February 2024

Investigator® Quantiplex® Pro FLX Handbook for Applied Biosystems 7500 and QuantStudio™ 5 Real-Time PCR Systems

For quantification of human and male DNA in forensic samples

Sample to Insight

Table of Contents

Kit Contents

Investigator Quantiplex Pro FLX Kit **Catalog number** **No. of 20 µL reactions**	 **387516** **576**
QuantiPlex Pro FLX Plate	6
Male Control DNA M1 (50 ng/µL)	0.2 mL
QuantiTect® Nucleic Acid Dilution Buffer	1 vial
Quick-Start Protocol	1

Shipping and Storage

The Investigator Quantiplex Pro FLX Kit is shipped at 2–8°C. The Quantiplex Pro FLX plates can be stored at room temperature (15–25°C), protected from light in the aluminum bags. Partially used plates can be put back into their aluminum bag, including the desiccant, and stored for up to 4 weeks until being used up completely. The QuantiTect Nucleic Acid Dilution Buffer and the Control DNA M1 should be stored at 2–8°C. Under these conditions, the components are stable until the expiration date indicated on the kit.

Intended Use

The Investigator Quantiplex Pro FLX 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 FLX Kits is tested against predetermined specifications to ensure consistent product quality. The Investigator Quantiplex Pro FLX Kit meets ISO 18385 requirements.

Introduction

Human identification is commonly based on the analysis of short tandem repeats (STRs), or single nucleotide polymorphisms (SNPs), 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 FLX 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 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 FLX 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.

The assay comes in a lyophilized 96-well plate format, which provides a high sample input flexibility of 1–18 µL, and up to approximately 10x higher sensitivity when using the maximum input. Breakable plates allow to adapt to individual throughput needs. Setup of reactions is fast and simple, and plates can be stored at room temperature before use.

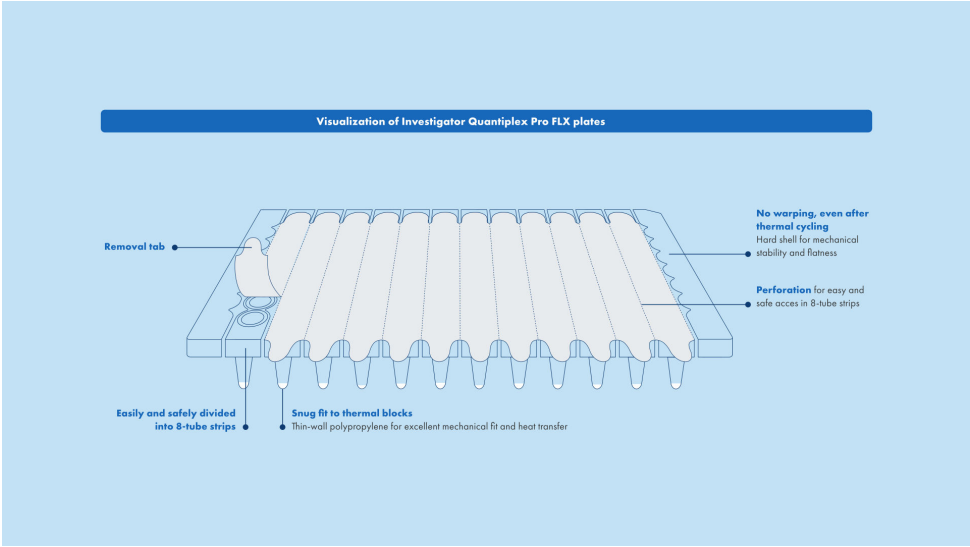


Figure 1. Features of the Investigator Quantiplex Pro FLX plate.

Principle and procedure

The Investigator Quantiplex Pro FLX 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

The Investigator Quantiplex Pro FLX Kit also 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 FLX Kit

Target	Amplicon length (bp)	Channel	Ploidy	Copy number
Human target, small autosomal (Human)	91	FAM	Diploid	Multi-copy
Human target, large autosomal (Degradation)	353	ATTO 550/TAMRA	Diploid	Multi-copy
Human male target (Male)	81	ATTO 647N/CY5	Haploid	Multi-copy
Internal PCR control	434	JOE	n/a	Synthetic fragment

Quantiplex Pro FLX Reaction Mix

The Quantiplex Pro FLX 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 3 minutes to 98°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 FLX 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 FLX 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 QuantStudio 5 Real-Time PCR System, Applied Biosystems 7500 Real-Time PCR System for Human Identification, and the Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems, QIAGEN has developed a set of template files. Download the template files from the **Resources** tab of the product page.

Description of protocols

Protocols for the following cyclers are provided in this handbook.

- QuantStudio 5 Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System for Human Identification
- Applied Biosystems 7500 Real-Time PCR System

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

- Microplate Vortex Mixer
- Real-time thermal cycler
  - QuantStudio 5 Real-Time PCR System
  - Applied Bioystems 7500 Real-Time PCR System for Human Identification
  - Applied Biosystems 7500 Real-Time PCR System

Material

- Pipettes and pipette tips
- qPCR adhesive plate foil, perforated (100) (cat. no. 386055)
- qPCR adhesive plate foil (100) (cat. no. 209003)
- Optical film compression pad (e.g., VWR cat. no. 76637-856)

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
- Nuclease and DNA-free water (e.g., PCR Water (10 x 1 mL), cat. no. 17000-10)

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 these have not been validated by QIAGEN for DNA quantification using the Investigator Quantiplex Pro FLX Kit.

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

Real-time thermal cycler	Protocol for cycler calibration	Protocol for quantification
QuantStudio 5 Real-Time PCR System	on page 24	on page 30
Applied Biosystems 7500 Real-Time PCR System for Human Identification	on page 50	on page 80
Applied Biosystems 7500 Real-Time PCR System	on page 66	on page 80

Contamination risks

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.

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.

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 FLX Kit is highly sensitive; despite the fact that the reagents contained in the Quantiplex Pro FLX 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 FLX plate.

Calibration of PCR Systems

When using the QuantStudio 5 Real-Time PCR System, or the Applied Biosystems 7500 Real-Time PCR System with Investigator Quantiplex Pro FLX, 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.

General guidelines for automation

Stick to the logic of the manual procedure described in this handbook:

- Open the seals of the plate just before starting a run.
- Do not operate the system in very humid areas (>60%), as lyophilized cakes rehydrate and resolution is problematic.
- Always apply the higher volume first to dissolve the cake.
- Do not move pipette tips into the cake during setup. Adjust z-heights to be above the cake for the first liquid addition. This height must accommodate for loosened cakes that may be upside down in the plate.
- Always vortex the finished plate after sealing. Do not fully rely on automated mixing during setup, as there might be spots of lyophilized material at the side of the walls.

Protocol: Setting Up Investigator Quantiplex Pro FLX Plates

This protocol describes the preparation of reactions in the Investigator Quantiplex Pro FLX plate.

Important points before starting

- Do not open plates in advance. Remove seals just before starting setup.
- Before removing the seals from the plate, make sure lyophilized cakes do not stick to the seals. Tap the plates on the bench to bring all cakes down.
- Loosened cakes, spots on the tube wall, or cakes with slightly varying appearance do not have impact on the performance of the assay.
- Avoid high humidity in the laboratory. We do not recommend opening plates in an environment with over 60% humidity.
- We strongly recommend to always start with the addition of the higher volume during setup. If using >9 µL sample input, add sample first to dissolve the lyophilized cake, and then fill up to the final volume with water. Pipette above the cake surface.
- 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.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored between 2–8°C for at least 1 week.
- Reaction setup can be done at room temperature (15–25°C).

Procedure: Preparing the optical plate for reaction setup

1. Prepare fresh serial dilutions of the 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 3. Serial dilution of Control DNA M1

Serial dilution of Control DNA M1 (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" on page 110.

2. Thaw samples for quantification if they were stored frozen.
3. When not using the entire plate, break the desired number of 8-tube strips from the plate ("Breaking of Investigator Quantiplex Pro FLX Plates." on page 22).

   Note: The unused part of the sealed PCR plate should be stored in the aluminum bag with desiccant and used up within 4 weeks.

4. Peel the perforated seals from the plate, or groups of 8-tube strips ("Peeling of Investigator Quantiplex Pro FLX 8-tube strips." on page 23). When using the entire plate, seals can be removed with the help of 1/3 of an adhesive foil ("Peeling of the entire Investigator Quantiplex Pro FLX Plate." on page 23). Start setting up reactions immediately.
5. If using less than 9 µL sample input, start the setup with adding the volume of water required to give a final volume of 18 µL added to the cake.
6. Add 18 µL water or Nucleic Acid Dilution Buffer to the NTC (No Template Control) wells.

7. Add 16 µL water or Nucleic Acid Dilution Buffer and 2 µL control DNA M1 dilutions to the individual wells of the standard row. It is required to run duplicates of the control DNA dilutions for each assay and on each reaction plate.

Table 4. Example plate setup of reactions

Well contents

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

All content in ng/mL. **NTC**, no-template control; **UNK**, unknown sample.

8. Add samples to reaction wells (variable input volumes from 1–18 µL).

Table 5. Reaction setup

Component	1 reaction
Quantiplex Pro FLX Reaction Mix and Primer Mix	Lyophilized cake
Sample	Variable (1–18 µL)
Water	Fill up to 18 µL
Total reaction volume	**20 µL***

* Lyophilized cake + 18 µL input volume will yield a final PCR reaction volume of 20 µL.

Note: If your sample input volume is not 2 µL, a correction factor needs to be applied to your sample concentration. You can also use the QIAGEN Quantification Assay Data Handling Tool for automated concentration correction calculation.

Table 6. Correction factors for calculations of sample concentrations

Sample input volume (µL)	Concentration correction factor (calculated concentration divided by ...)
1	0.5
2	–
3	1.5
4	2
5	2.5
6	3
7	3.5
8	4
9	4.5
10	5
11	5.5
12	6
13	6.5
14	7
15	7.5
16	8
17	8.5
18	9

The concentration correction factor can be obtained by dividing the sample input volume by 2.

9. Add water where still needed to all samples or controls to result in a total liquid volume of 18 µL. Never run reactions with less than 18 µL.
10. Seal the plate or the 8-tube strips with the qPCR adhesive plate foil and vortex for 5 s.
11. Centrifuge briefly.
12. Continue with the amplification and analysis as described for the corresponding thermal cycler.

    Note: Always use a compression pad if using the entire plate. If using 8-tube strips, please follow the PCR cycler manufacturer's recommendation for the use of strips.

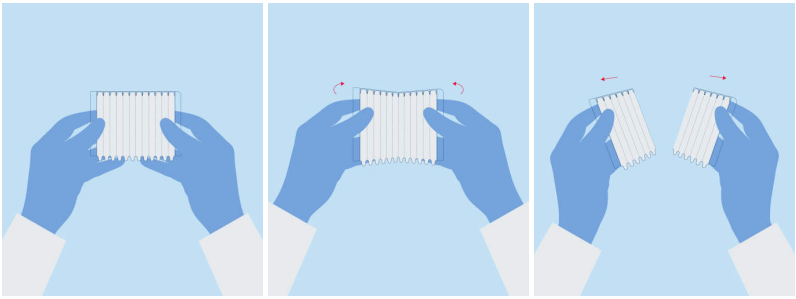


Figure 2. Breaking of Investigator Quantiplex Pro FLX Plates.

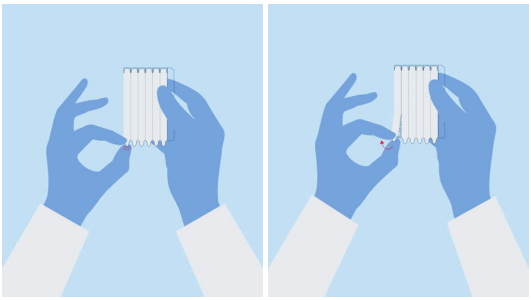


Figure 3. Peeling of Investigator Quantiplex Pro FLX 8-tube strips.

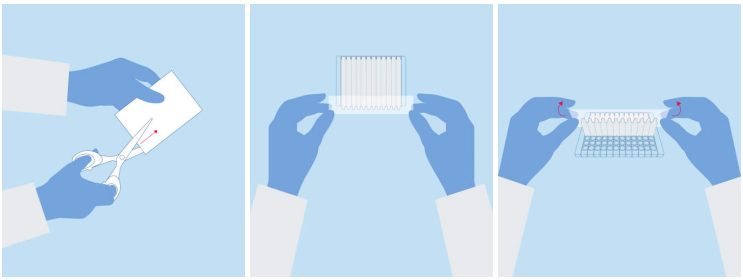


Figure 4. Peeling of the entire Investigator Quantiplex Pro FLX Plate.

Protocol: Cycler Calibration Using the Investigator Quantiplex Pro Calibration Kit and QuantStudio 5 Real-Time PCR System

This protocol is optimized for use of the Investigator Quantiplex Pro Calibration Kit on the QuantStudio 5 Real-Time PCR System using QuantStudio Design and Analysis Software (v1.4.3 or higher).

For general instructions on instrument calibration, refer to the *QuantStudio 5 Real-Time PCR Instrument User Guide*.

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 QuantStudio 5 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. Avoid touching plate wells, optical adhesive films, and bottoms of the plates.
- We strongly recommend performing the Region-of-Interest (ROI) calibration and background calibration before you perform the custom dye calibration with the Investigator Quantiplex Pro Calibration Kit. Further details on how to perform ROI calibration and

background calibration can be found in the *QuantStudio 5 Real-Time PCR Instrument User 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 7.

Table 7. Dilution scheme for Investigator Quantiplex Pro Calibration Kit standards

Component					
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°C 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 QuantStudio 5 Real-Time PCR System

1. Start cycler and enter **Settings** menu on the Home screen. Select **Maintenance and Service**.
2. Click **Calibrations**, then **Custom**, and **Custom Dye**.
3. Select **Add Custom Dye**.
4. Enter **QPP_FAM** as a new dye name, confirm that **Reporter** is selected, and click **Save**.
5. Load the **QPP_FAM** plate prepared in Procedure A into the instrument.
6. Enter "60°C" for the calibration temperature.
7. Press **Start**.

8. When the calibration is complete, the screen will display "Calibration Complete". Press **View Results** to review details. The QPP_FAM calibration spectra should show the highest signals in filter x1-m1. For other QPP dyes see Table 8.

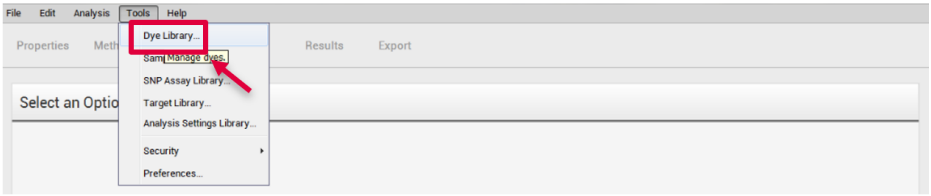
Table 8. Calibrated dye filter signal

Custom dye	Highest signal in filter
QPP_FAM	x1-m1
QPP_JOE	x2-m2
QPP_ATTO550	x3-m3
QPP_ROX	x4-m4
QPP_ATTO647N	x5-m5

9. Repeat steps 1 to 8 to calibrate for the following dyes:
   - QPP_JOE
   - QPP_ROX
   - QPP_ATTO550
   - QPP_ATTO647N

Procedure C: Adding the Quantiplex Pro dyes to QuantStudio Design and Analysis Software (v1.4.3 or higher)

1. Open the QuantStudio Design and Analysis Software and select **Tools** > **Dye Library**.

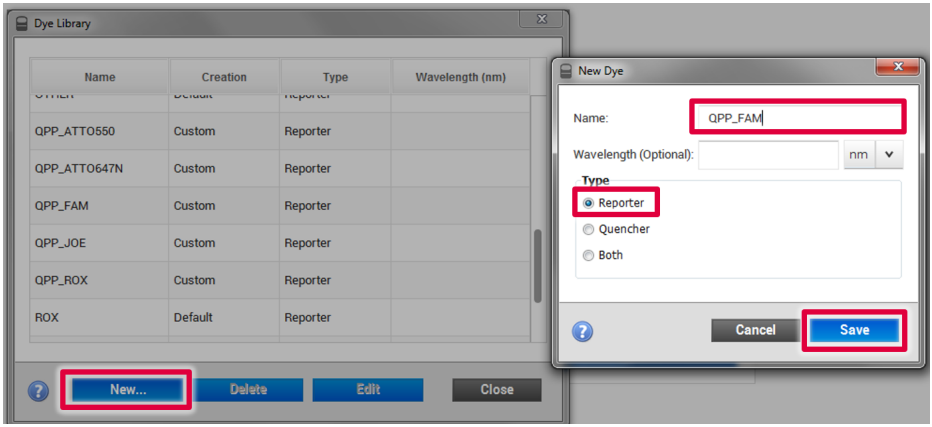


2. Click **New** and add all QPP dyes:

   - QPP_FAM
   - QPP_JOE
   - QPP_ROX
   - QPP_ATTO550
   - QPP_ATTO647N

   Important: Dye names entered in the Dye Library must match exactly the dye names entered on the QuantStudio 5 during custom dye calibration.

