be conducted. For proper functioning of the Data Handling Tool please ensure that the QPP dyes have been named **exactly** on your cycler as described in the calibration section of this handbook. The one-time update will set proper dye naming in the Data Handling Tool. Save the Data Handling Tool afterwards on your computer. If you are using the Data Handling Tool the first time on your computer proceed to step 3. If the one-time update has already been done and the changes have been saved proceed to step 4.
3. Click on the blue **One Time update for ABI 7500 (non SDS) Software** button (Figure 44, next page).

4. Click the **Configuration** worksheet tab (Figure 44).

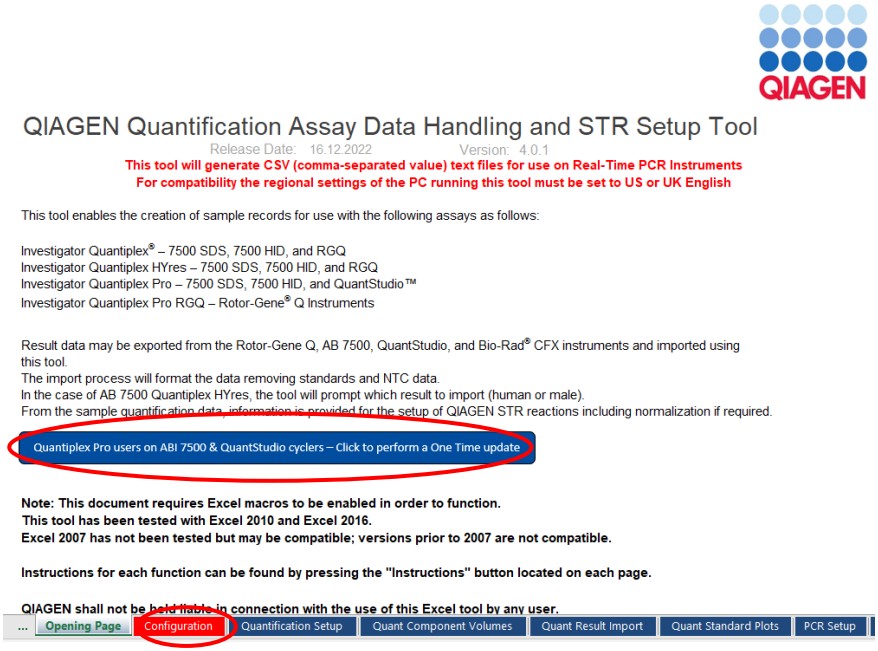


Figure 44. Performing one-time update and opening the Configuration worksheet.

5. Set the root/home directory to save Quant batch files (Figure 45, next page).
6. Set the root/home directory to import Quant result files (Figure 45, next page).
7. Target names should be assigned for the Applied Biosystems 7500 Real-Time PCR System. Click on the **Enter Defaults** button and select **ABI 7500**. Default names for the targets are "Human" (Human Target), "Male" (Male Target), "Degradation "(Degradation Target), and "IC" (Internal Positive Control). Defaults can be restored by clicking the **Enter Defaults** button (Figure 45, next page).

   Note: The Male Degradation Marker is only available in the Investigator Quantiplex Pro RGQ Kit.