Confirm that **Reporter** is selected for each dye and then click **Save**.



Protocol: Quantification of DNA Using the QuantStudio 5 Real-Time PCR System

This protocol is optimized for use of the Investigator Quantiplex Pro FLX Kit on the QuantStudio 5 Real-Time PCR System using QuantStudio Design and Analysis Software (v1.4.3 or higher).

For general instructions on instrument setup and other software versions, refer to the *QuantStudio 5 Real-Time PCR Instrument User Guide*.

Important points before starting

- When using the QuantStudio 5 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.
- Use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (e.g., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure A: Create an experiment

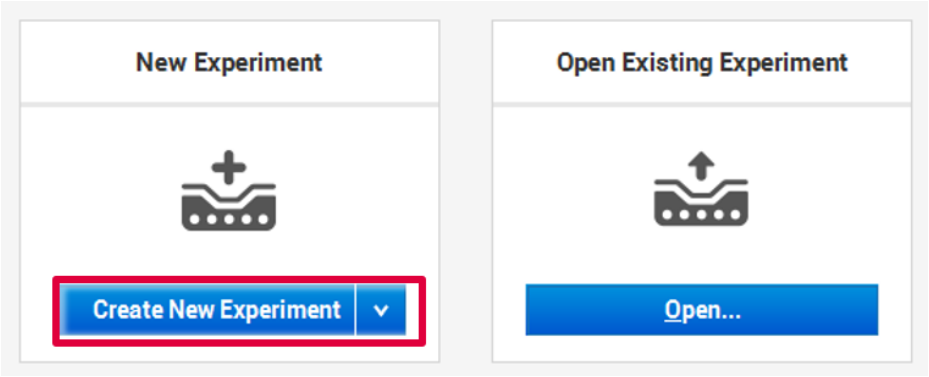
1. Open the QuantStudio Design and Analysis Software (v1.4.3 or higher).
2. Several options to set up a run are described:
    - If you are using a template file and doing a manual plate setup, proceed to step 18 to define DNA sample names and assign to the plate layout. Then proceed to step 23 to

start the run.

- If you are using a template file and also use a **.txt** setup file for plate setup, proceed to "Procedure B: Run setup using a template file and a plate setup file" on page 44.
- If you are not using a template file, proceed with step 8 on page 36.

The template file loads all of the settings needed to start an Investigator Quantiplex Pro FLX run, including the standard curve settings, the cycling profile, and the targets needed for fluorescence acquisition. Download the template files from the **Resources** tab of the product page.

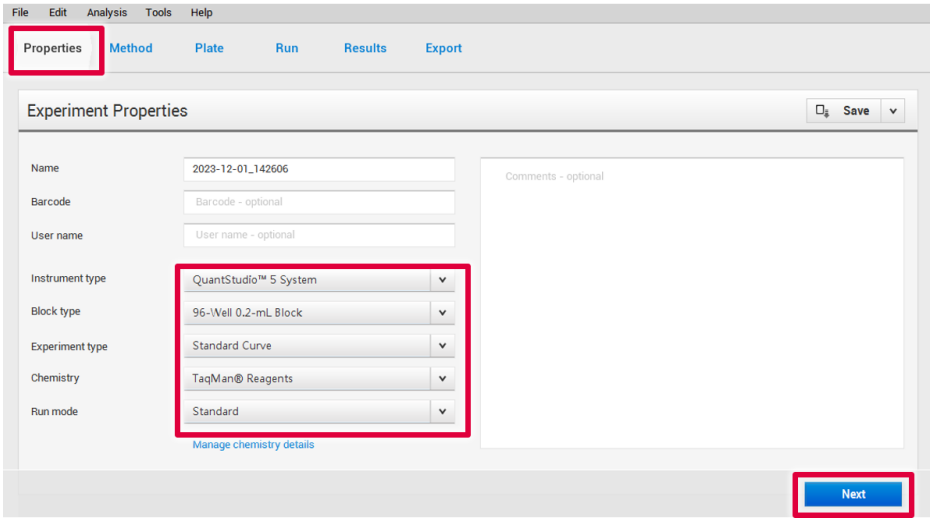
3. If you are not using a template file, select **Create New Experiment**.



4. In the **Properties** tab, confirm the following settings in **Experiment Properties**:

   - Instrument type: **QuantStudio™ 5 System**
   - Block type: **96-Well 0.2 mL Block**
   - Experiment type: **Standard Curve**
   - Chemistry: **TaqMan® Reagents**
   - Run mode: **Standard**

   And then, click **Next**.



5. In the **Method** tab, adjust thermal profile by changing the holding times to those in Table 9. Change **Volume** to **20 µL**.

   Data acquisition should be performed during the combined annealing/extension step. Clicking the camera symbol enables data acquisition for the step.

   Click **Next**.

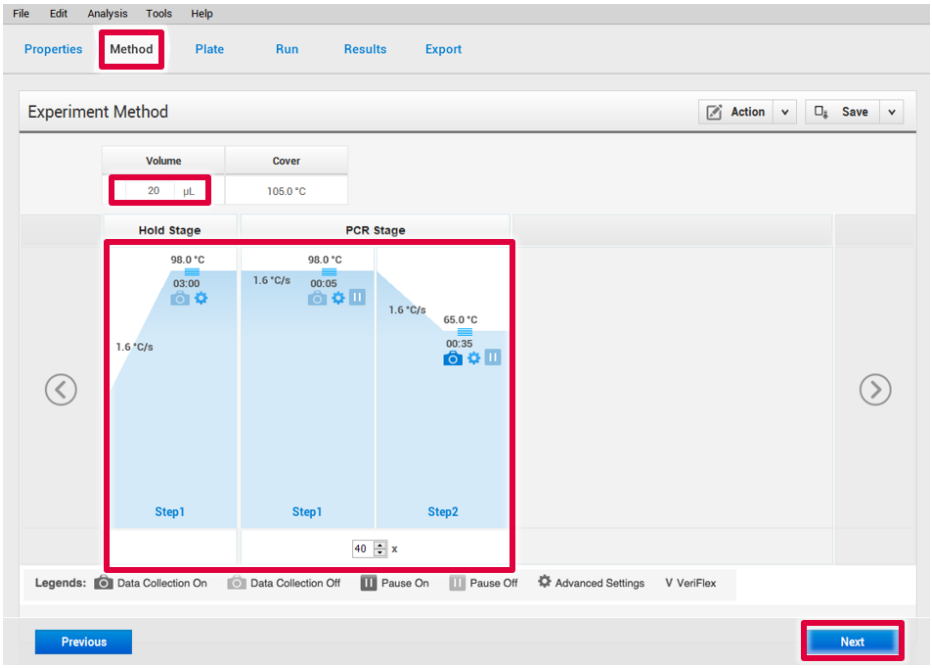
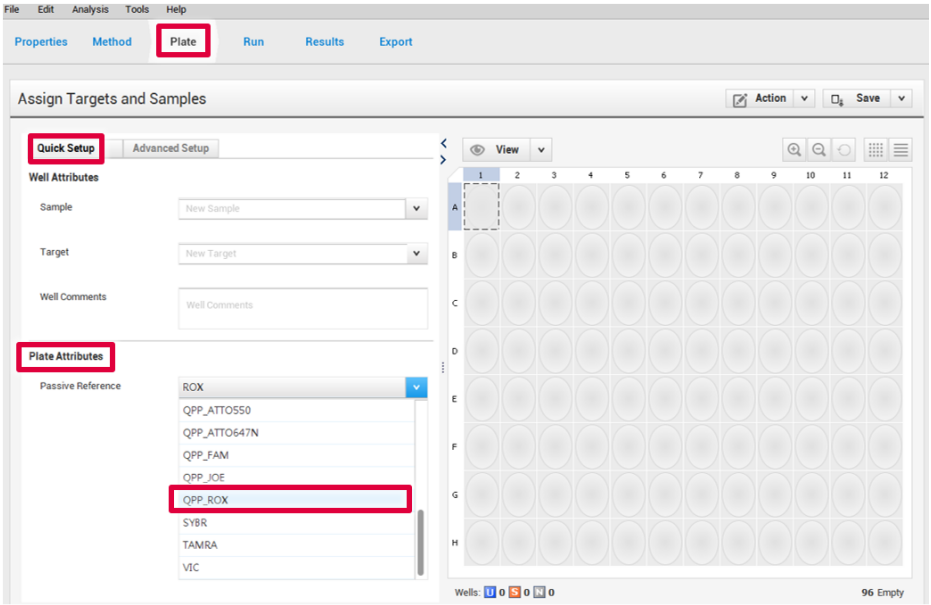


Table 9. Cycling protocol using QuantStudio 5 Real-Time PCR System

Step	Temperature (°C)	Time	No. of cycles	Remarks
Initial PCR activation step	98	3 min	–	PCR requires an initial incubation at 98°C to activate the DNA polymerase
Denaturation	98	5 s	40	–
Combined annealing/extension	65	35		Perform fluorescence data collection

Note: Always use a compression pad if using the entire plate. If using 8-tube strips, please follow the PCR cycler manufacturer's recommendation for the use of strips.

6. In the **Plate** tab, select **Quick Setup**. And then, in **Plate Attributes** > **Passive Reference**, select **QPP_ROX**.



7. Still in the **Plate** tab, select **Advanced Setup**. Click **Add** 3 times, and then add the targets listed in Table 10 below.

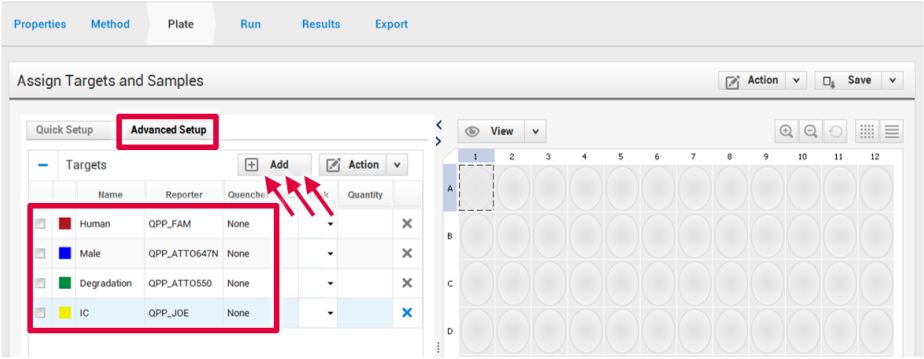
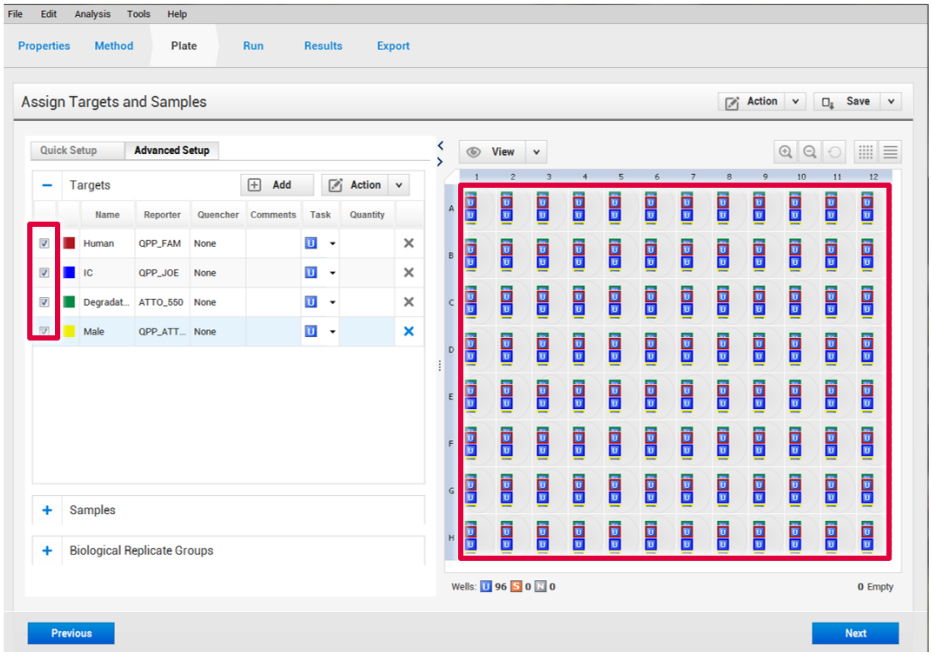


Table 10. Assign targets and samples

Name	Reporter	Quencher
Human	QPP_FAM	None
Male	QPP_ATTO647N	None
Degradation	QPP_ATTO550	None
IC	QPP_JOE	None

8. Select the wells in use and assign all 4 targets by marking the boxes on their left.

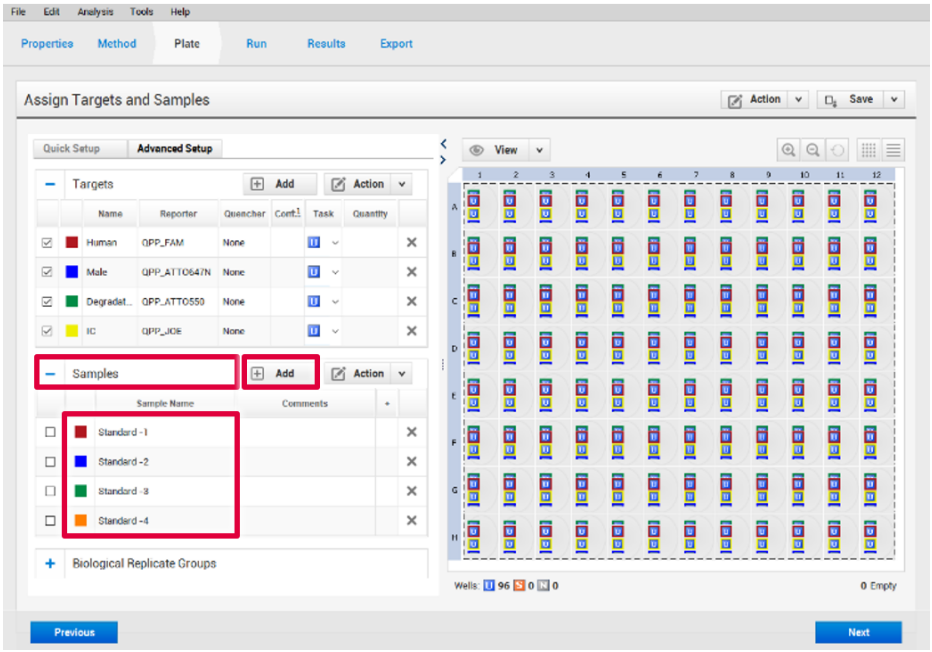
   Important: Do not highlight the wells that are not in use (i.e., those without reaction mix). Including unused wells will significantly impact the scale of the x and y axes when viewing the data.



9. While in **Advanced Setup**, open the **Samples** section and click **Add** to enter the sample names for the standards (e.g., Standard -1, Standard -2, etc.; or Std1, Std2, etc.) and NTCs.

   Note: Precise naming of standards is required for proper subsequent analysis with the QIAGEN Quantification Assay Data Handling Tool.

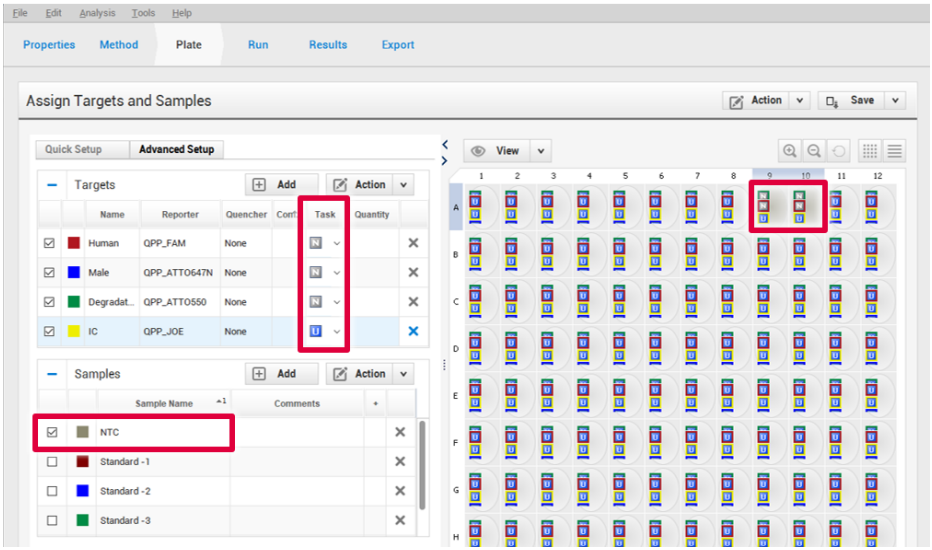
   Important: If replicates are needed, they should be assigned before you proceed to the next step. Define replicates by using the same sample name for 2 or more wells or by using the **Biological Replicate Groups** panel.[1]



[1] Instructions for using the Biological Replicate Groups panel are not covered by this handbook.

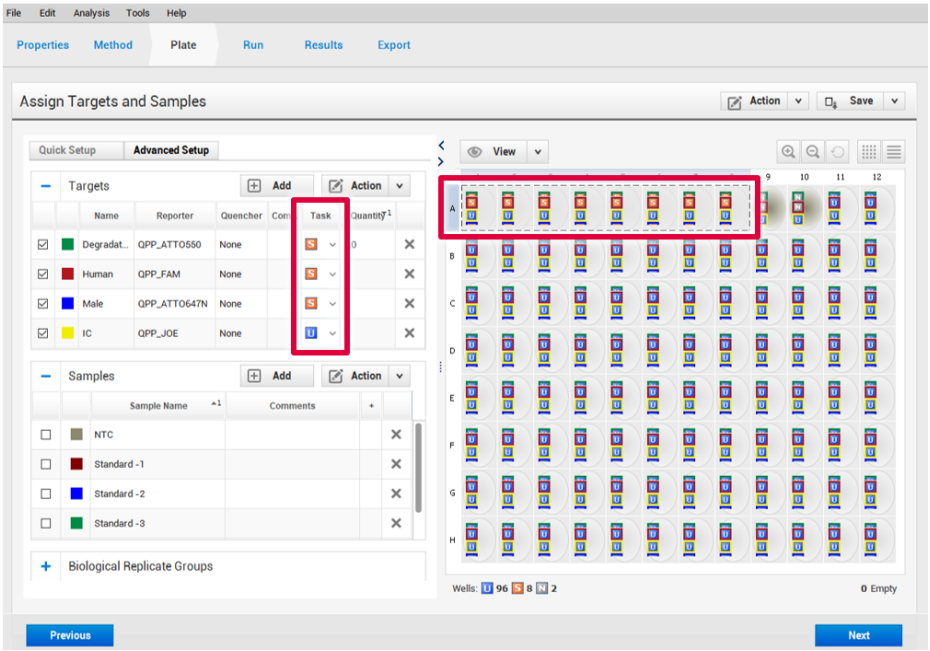
10. Select the wells for the no-template controls (NTC) and flag them as negative control in **Task** by selecting the gray **N** button.

    Note: Leave the **IC** (**QPP_JOE**) task for NTC reactions set to **U** ("unknown"). In **Samples**, select the sample name **NTC**.

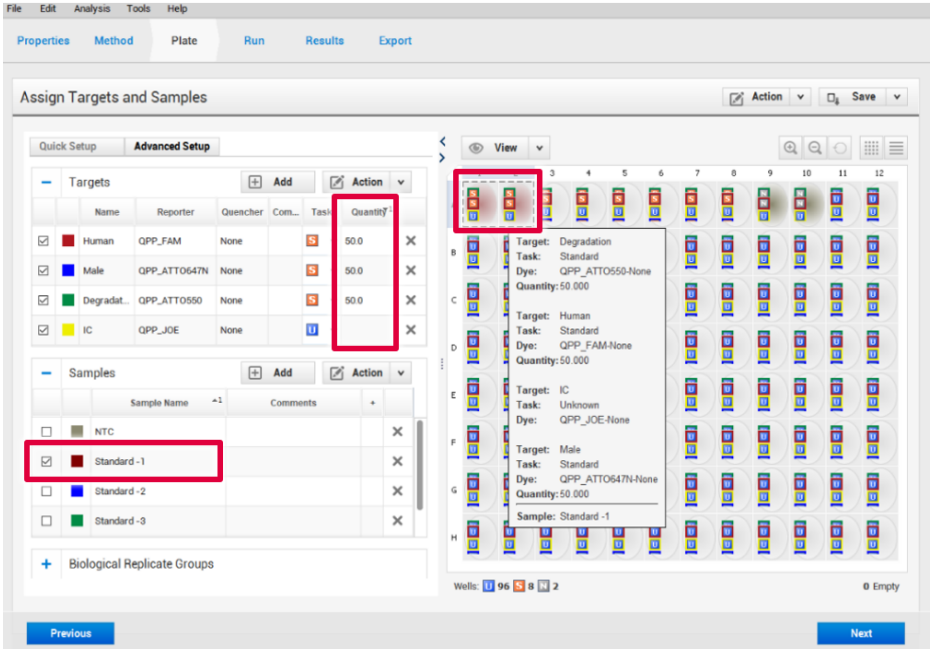


11. Select the wells for the standard curve and flag them in the **Task** column as standard by selecting the orange **S** button.

    Note: Leave the **IC** (**QPP_JOE**) task for standard reactions set to **U** ("unknown").

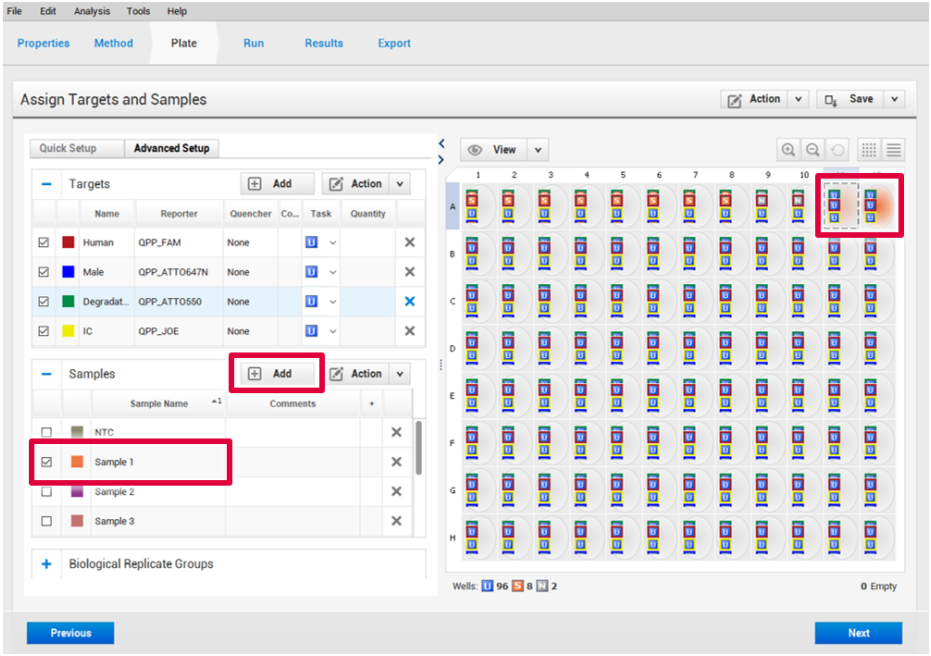


12. Enter the concentration and select a sample name for each standard. Enter the quantity of DNA in the wells according to "Protocol: Setting Up Investigator Quantiplex Pro FLX Plates" on page 18.

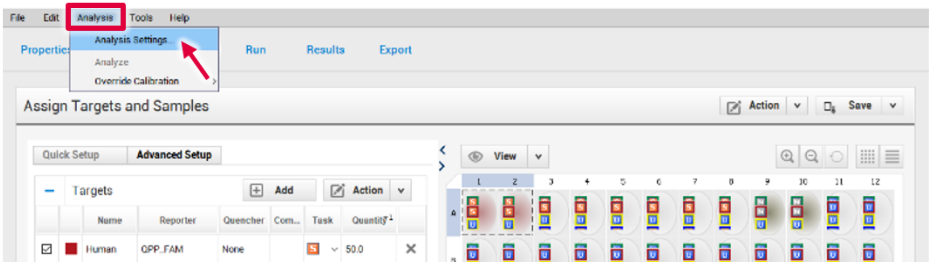


13. In the **Samples** section, click **Add** to enter the names of the DNA samples.

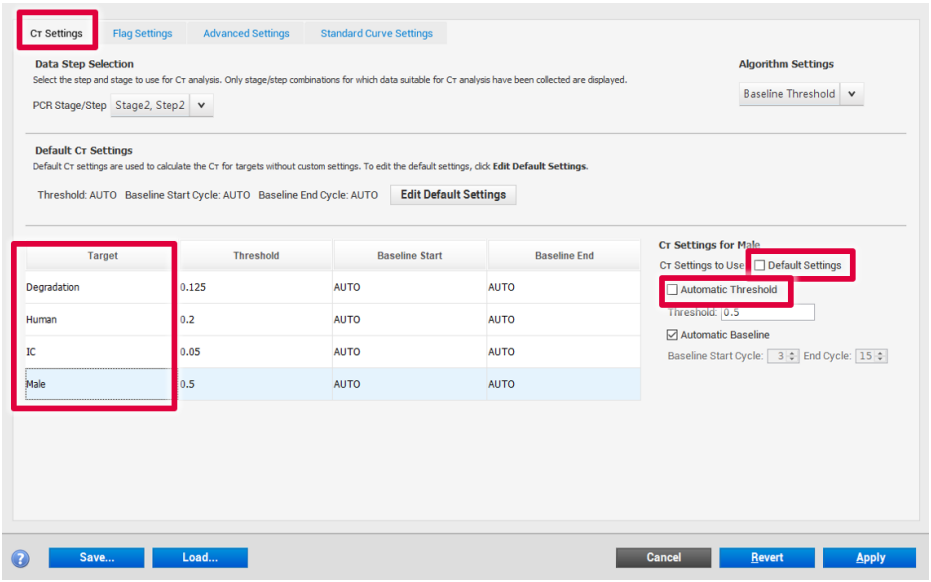
    Assign DNA samples to the plate layout by clicking on the wells and checking the appropriate box on the left **Samples** panel.



14. At the top toolbar, select **Analysis** > **Analysis Settings**.



15. In the **CT Settings** tab, under **Default CT Settings**, select the first Target. At the leftmost side of the window, uncheck the boxes beside **Default Settings** and **Automatic Threshold**. Do the same for the rest of the targets.

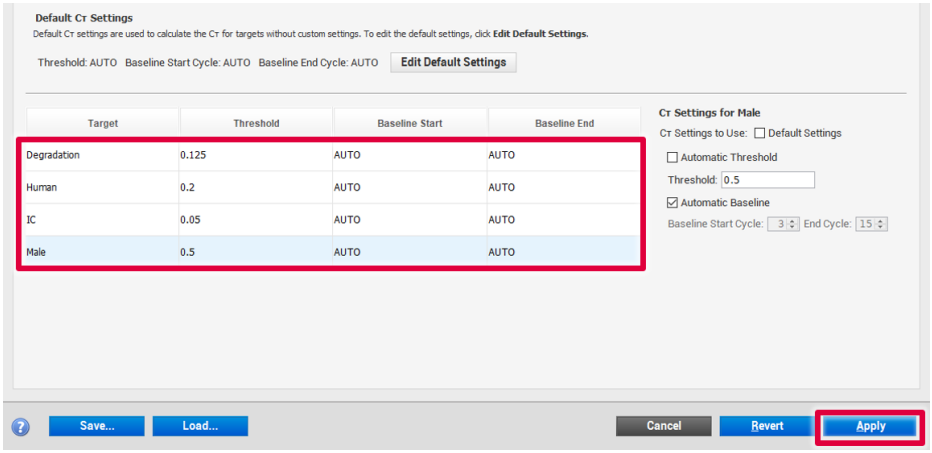


16. Enter the following settings for each target (Table 11), and then click **Apply**:

Table 11. CT settings

Target	Threshold	Baseline
Degradation	0.125	**Auto**
Human	0.2	**Auto**
IC	0.05	**Auto**
Male	0.5	**Auto**

Important: Verify that option for **Automatic Threshold** is deselected for all targets. Setting the appropriate threshold value may require further internal validation in your facility.



17. **Optional**: Before running the reaction plate, you can save the setup as an EDT template file:

a. Click **File** > **Save as**.

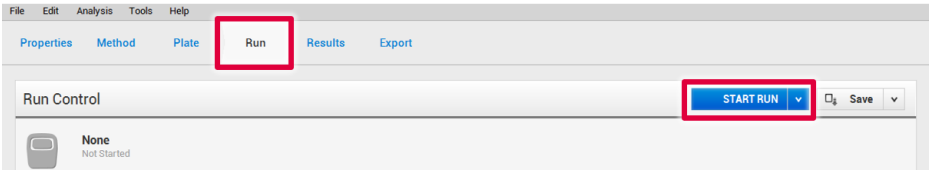
b. Enter a name for the template document.

c. Click **Save** again.

If you do not want to save the setup as a template, proceed to the next step.

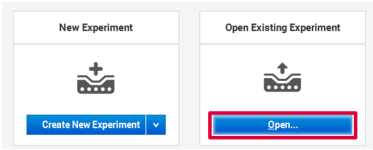
18. Load the plate into the instrument. Ensure that position A1 on the plate is on the top-left side of the tray.

19. Select the **Run** tab, and then click **Start Run**.



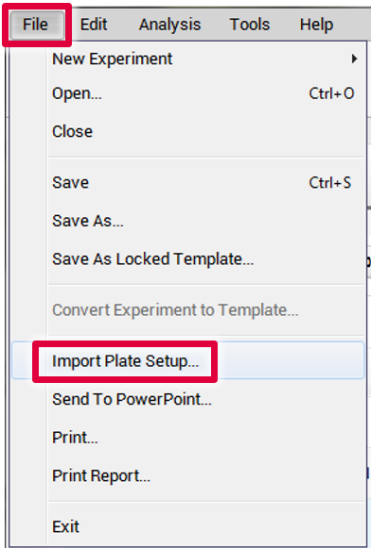
Procedure B: Run setup using a template file and a plate setup file

1. Open the QuantStudio Design and Analysis Software (v1.4.3 or higher). Select **Open**.



2. Select the EDT template file.

3. Click **File** > **Import Plate Setup**, select the plate setup **.txt** file, and then click **Apply**.



4. After successfully importing the plate setup, load the plate into the instrument. Ensure that position A1 of the plate is on the top-left side of the tray.
5. Select the **Run** tab, and then click **Start Run**.

Procedure C: Data analysis

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

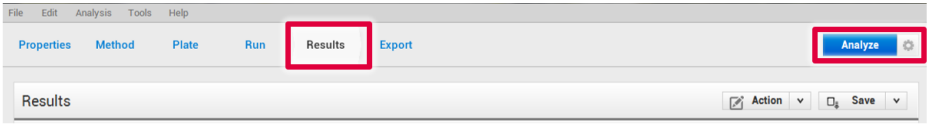
1. Open the run file using the QuantStudio Design and Analysis Software (v1.4.3 or higher).
2. Before a standard curve can be created, standards must first be defined.

Note: If standards had been defined before the run was started, proceed to step 4, below.

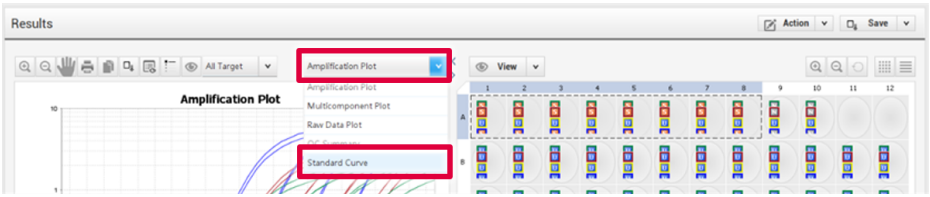
3. Go to **Plate** and select **Advanced Setup**. Define the wells that contain DNA standards as explained in steps 10 and 11 of "Procedure A: Create an experiment" (starting).

4. Select **Analysis** > **Analysis Settings** from the top toolbar and confirm that settings are set as described in Table 8.