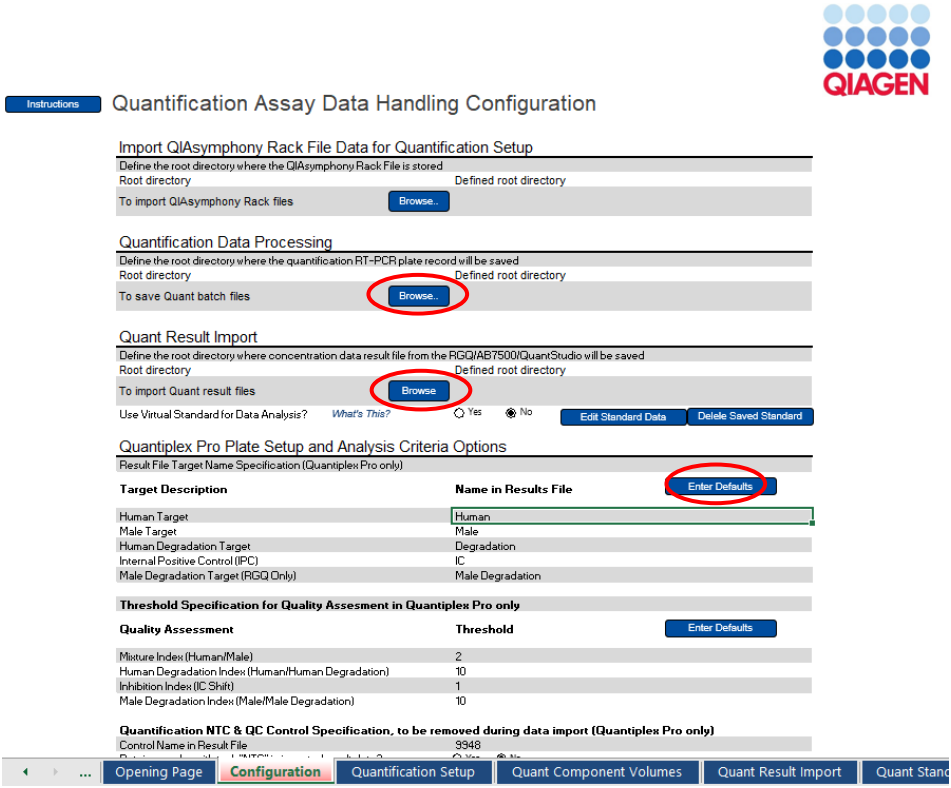


Figure 45. Setting the Quant batch and Quant result files.

8. Threshold setting for the Quality Assessment can be changed/adjusted as needed. The default threshold settings are

   - Mixture index (Human/Male): **2**
   - Degradation Index (Human/Degradation): **10**
   - Inhibition Index (IC Shift): **1**

   Note: Setting the appropriate threshold values may require further internal validation at your facility. 9948 will be filtered from the import, provided it is included in the section

"Quantification QC Control Specification". Removing it allows it to be kept in the final data set.

Defaults can be restored by clicking the **Enter Defaults** button.

9. To import quantification results click the **Quant Result Import** worksheet tab (Figure 46).

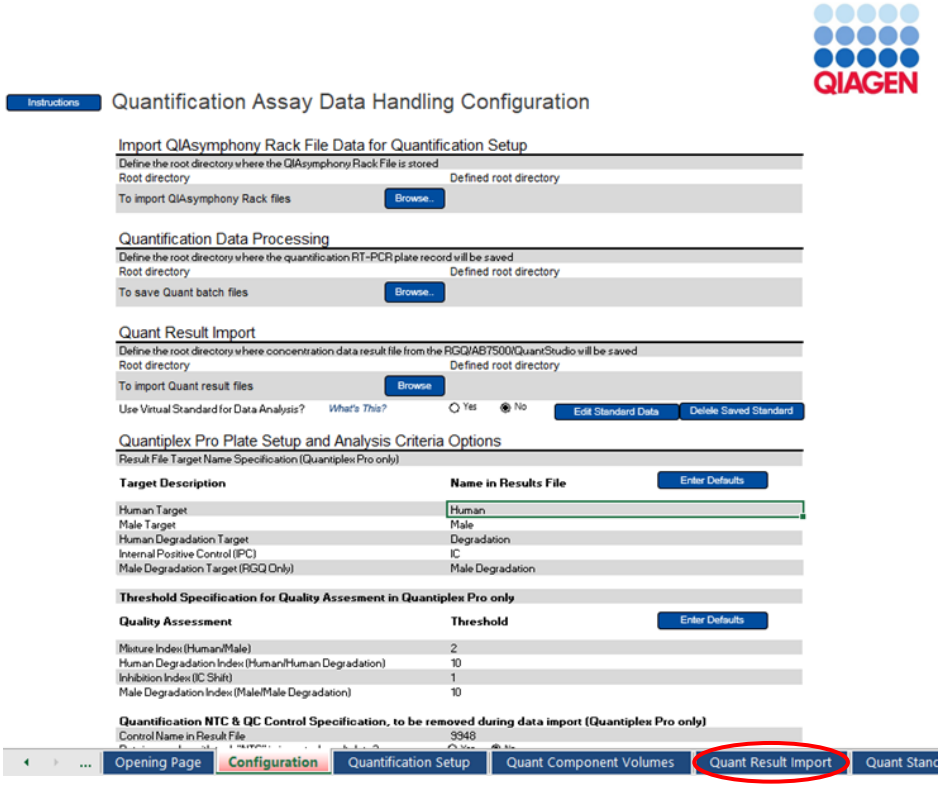


Figure 46. Opening the Quant Result Import tab.