5. Click the **Results** tab, select the wells to be analyzed, and click **Analyze**.

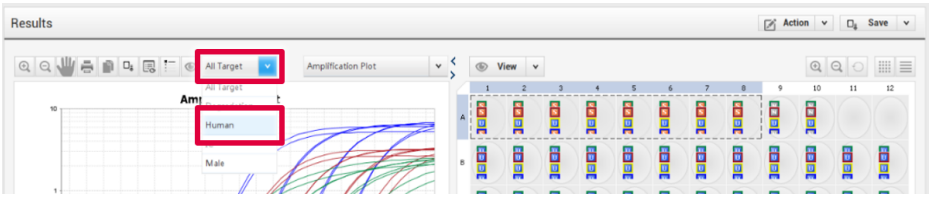


6. To view the standard curve, select **Amplification Plot** > **Standard Curve** from the drop down menu.

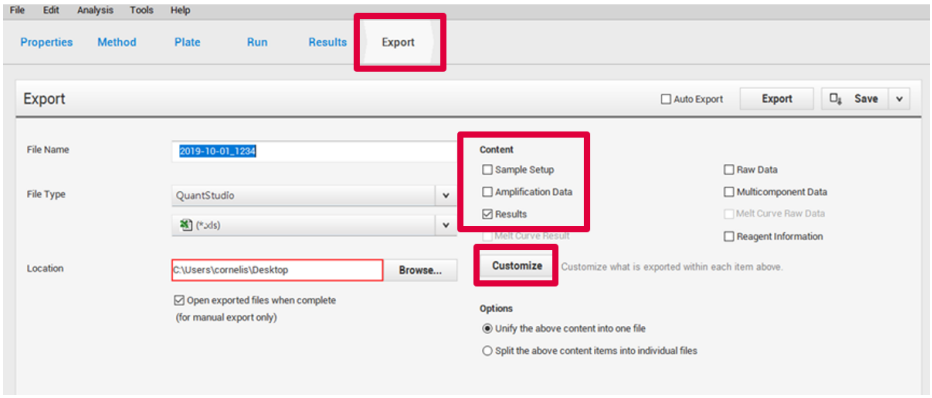


7. Review standard curves for each target by selecting **All Target**.

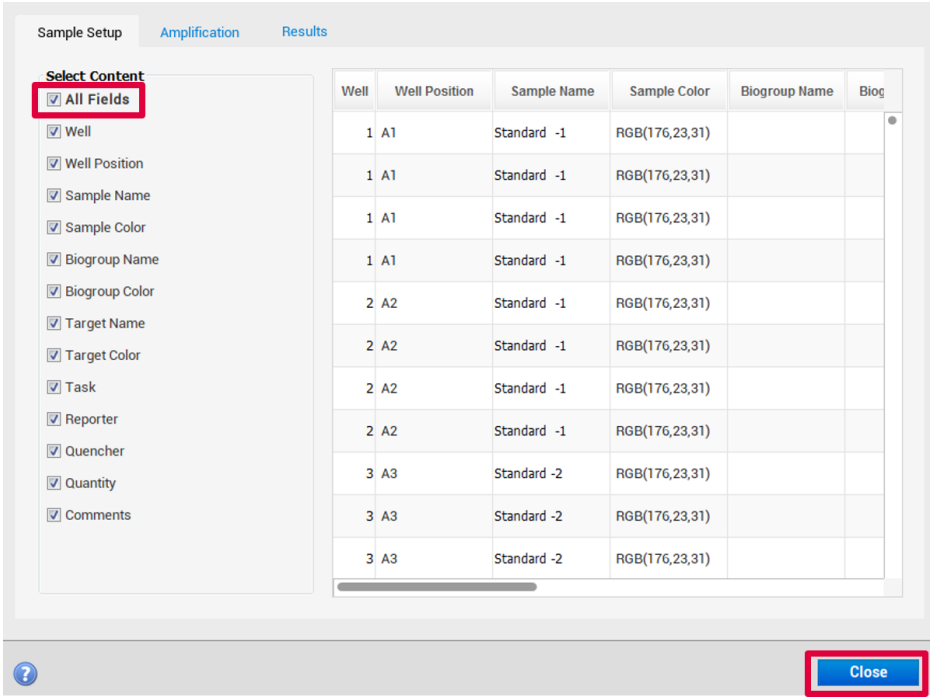
   View the C_T values for the quantification standard reactions, and the calculated regression line, slope, y-intercept, and R^2 values.



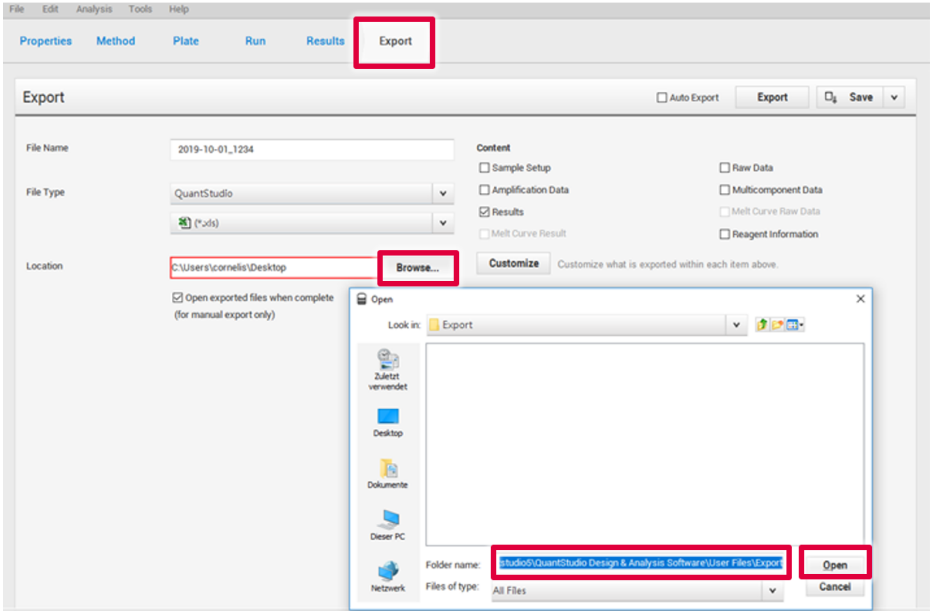
8. To export and save the results report, go to **Export** in the top bar. Ensure that the **Results** box is checked under **Content**. Click **Customize**.



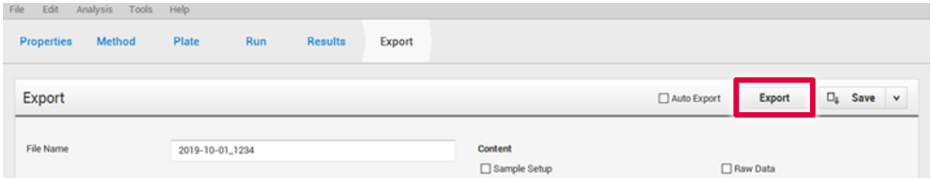
9. Ensure **All Fields** are selected for export. Click **Close**.



10. In **Export**, click **Browse**, choose where you want to save your file, then click **Open**.



11. Click **Export**. Save the file in ***.xls** format.



12. To interpret results, see “Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool” on page 93.

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 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 12.

Table 12. Dilution scheme for Investigator Quantiplex Pro Calibration Kit standards

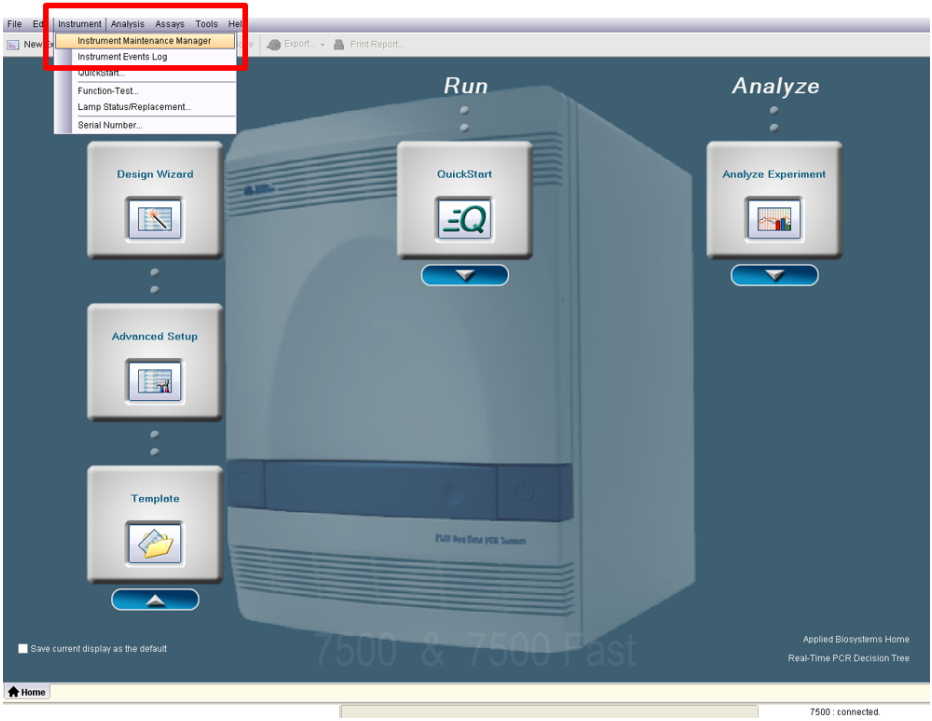
Component	Volume (µL)				
Calibration Standard FAM	23	–	–	–	–
Calibration Standard JOE	–	23	–	–	–
Calibration Standard ATTO 550	–	–	23	–	–
Calibration Standard ROX	–	–	–	23	–
Calibration Standard ATTO 647N	–	–	–	–	23
Quantiplex Pro Calibration Buffer	2277	2277	2277	2277	2277
Total volume	**2300**	**2300**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

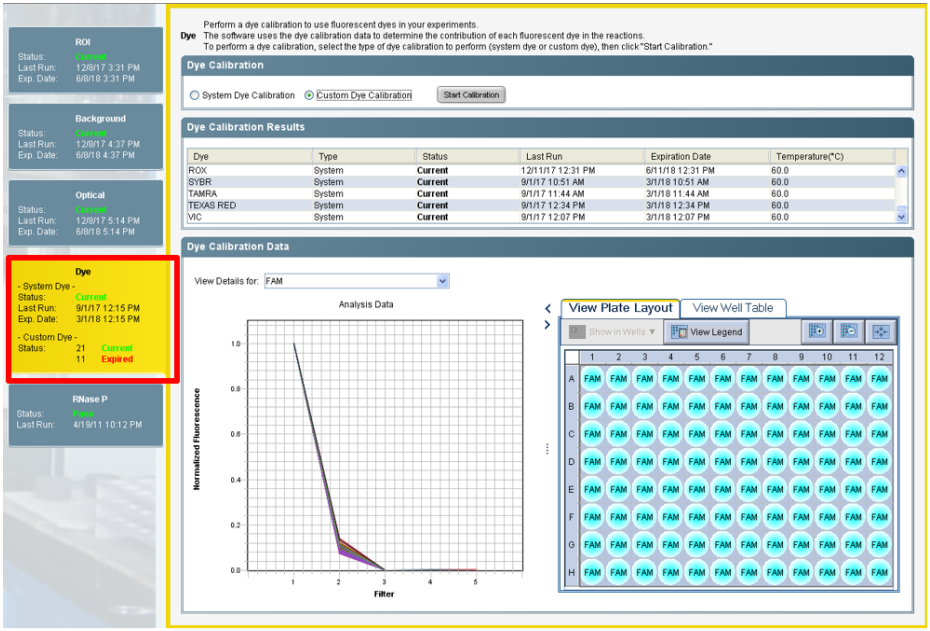
6. Dispense 20 µL of diluted Investigator Quantiplex Pro Calibration Kit standard FAM into the assigned dye-specific optical 96-well reaction plate.
7. Close the plate with an optical adhesive film.
8. Repeat these steps for each of the other remaining Investigator Quantiplex Pro Calibration Kit standards (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°C 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

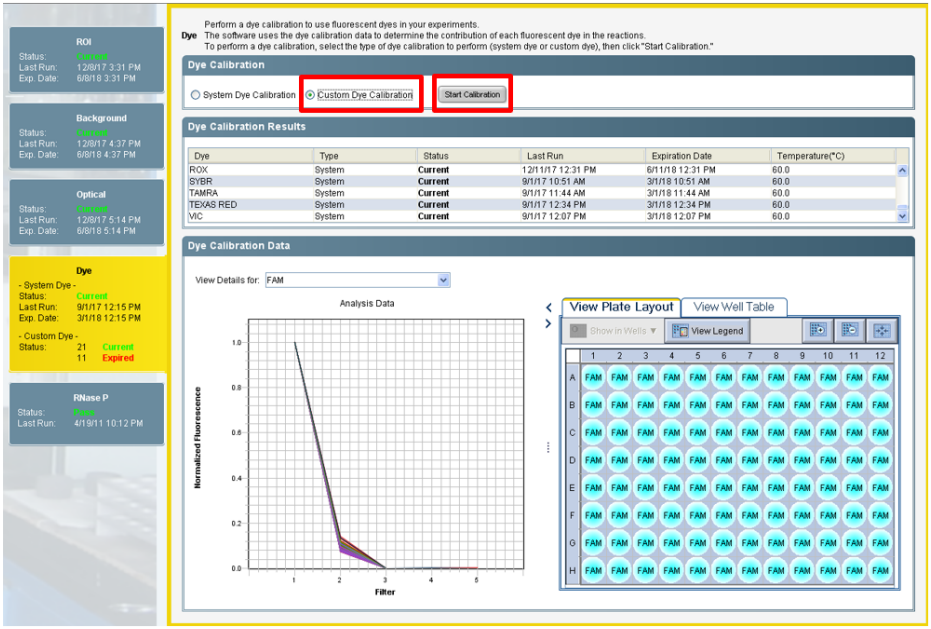
1. Open the HID Real-Time PCR Analysis Software v1.1 or 1.2 and select the **Instrument Maintenance Manager** from the instrument menu.



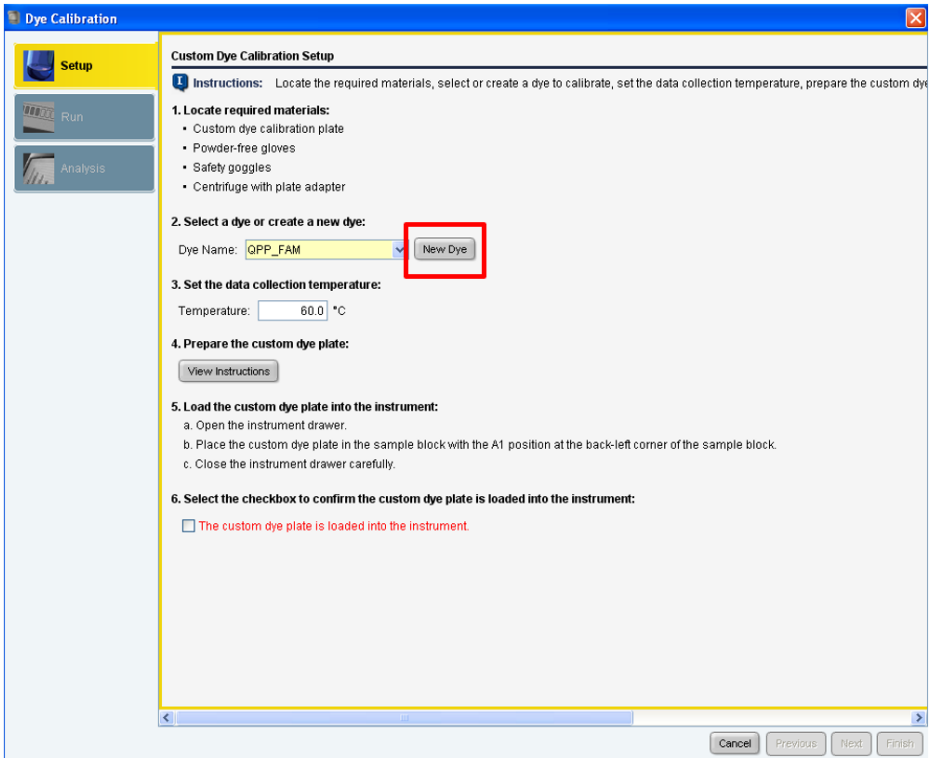
2. Click the **Dye** section on the left panel.



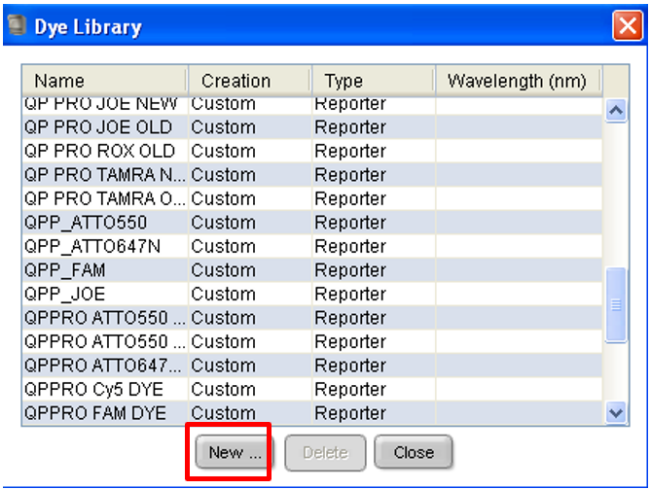
3. Select **Custom Dye Calibration** and then click **Start Calibration**.



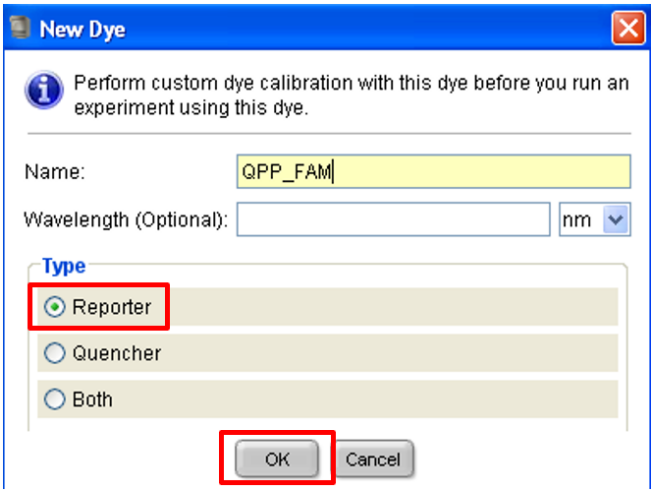
4. Select **New Dye** in the **Dye Calibration** window.



5. In the **Dye Library** window click **New**.

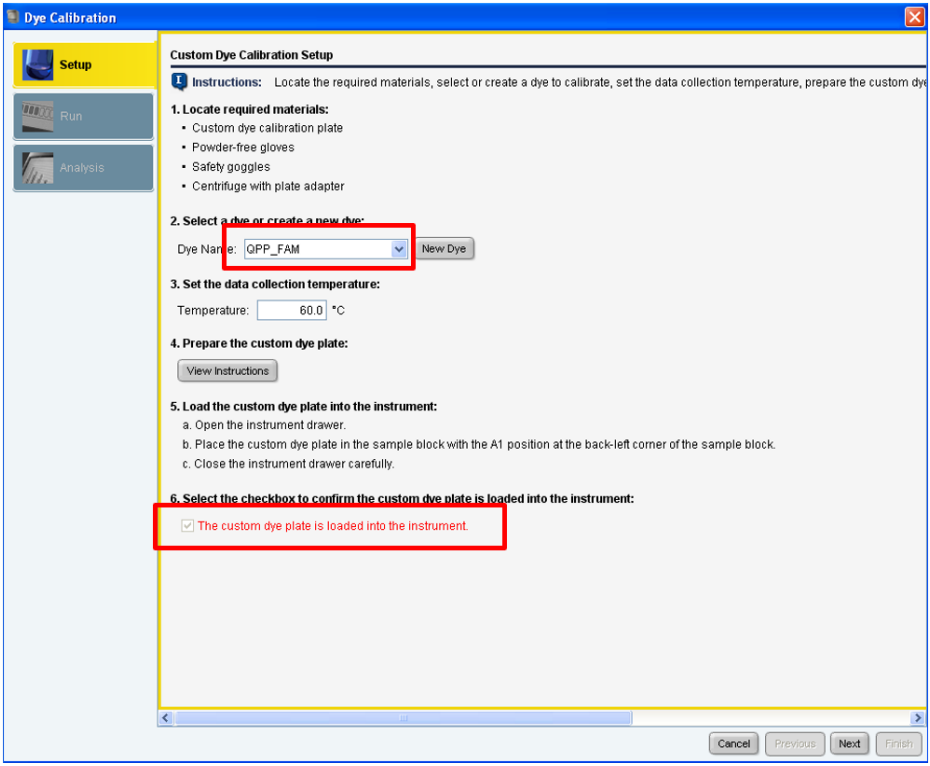


6. Set names for the new custom dyes. To differentiate the new dyes from standard dyes, we recommend using the prefix QPP (for Quantiplex Pro). Enter "QPP_FAM" as a new dye name, select **Reporter**, and click **OK**.

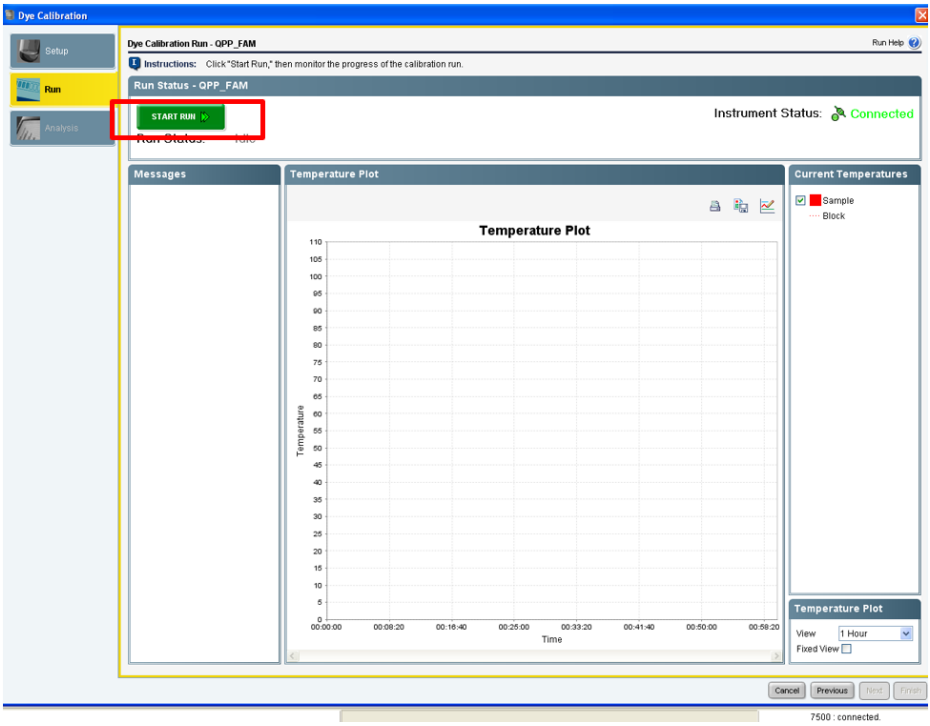


7. Repeat step 6 to set new dye names for:
   - QPP_JOE
   - QPP_ATTO550
   - QPP_ATTO647N

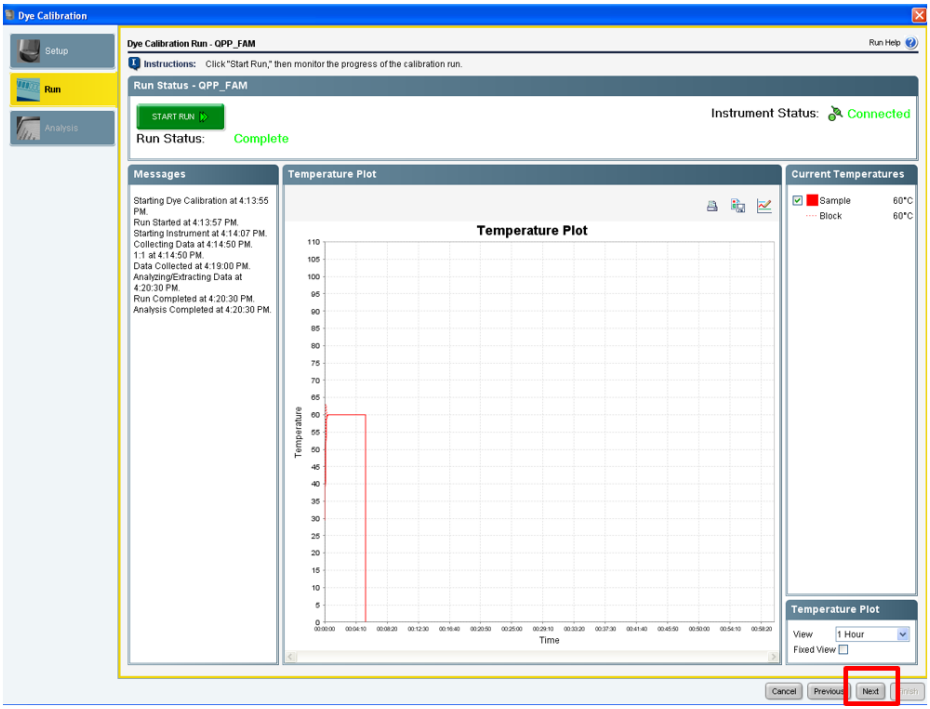
8. Select **QPP_FAM** in the **Custom Dye Calibration Setup** window. Load the QPP_FAM plate prepared in Procedure A into the instrument. Confirm by checking the tick box for **The custom dye plate is loaded into the instrument**.



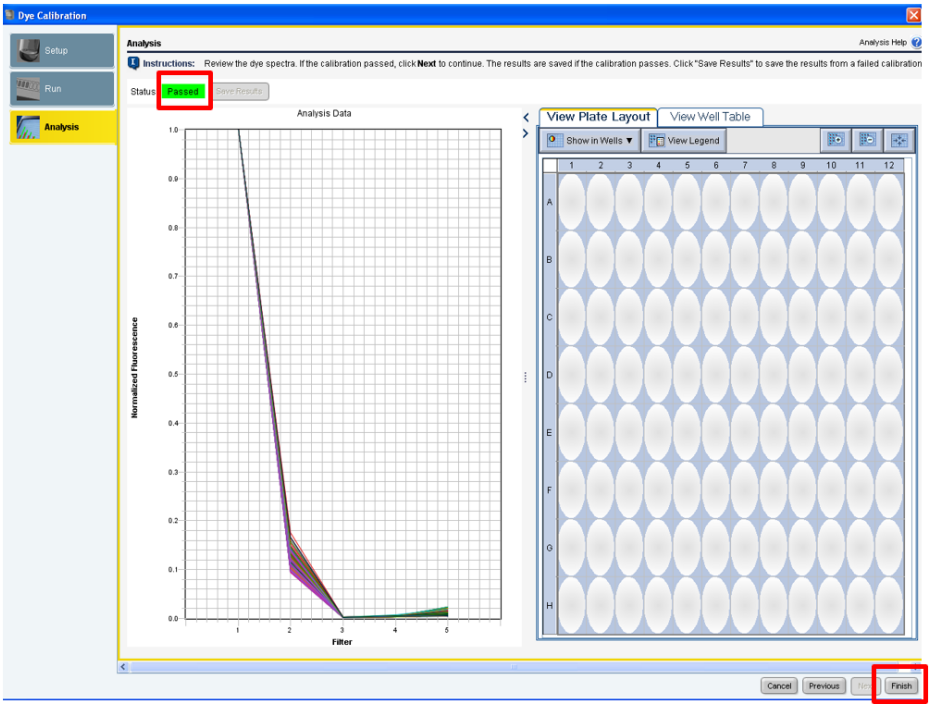
9. Click **Next** and then **Start Run**.



10. After the calibration run has finished, click **Next**.



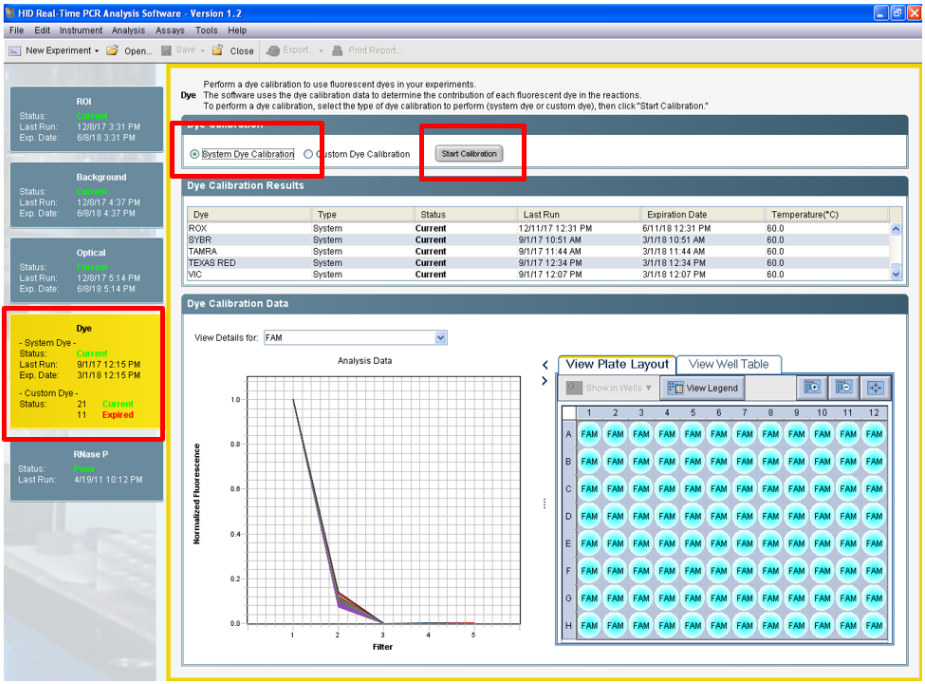
11. The spectra and the status (e.g., "Passed") will be displayed. Click **Finish**.



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

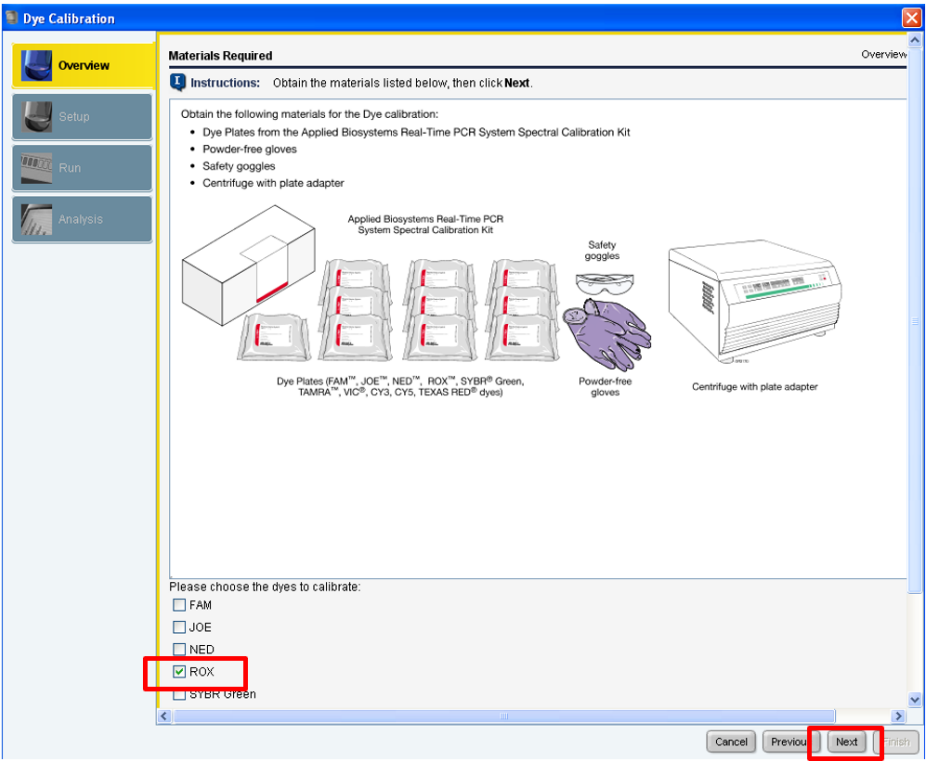
    - QPP_JOE
    - QPP_ATTO550
    - QPP_ATTO647N

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



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

15. In the **Materials Required** window select only **ROX** and click **Next**.



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 lower 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 13.

Table 13. 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 or Higher

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 or higher.

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 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 14.