10. Click the **Import QuantData** button (Figure 47).

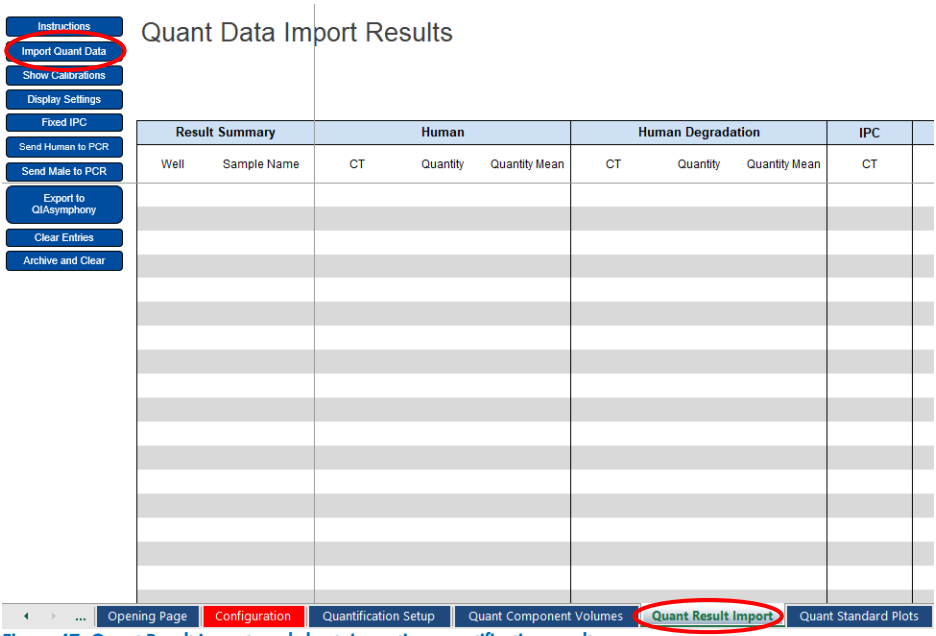


Figure 47. Quant Result Import worksheet: importing quantification results.

11. Confirm that your data are in the necessary format (Figure 48).

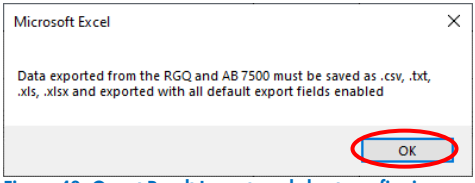
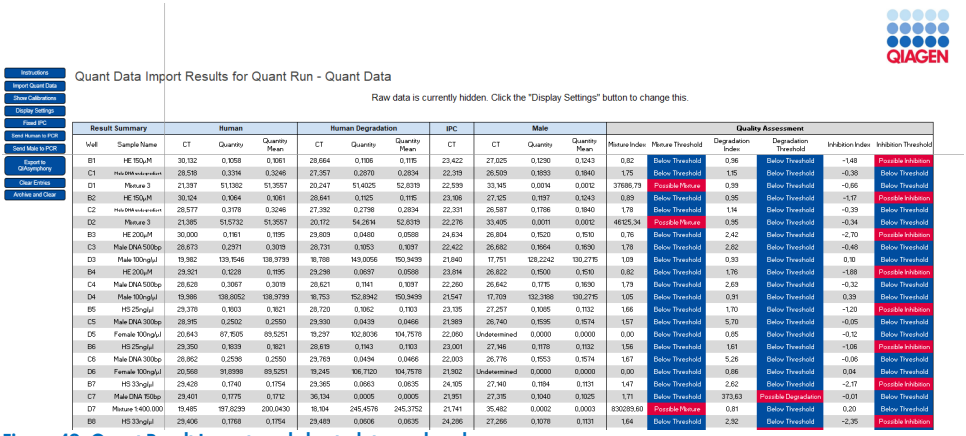


Figure 48. Quant Result Import worksheet: confirming your data format.

Your quantification data are now imported and the data analyzed. The Mixture Index, Degradation Index, and Inhibition Index are calculated and tagged as "Below Threshold", "Possible Mixture", "Possible Degradation", or "Possible Inhibition" (Figure 49).