Table 14. Dilution scheme for Investigator Quantiplex Pro Calibration Kit standards

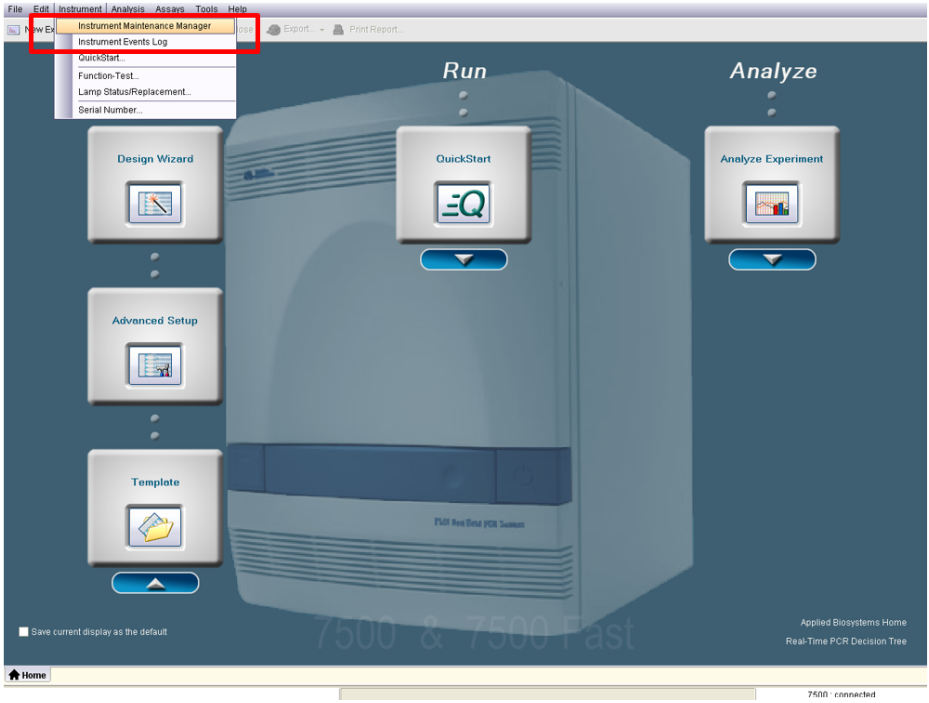
Component	Volume (µL)				
Calibration Standard FAM	23	–	–	–	–
Calibration Standard JOE	–	23	–	–	–
Calibration Standard ATTO 550	–	–	23	–	–
Calibration Standard ROX	–	–	–	23	–
Calibration Standard ATTO 647N	–	–	–	–	23
Quantiplex Pro Calibration Buffer	2277	2277	2277	2277	2277
Total volume	**2300**	**2300**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

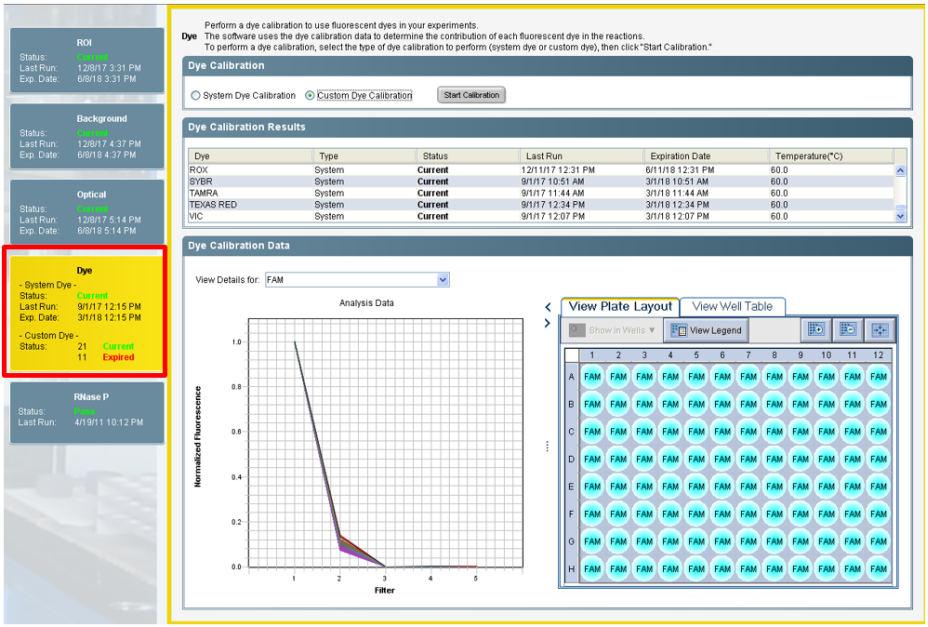
8. Repeat these steps for each of the other remaining Investigator Quantiplex Pro Calibration Kit standards (JOE, ATTO 550, ROX, and ATTO 647N).
9. Centrifuge plates briefly. Always protect plates from light.
10. After calibration, store the calibration plates at –30°C to –15°C in a constant-temperature 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 or higher

1. Open the 7500 Software v2.0.6 or higher and select the **Instrument Maintenance Manager** from the **Instrument** menu.

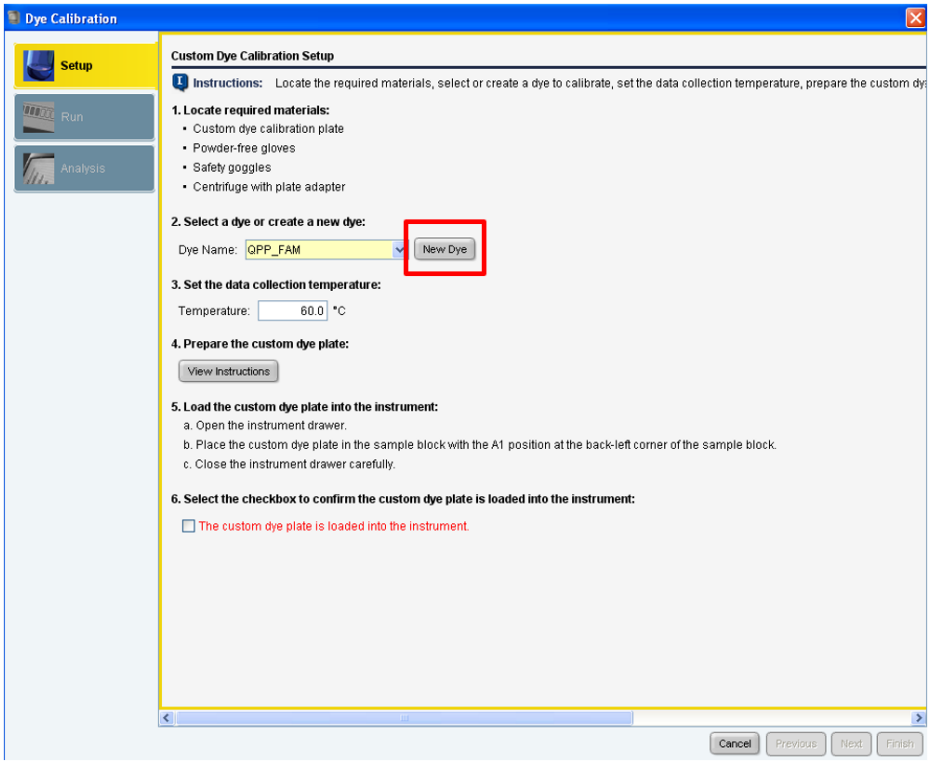


2. Click the **Dye** section in the left panel.

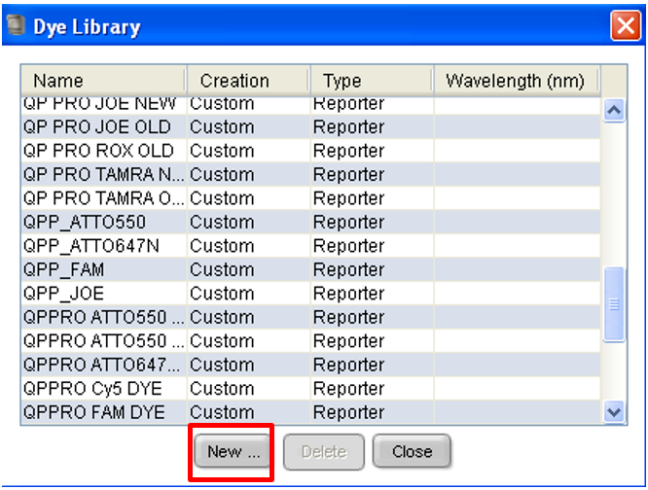


3. Select **Custom Dye Calibration** and then click **Start Calibration**.

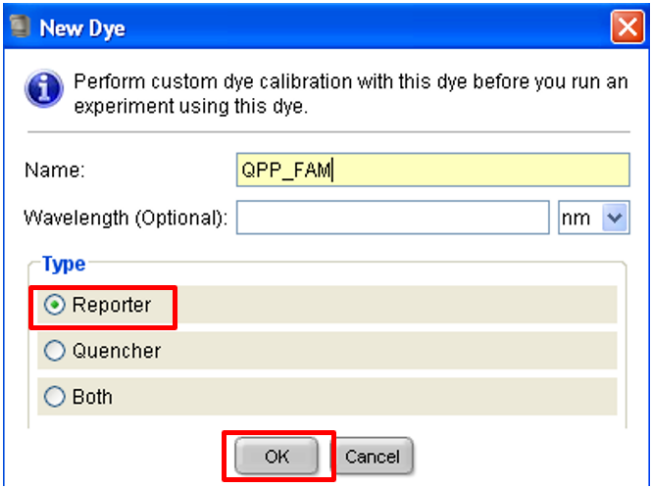
4. Select **New Dye** in the **Dye Calibration** window.



5. In the **Dye Library** window click **New**.

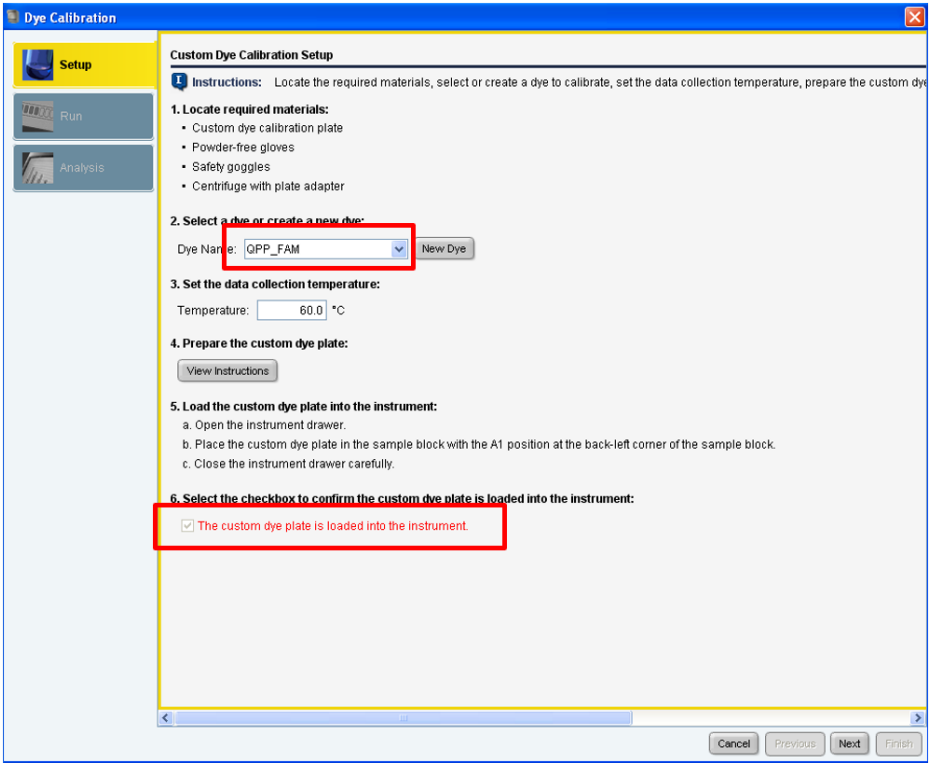


6. Set names for the new custom dyes. To differentiate the new dyes from standard dyes, we recommend using the prefix QPP (for Quantiplex Pro). Enter "QPP_FAM" as a new dye name, select **Reporter**, and click **OK**.

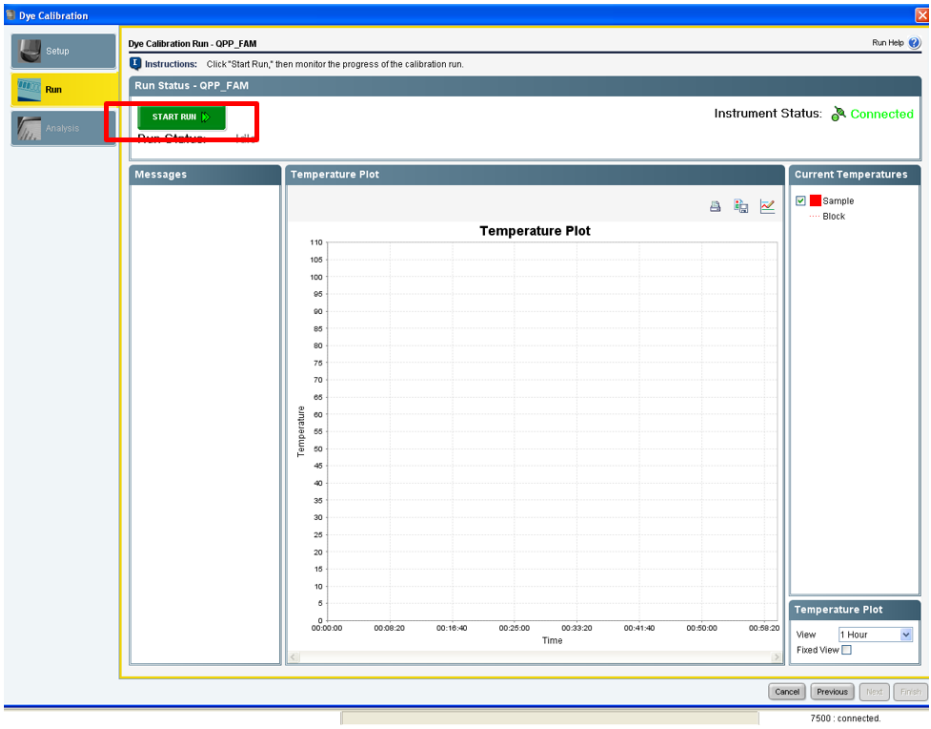


7. Repeat step 6 to set new dye names for:
   - QPP_JOE
   - QPP_ATTO550
   - QPP_ROX
   - QPP_ATTO647N

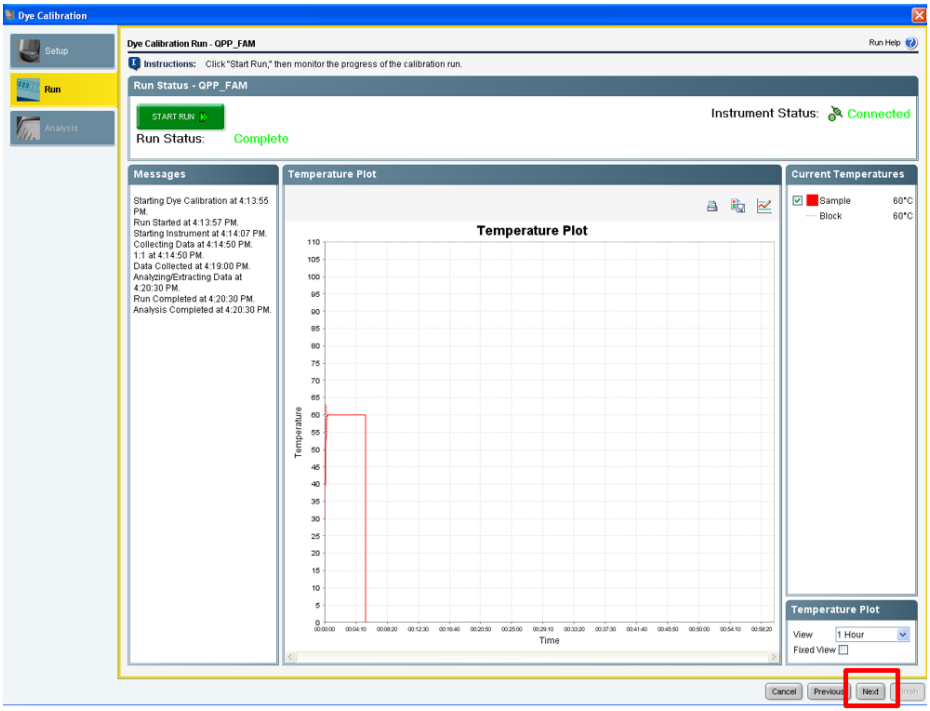
8. Select **QPP_FAM** in the **Custom Dye Calibration Setup** window. Load the QPP_FAM plate prepared in Procedure A into the instrument. Confirm by checking the box for **The custom dye plate is loaded into the instrument**.



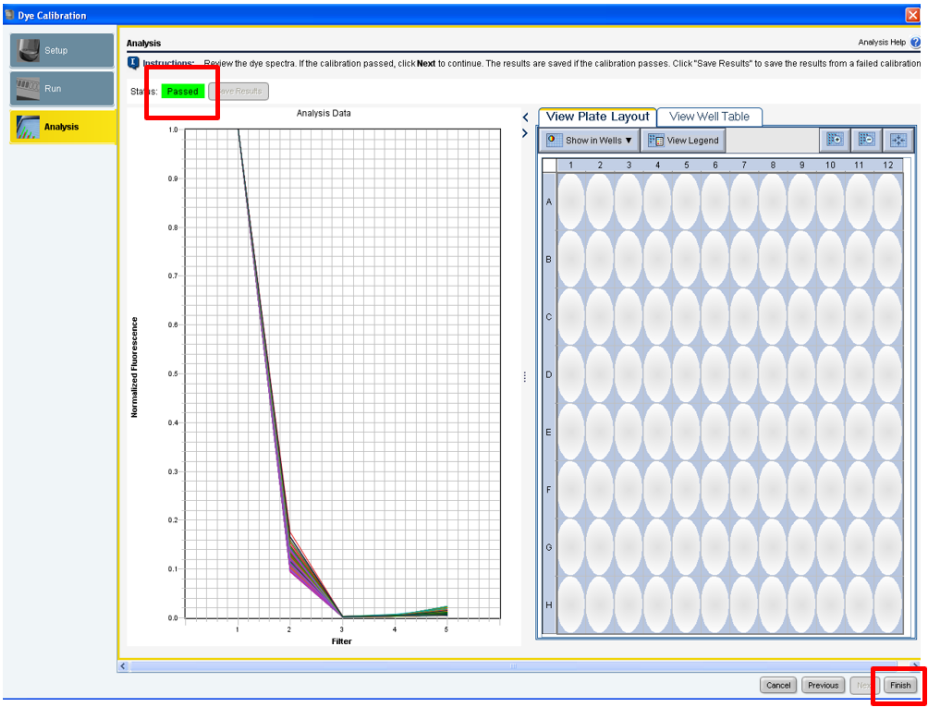
9. Click **Next** and then **Start Run**.



10. After the calibration run has finished, click **Next**.



11. The spectra and the status (e.g., "Passed") will be displayed. Click **Finish**.



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 15.

Table 15. 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: Quantification of DNA Using the Applied Biosystems 7500 Real-Time PCR System for Human Identification or Applied Biosystems 7500 Real-Time PCR System

This protocol is optimized for use of the Investigator Quantiplex Pro FLX 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 the Applied Biosystems 7500 Real-Time PCR System using 7500 Software v2.0.6 or v2.3.

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* or *Applied Biosystems 7500 Real-Time PCR System User Manual*.

Important points before starting

- When using the Applied Biosystems 7500 Real-Time PCR System for Human Identification or the Applied Biosystems 7500 Real-Time PCR System with Investigator Quantiplex Pro FLX Kit, 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.
- Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., threshold values) for analysis of every reporter dye channel in every run.

Procedure A: Creating an experiment

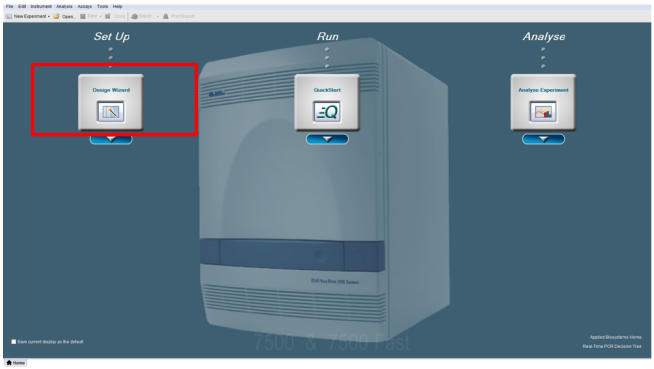
Open the HID Real-Time PCR Analysis Software v1.1 or v1.2 in the Custom Assays mode. If using the Applied Biosystems 7500 Real-Time PCR System, launch the 7500 Software v2.0.6 or v2.3.

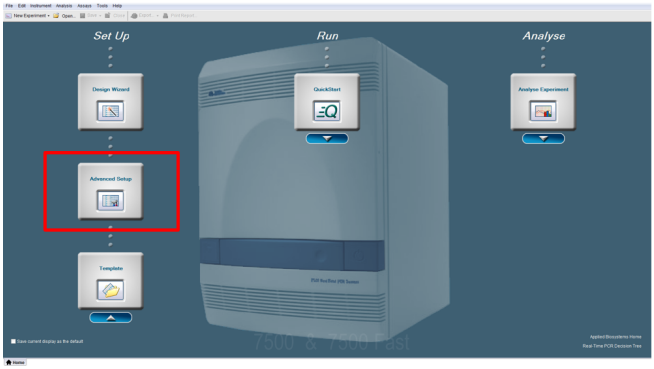
1. If you are using a template file, select **New Experiment** > **From Template** and proceed to step 6 to assign the Targets to the Plate Layout. Then proceed to step 12 on page 88 to save and start the run.

   If you are not using a template file, proceed to step 2.

   The template file loads all of the settings needed to start an Investigator Quantiplex Pro FLX run, including the standard curve settings, the cycling profile, and the targets needed for fluorescence acquisition. Download the template files from the **Resources** tab of the product page.

2. If you are not using a template file, select **Advanced Setup** by clicking the arrow below **Design Wizard**.

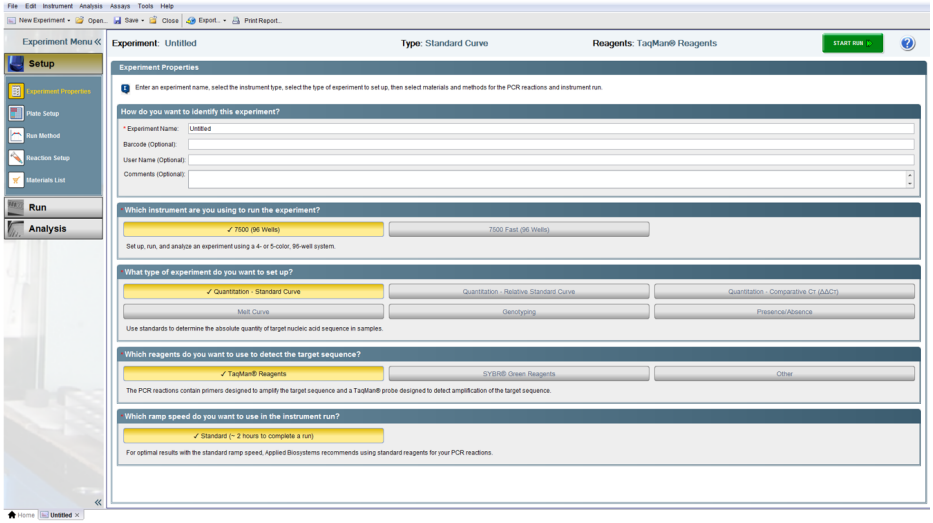




3. Once the new window opens, enter a new **Experiment Name** in the appropriate field. Select the following settings:

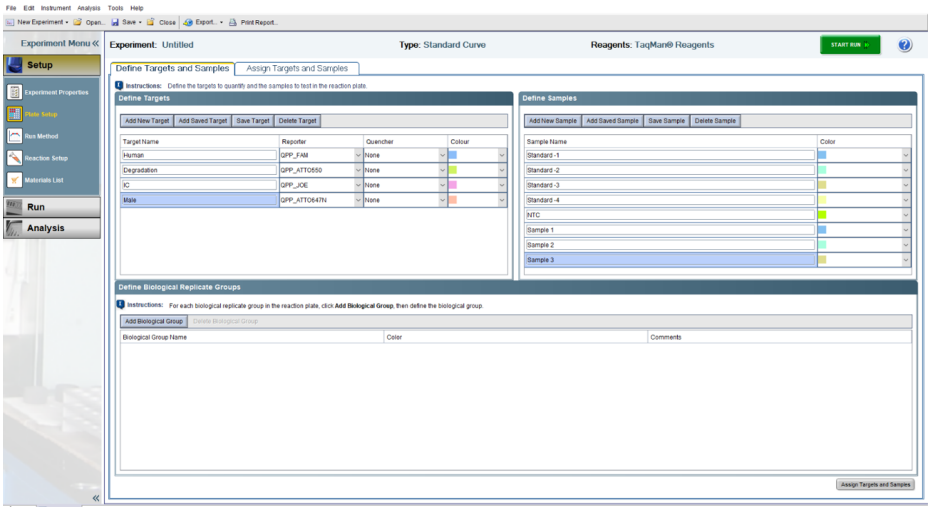
   - Instrument: 7500 (96 Wells)
   - Experiment Type: Quantitation – Standard curve
   - Reagents: TaqMan Reagents
   - Ramp Speed: Standard

   Deselect the **Include Melt Curve** option.



4. Click **Plate Setup** and define 4 Targets by clicking twice on **Add New Target**. Select the following settings.

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



5. 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. Precise 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.

6. Switch to the tab **Assign Targets and Samples**. In the **Plate Layout**, select the wells in use and assign all 4 Targets by checking the boxes.

   If using Applied Biosystems 7500 Real-Time PCR System with Software v2.0.6 or higher, make sure to change passive reference dye to **QPP_ROX**.

   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.

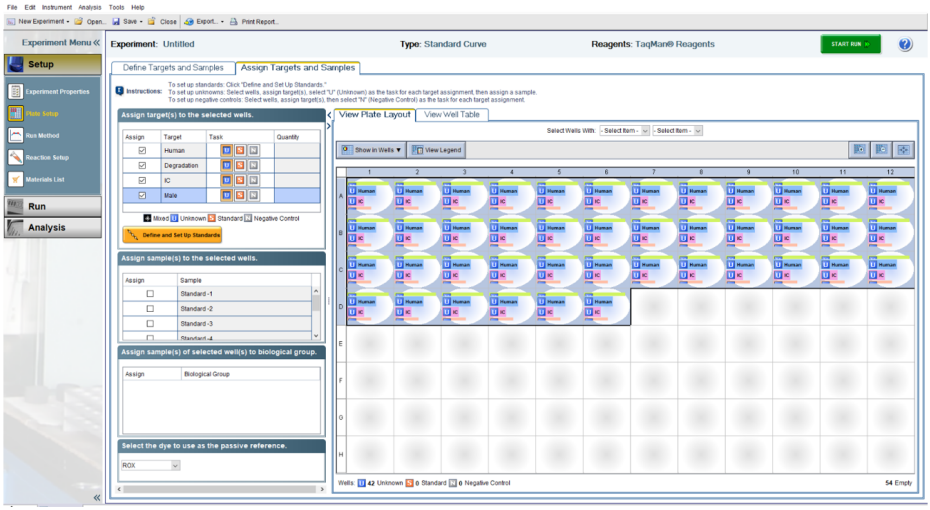


Figure 5. Settings for HID Real-Time PCR Analysis Software v1.1 or v1.2.

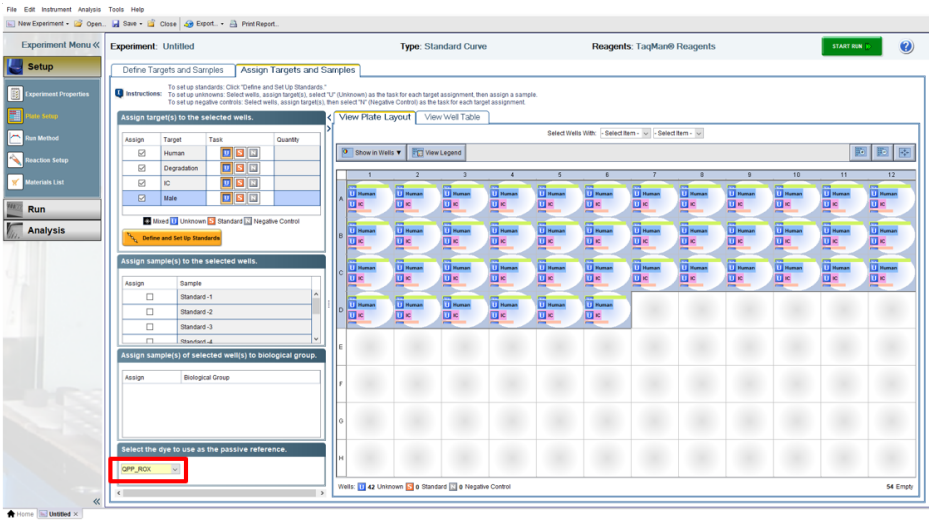


Figure 6. Settings for Applied Biosystems 7500 Real-Time PCR System with Software v2.0.6 or higher.

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

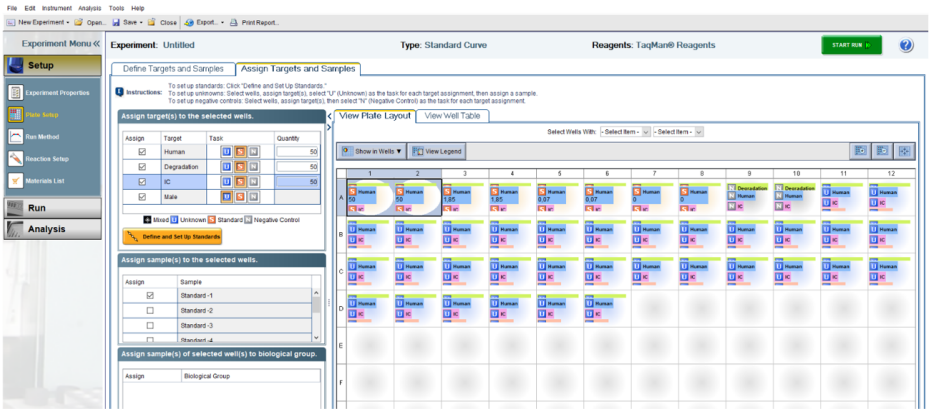
   Note: Leave the IC (QPP_JOE) Task for NTC reactions set to **Unknown**. Enter the sample name.

8. 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 3 (on page 19).

   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**.

9. Assign the samples to the plate layout by clicking on the wells and checking the appropriate box on the left panel.



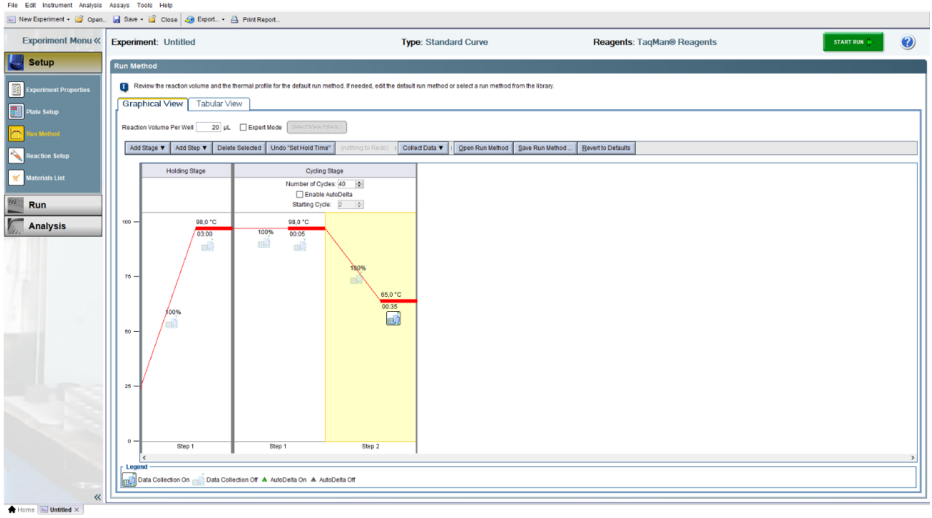
10. Click **Run Method**. Program the cycler according to Table 16.

Table 16. Cycling protocol using an Applied Biosystems 7500 Real Time PCR System for Human Identification or Applied Biosystems 7500 Real Time PCR System

Step	Temperature (°C)	Time	No. of cycles	Remarks
Initial PCR activation step	98	3 min	–	PCR requires an initial incubation at 98°C to activate the DNA polymerase
Denaturation	98	5 s	40	–
Combined annealing/extension	65	35 s		Perform fluorescence data collection

Note: Always use a compression pad if using the entire plate. If using 8-tube strips, please follow the PCR cycler manufacturer's recommendation for the use of strips.

11. On the thermal profile, change the **Reaction Volume Per Well** to **20 µL**. Data acquisition should be performed during the combined annealing/extension step.



12. 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.
13. Load the plate into the instrument. Ensure that position A1 on the plate is on the top-left side of the tray.
14. 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., 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.

   If using Applied Biosystems 7500 Real-Time PCR System open the run file by using 7500 Software v2.0.6 or higher. 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 8 on page 86.

   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.

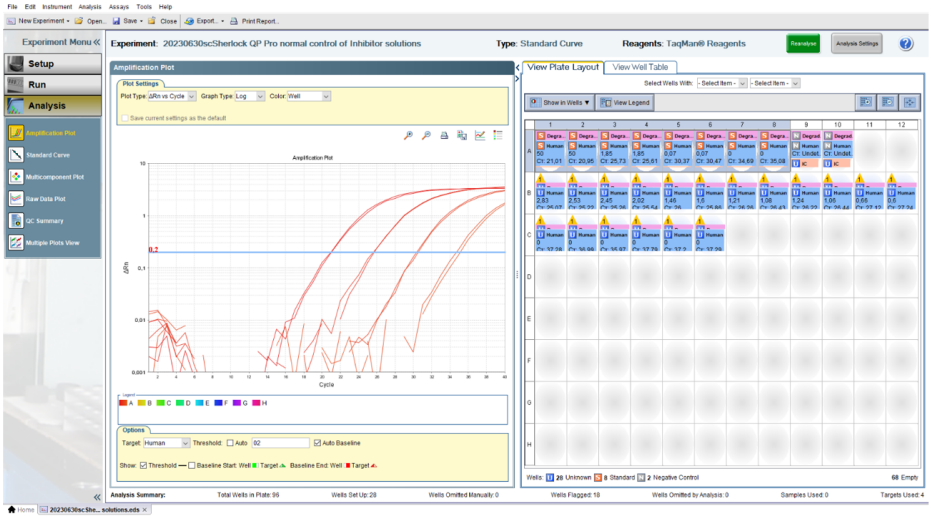
   Note: Leave the IC (QPP_JOE) Task for standard reactions set to **Unknown**. Enter the sample name (e.g., Standard 50 ng/µL).