Result Summary		Human			Human Degradation			IPC	Male			Quality Assessment					
Well	Sample Name	CT	Quantity	Quantity Mean	CT	Quantity	Quantity Mean	CT	CT	Quantity	Quantity Mean	Mixture Index	Mixture Threshold	Degradation Index	Degradation Threshold	Inhibition Index	Inhibition Threshold
B1	HE 150µM	30,132	0,1058	0,1061	28,664	0,1106	0,1115	23,422	27,025	0,1230	0,1243	0,82	Below Threshold	0,96	Below Threshold	-1,48	Possible Inhibition
C1	Hela DNA independent	28,518	0,3314	0,3246	27,357	0,2870	0,2834	22,319	26,503	0,1893	0,1940	1,75	Below Threshold	1,15	Below Threshold	-0,38	Below Threshold
D1	Mixture 3	21,397	51,1382	51,3557	20,247	514025	52,8319	22,599	33,145	0,0014	0,0012	37686,79	Possible Mixture	0,99	Below Threshold	-0,66	Below Threshold
B2	HE 150µM	30,124	0,1064	0,1061	28,641	0,1125	0,1115	23,106	27,125	0,1197	0,1243	0,89	Below Threshold	0,95	Below Threshold	-1,17	Possible Inhibition
C2	Hela DNA independent	28,577	0,3178	0,3246	27,392	0,2798	0,2834	22,331	26,587	0,1786	0,1940	1,78	Below Threshold	1,14	Below Threshold	-0,39	Below Threshold
D2	Mixture 3	21,365	51,5732	51,3557	20,172	54,2614	52,8319	22,276	33,405	0,0011	0,0012	46125,34	Possible Mixture	0,95	Below Threshold	-0,34	Below Threshold
B3	HE 200µM	30,000	0,1161	0,1195	29,609	0,0492	0,0588	24,634	26,894	0,1520	0,1510	0,76	Below Threshold	2,42	Below Threshold	-2,70	Possible Inhibition
C3	Male DNA 500bp	28,673	0,2971	0,3019	28,731	0,1053	0,1097	22,422	26,692	0,1964	0,1930	1,78	Below Threshold	2,82	Below Threshold	-0,48	Below Threshold
D3	Male 100ng/µl	19,382	139,1546	138,9799	18,788	149,0056	150,9499	21,840	17,751	128,2242	130,2715	1,09	Below Threshold	0,93	Below Threshold	0,10	Below Threshold
B4	HE 200µM	29,521	0,1228	0,1195	29,298	0,0697	0,0588	23,814	26,822	0,1500	0,1510	0,82	Below Threshold	1,76	Below Threshold	-1,88	Possible Inhibition
C4	Male DNA 500bp	28,626	0,3067	0,3019	28,621	0,1141	0,1097	22,260	26,642	0,1715	0,1930	1,79	Below Threshold	2,69	Below Threshold	-0,32	Below Threshold
D4	Male 100ng/µl	19,986	138,8052	138,9799	18,753	152,8942	150,9499	21,547	17,769	132,3188	130,2715	1,05	Below Threshold	0,91	Below Threshold	0,39	Below Threshold
B5	HS 25ng/µl	29,376	0,1803	0,1821	28,720	0,1062	0,1103	23,135	27,257	0,1065	0,1132	1,66	Below Threshold	1,70	Below Threshold	-1,20	Possible Inhibition
C5	Male DNA 300bp	28,915	0,2502	0,2550	29,330	0,0439	0,0466	21,989	26,740	0,1535	0,1574	1,57	Below Threshold	5,70	Below Threshold	-0,05	Below Threshold
D5	Female 100ng/µl	20,843	67,1505	69,5251	19,297	102,8036	104,7578	22,060	Undetermined	0,0000	0,0000	0,00	Below Threshold	0,65	Below Threshold	-0,12	Below Threshold
B6	HS 25ng/µl	29,350	0,1839	0,1821	28,678	0,1143	0,1103	23,001	27,146	0,1178	0,1132	1,56	Below Threshold	1,61	Below Threshold	-1,06	Possible Inhibition
C6	Male DNA 300bp	28,862	0,2536	0,2550	29,769	0,0494	0,0466	22,003	26,776	0,1553	0,1574	1,67	Below Threshold	5,28	Below Threshold	-0,06	Below Threshold
D6	Female 100ng/µl	20,568	91,8998	69,5251	19,245	106,7120	104,7578	21,902	Undetermined	0,0000	0,0000	0,00	Below Threshold	0,66	Below Threshold	0,04	Below Threshold
B7	HS 33ng/µl	29,428	0,1740	0,1754	29,365	0,0663	0,0635	24,105	27,140	0,1184	0,1131	1,47	Below Threshold	2,62	Below Threshold	-2,17	Possible Inhibition
C7	Male DNA 150bp	29,401	0,1775	0,1712	36,134	0,0005	0,0005	21,951	27,315	0,1040	0,1025	1,71	Below Threshold	373,63	Possible Degradation	-0,01	Below Threshold
D7	Mixture 1:400.000	19,485	197,8299	200,0430	18,104	245,4576	245,3752	21,741	35,482	0,0002	0,0003	830289,60	Possible Mixture	0,81	Below Threshold	0,20	Below Threshold
B8	HS 33ng/µl	29,406	0,1768	0,1754	29,483	0,0606	0,0635	24,286	27,266	0,1078	0,1131	1,64	Below Threshold	2,92	Below Threshold	-2,35	Possible Inhibition

Figure 49. Quant Result Import worksheet: data analyzed.

12. Display options can be adjusted by clicking **Display Settings**:

    - Show Raw Data
    - Show Quantity Mean Values
    - Show Ct Values

    Note: The Degradation Index is set to 10 as a default. Full STR profiles can be obtained with DNA fragmented to an average fragment size of approximately 300 bp. The default Degradation Index of 10 should allow differentiation between DNA fragments larger or smaller than 300 bp.

    Note: The Inhibition Index is set to 1 as a default. The IC acts as a quality sensor and reports the presence of inhibitors with a C_T shift while quantification remains reliable. The default value can be changed and adjusted for relevant degrees of inhibition. Therefore, laboratory validation should be performed to determine criteria for detecting inhibition.

General Interpretation of Results

General considerations for data analysis

Real-time PCR data are produced as sigmoidal-shaped amplification plots (when using a linear scale), in which fluorescence is plotted against the number of cycles.

The threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence.

The optimal threshold setting depends on the reaction chemistries used for PCR. Therefore, an optimal threshold setting that has been established for another kit may not be suitable for the Investigator Quantiplex Pro Kit and may need to be adjusted.

For DNA quantification using the Investigator Quantiplex Pro Kit, the analysis settings must be adjusted for both reporter dyes.

Standard curve

The standard curve is the best fit for a linear regression to the standard dilution series data. The equation is in the form

$$y = mx + b$$

where x = log concentration and y = C_T.

The slope

The slope (m) describes the PCR efficiency. A slope of –3.3 indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). Typically, the slope ranges between –3.0 and –3.6. If the values fall outside of this range, see the Troubleshooting Guide, page 81, for more information.

The Y-intercept

The Y-intercept (b) indicates the expected C_T value for a sample with a quantity value of 1 (for example, 1 ng/µL).

The R^2 value

The R^2 value is a measure of the fit of the data points to the regressed line. In general, the standard curve has an R^2 value ≥0.990. Low R^2 values (R^2 ≤0.98) may occur for many different reasons. In case of low R^2 values, see the Troubleshooting Guide, page 81, for more information.

Internal control

The internal control (IC)is intended to report chemistry or instrument failure, errors in assay setup, and the presence of inhibition in the sample. The IC system is designed to be more sensitive to inhibition than the specific target for human DNA. Therefore, the quantification will be valid even if some inhibition is present in the sample. In this case, the operator will get information both about the concentration of DNA in the sample and about the presence of inhibitors. Comparison of the C_T value of the IC system for DNA standards with the C_T values of the IC system for unknown samples can provide an indication of potential inhibition. At higher concentrations of inhibitor, the quantification data may be affected, and this must be

considered for downstream applications. In general, the internal control can be interpreted in the following manner:

a)	IC system shows normal amplification. No IC shift greater than specified is observed. No amplification of the Human, Degradation, and Male Targets is detectable.	No or insufficient DNA was present.
b)	IC shift is greater than specified. Degradation Index is below threshold.	Sample contains inhibitors. DNA is not degraded.
c)	IC shift is greater than specified. Degradation Index is above threshold.	Sample contains inhibitors. DNA is possibly degraded. **Note**: Extremely high concentrations of inhibitors can inhibit amplification of the Degradation target and trigger the Degradation Index.

Important: Internal laboratory validation with relevant inhibitors should be performed to determine criteria for detecting inhibition.

Quantification of unknowns

The Investigator Quantiplex Pro Kit can quantify a broad range of DNA amounts in a sample, from 200 ng/µL to approximately 0.5 pg/µL of human genomic DNA. When 2 µL of a sample with very low concentrations is loaded in a reaction, the well probably contains less than 1 diploid human genome equivalent. In the low DNA concentration range, statistical effects, known as stochastic variations, can significantly affect the assay result. When using samples with low concentrations of DNA, make sure that as many replicates as possible are assayed in order to confirm the result.

Quantification of female/male mixtures

The Investigator Quantiplex Pro Kit provides high sensitivity to detect low amounts of male DNA even in a very high background of female DNA. The Mixture Index provides information on whether a sample is a female/male mixture. In general, the Mixture Index can be interpreted in the following manner:

a)	The sample has a Mixture Threshold below the index specified.	The sample contains only male DNA or only low levels of female DNA.
b)	The sample has a Mixture Threshold above the index specified.	The sample contains a possible male DNA/female DNA mixture.

Degradation status assessment

Environmental degradation may occur with forensic casework samples and is a typical challenge in routine genetic fingerprinting. The Investigator Quantiplex Pro Kit contains a newly developed system for detection of DNA degradation. In general, the Degradation Index can be interpreted in following manner:

a)	The sample has a Degradation Threshold below the index specified. No IC shift is detected.	DNA is most likely not degraded. The sample most likely contains no inhibitors.
b)	The sample has a Degradation Threshold below the index specified. IC shift is detectable above the threshold.	DNA is most likely not degraded. The sample may contain inhibitors.
c)	The sample has a Degradation Threshold above the index specified. No IC shift is detected.	DNA is most likely degraded. The sample most likely contains no inhibitors.
d)	The sample has a Degradation Threshold above the index specified. IC shift is detectable above the threshold.	DNA may or may not be degraded. The sample contains inhibitors.

Note: When 2 µL of a sample with very low concentrations is loaded in a reaction, the well probably contains less than 1 diploid human genome equivalent. In the low DNA concentration range, statistical effects, known as stochastic variations, can affect the assay. In case of degraded DNA with a very low DNA concentration, the Degradation target can be affected. If the Degradation target has an undetermined value, the sample will be tagged with "Possible Degradation". Extremely high inhibitor concentrations can also affect the Degradation target and lead to a "Possible Degradation" flag.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

	Comments and suggestions	
No signal or one or more signals detected late in PCR		
a)	Incorrect cycling conditions	Always use the optimized cycling conditions specified in the protocols. Make sure to select ROX as the passive dye on Applied Biosystems instruments.
b)	Pipetting error, missing or degraded reagent	Check the storage conditions of the reagents. Repeat the assay.
c)	Incorrect or no detection step	Make sure that fluorescence detection takes place during the combined annealing/extension step.
d)	Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the template DNA are present in the sample.
e)	Problems with starting template	Check the storage conditions of the starting template DNA. Efficient removal of PCR inhibitors is essential for optimal results. Purify nucleic acids from your sample using an appropriate purification method. Ensure that all reagents, buffers and solutions used for isolating and diluting template nucleic acids are free from nucleases.
f)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Ensure that the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.
g)	Degraded control DNA	Make new serial dilutions of the control DNA from the stock solution. Repeat the assay using the new dilutions.

		Comments and suggestions
Differences in C_T values or in PCR efficiencies between runs		
a)	Incorrect cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Ensure that the cycling conditions include the initial step for activation of the DNA polymerase and the specified times for denaturation and annealing/extension.
b)	Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.
No linearity in ratio of C_T value/crossing point to log of the template amount		
	Amount of template in unknown sample too high	Linearity is guaranteed within the range of the standard curve. If signals appear at very early C_T values, dilute the sample and repeat the reaction.
Increased fluorescence or C_T value for no-template control		
a)	Contamination of reagents	Discard all the components of the assay (e.g., master mix). Repeat the assay using new components.
b)	Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.
c)	Crosstalk problems	Depending on the instrument, different techniques are used to avoid spectral crosstalk when using multiple fluorophores for multiplex assays. However, minimal crosstalk, as a result of residual spectral overlap, may be observed in the NTC wells, especially if the instrument is in need of calibration.
Varying fluorescence intensity		
a)	Contamination of real-time cycler	Reactions were contaminated with target DNA. Decontaminate the real-time workstations and the cycler according to the manufacturer's instructions. Use new reagents and solutions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.

		Comments and suggestions
c)	Wavy curve at high template amounts for highly concentrated targets	In the analysis settings, reduce the number of cycles used for background calculation (if the real-time cycler allows this) or reduce the amount of template.
Slope for the standard curve differs significantly from –3.33 or R^2 value is significantly less than 0.98–0.99		
a)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b)	Real-time cycler and/or pipettes no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions. Calibrate pipettes to minimize pipetting variability.
c)	Wavy curve at high template amounts for highly concentrated targets	In the analysis settings, reduce the number of cycles used for background calculation or reduce the amount of template.
d)	Problem with dilution of standards	Ensure that the DNA standard is completely thawed and mixed thoroughly before use. Ensure that dilutions of the DNA standard are mixed thoroughly before removing each aliquot for the serial dilution. Always use a sample volume of 2 µL. Change pipette tips between each dilution step.
e)	Plate not sealed	Carefully seal the plates to avoid evaporation.
f)	Error made during dilution of the DNA standard	Verify all calculations, and repeat dilution of the DNA standard.
g)	Incorrect concentration values entered in the software	Verify the concentrations for all samples used to generate the standard curve.
h)	Abnormal fluorescence	Do not write on the plate. Use caution when handling plates. Wear gloves.
i)	Statistical variation	Some variation in the reaction is normal, particularly when the DNA target is present at a low copy number. Perform at least duplicates for the standard curve to minimize the effect of this variation.

Appendix: Alternative Standard Curves

Table 15. Alternative 5-point standard curve (10x dilution)

Serial dilution of control DNA (ng/µL)	Amount of control DNA (µL)	QuantiTect Nucleic Acid Dilution Buffer (µL)
50	Undiluted DNA	–
5	5	45
0.5	5	45
0.05	5	45
0.005	5	45

Table 16. Alternative 6-point standard curve (9x dilution)

Serial dilution of control DNA (ng/µL)	Amount of control DNA (µL)	QuantiTect Nucleic Acid Dilution Buffer (µL)
50	Undiluted DNA	–
7.1429	5	40
1.0204	5	40
0.0686	5	40
0.0076	5	40
0.0030	5	40

Table 17. Alternative 7-point standard curve (5x dilution)

Serial dilution of control DNA (ng/µL)	Amount of control DNA (µL)	QuantiTect Nucleic Acid Dilution Buffer (µL)
50	Undiluted DNA	–
10	10	40
2	10	40
0.4	10	40
0.08	10	40
0.016	10	40
0.0032	10	40

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Pro Kit (200)	Quantiplex Pro Reaction Mix, Quantiplex Pro Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387216
Investigator Quantiplex Pro Calibration Kit	Calibration Standard FAM, JOE, ATTO 550, ROX, ATTO 647N, and Quantiplex Pro Calibration Buffer	387416
Related products		
Invetsigator Quantiplex Pro RGQ kit (200)	Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316
Investigator 24plex QS Kit (100)*	Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder, DNA size standard, and Nuclease-free water	382415
Investigator 26plex QS Kit (100)*	Primer mix, Fast Reaction Mix 3.0, Control DNA, allelic ladder, and Nuclease-free water	382615
Investigator ESSplex SE QS Kit (100)*	Primer Mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder, DNA size standard, and Nuclease-free water	381575

* Larger kit sizes available; please inquire

Product	Contents	Cat. no.
Investigator Argus X-12 QS Kit (25)*	Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder, DNA size standard, and Nuclease-free water	383223
Investigator Argus Y-28 QS Kit (100)	Primer mix, Fast Reaction Mix 3.0, Control DNA, allelic ladder, DNA size standard, and Nuclease-free water	383625

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Document Revision History

Date	Changes
03/2018	First revision of document. Addition of protocols for Applied Biosystems 7500 Real-Time PCR cycler calibration pages 13–39. Content updates for quantification protocols and data/results interpretation. Updated figures and tables. Minor text modifications.
01/2023	ISO18385 information added. New information on targets, amplicons, and dyes added (Table 1). Minor updates in introduction and section "Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool".

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