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** and then press **Analyze**. Repeat this for Male, Degradation, and IC with the following thresholds listed:

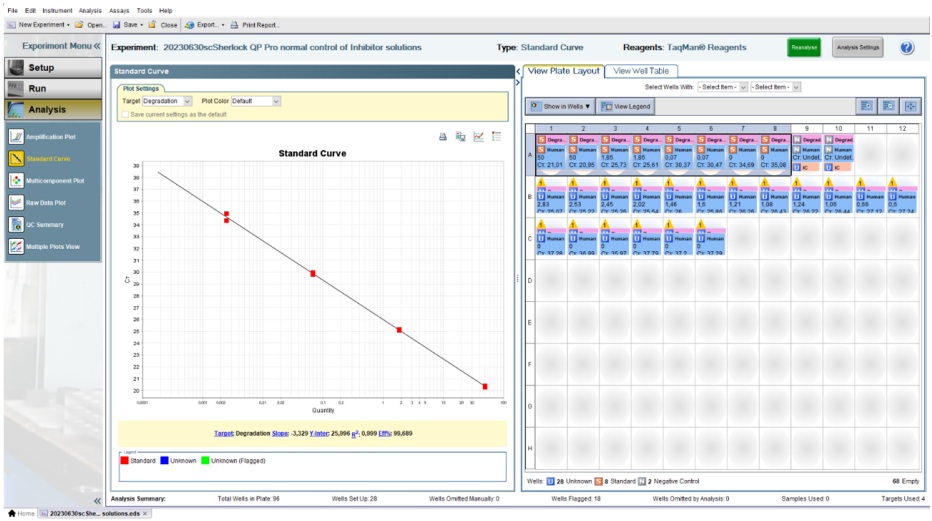
   Note: Alternatively, click the **Analysis Settings** button in the upper right corner to set all analysis settings.

   - Human: **0.2**
   - Male: **0.5**
   - Degradation: **0.2**
   - IC: **0.05**
   - Auto Baseline for all targets

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



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.

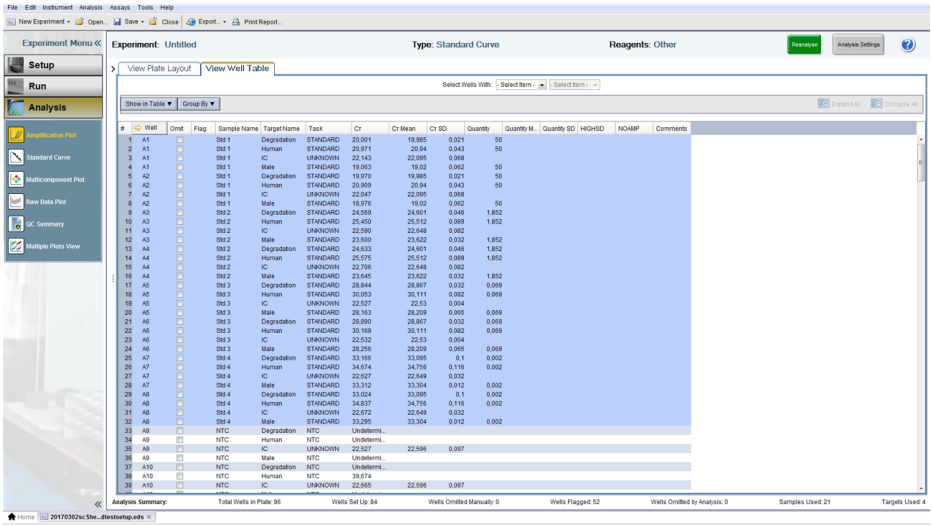


6. View the concentrations of the unknown samples.

   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 QIAGEN Quantification Assay

Data Handling Tool. Mean quantification values should be displayed if replicates have been assigned.

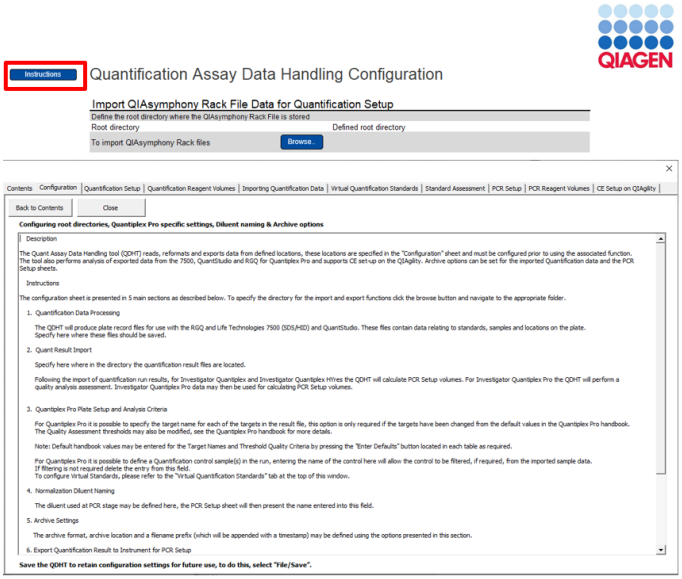


7. To export and save the results report, go to **File**, followed by **Export** and then **Results**. The analysis settings must be saved first, then the results may be saved in the format **Results Export Files *.csv**.

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

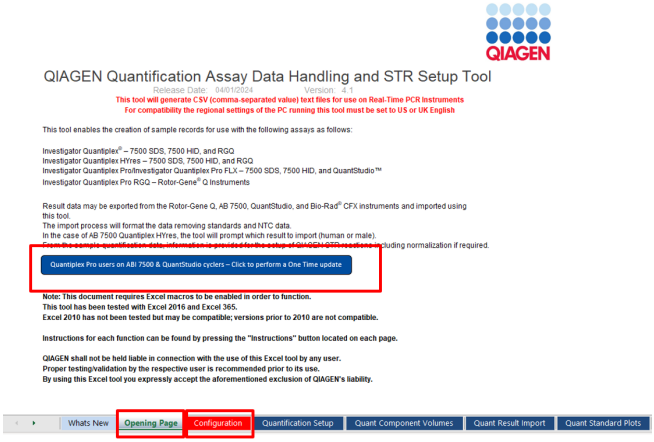
Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool

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.



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** button.
4. Click the **Configuration** worksheet tab.

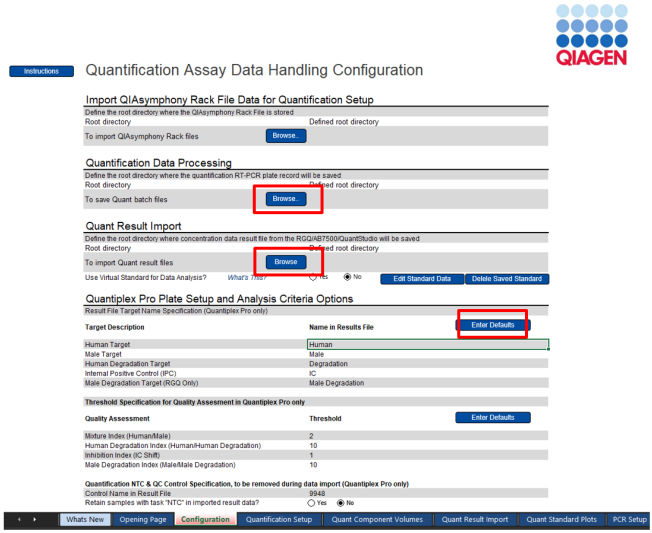


5. Set the root/home directory to save Quant batch files.

6. Set the root/home directory to import Quant result files.

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/Quantstudio**. 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.

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



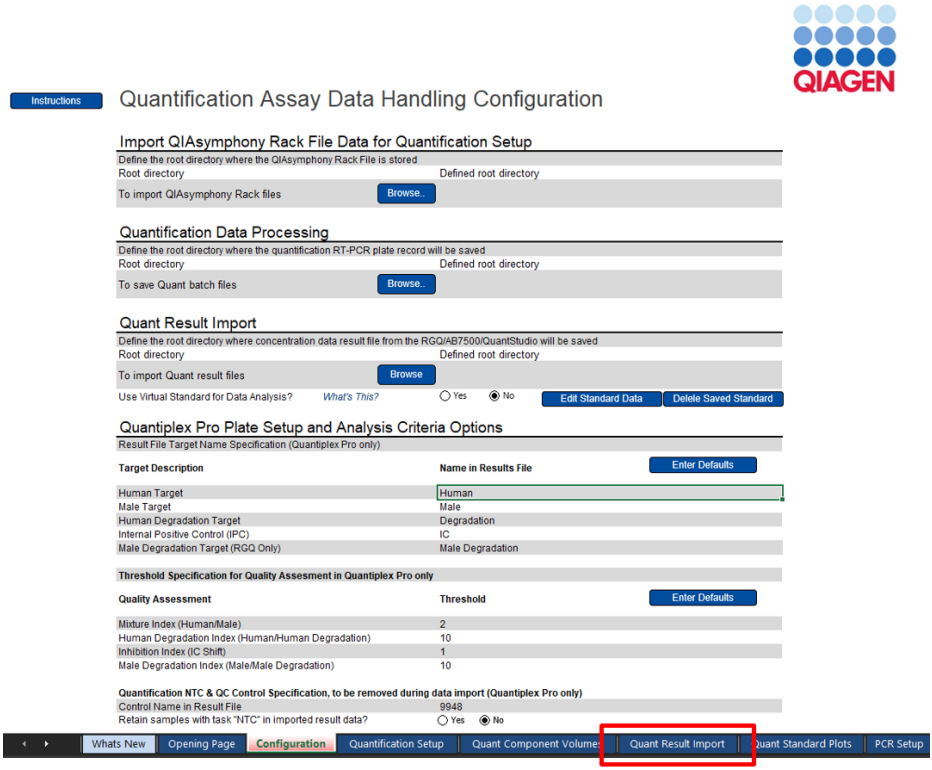
8. Threshold setting for the Quality Assessment can be changed/adjusted as needed. The default threshold settings are

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

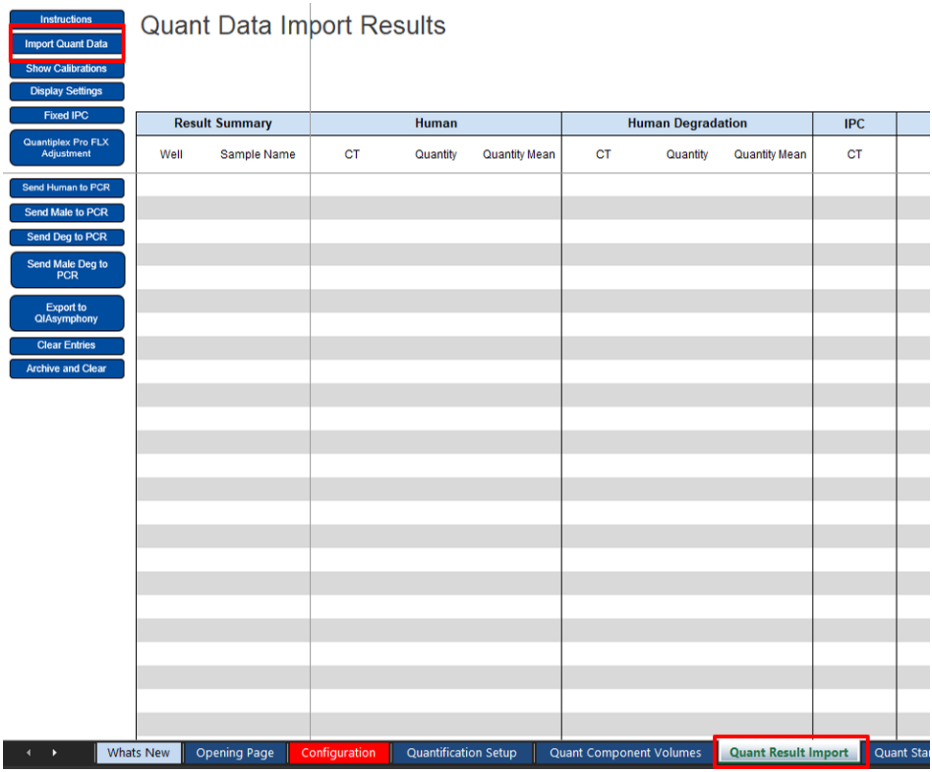
Note: Setting the appropriate threshold values may require further internal validation at your facility. 9948 will be filtered from the import, provided it is included in the section "Quantification QC Control Specification". Removing it allows it to be kept in the final data set.

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

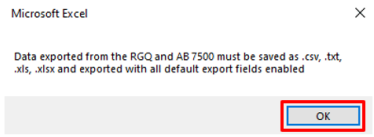
9. To import quantification results click the **Quant Result Import** worksheet tab.



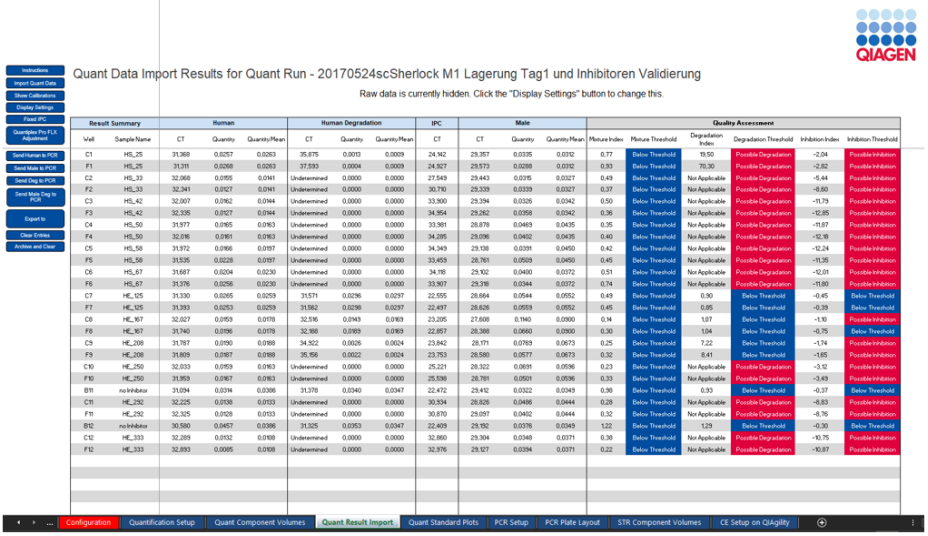
10. Click the **Import QuantData** button.



11. Confirm that the exported data are in the appropriate 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”.



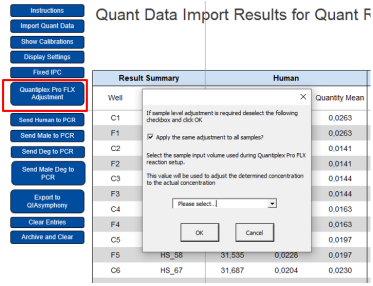
12. Display options can be adjusted by clicking **Display Settings**:

    - Show Raw Data
    - Show Quantity Mean Values
    - Show C_T 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.

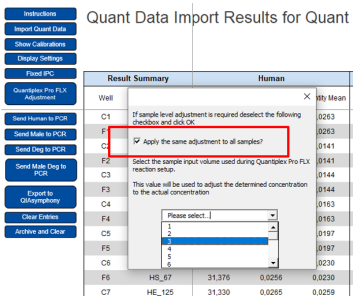
Note: The Inhibition Index is set to 1 as a default. The IC acts as a quality sensor and reports the presence of inhibitors with a C_T shift while quantification remains reliable. The default value can be changed and adjusted for relevant degrees of inhibition. Therefore, laboratory validation should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

Apply concentration correction factor (only necessary if sample volume was not 2 µL) by clicking **Quantiplex Pro FLX Adjustment**.



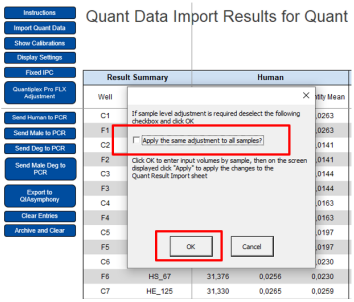
By checking or unchecking the box, you can choose between:

a. the same correction factor is applied to all samples

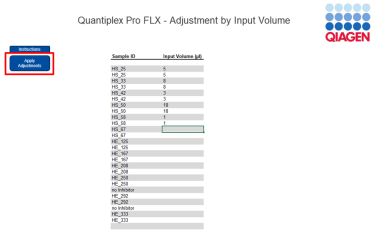


or;

b. a separate correction factor is used for each sample individually (if the input volume of the samples differs).



After entering the sample volume, click on **Apply Adjustments**.



Back in the **Quant Result Import** sheet, the samples are corrected with the selected correction factor.

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 FLX Kit and may need to be adjusted.

For DNA quantification using the Investigator Quantiplex Pro FLX Kit, the analysis settings must be adjusted for all 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 represents the log concentration and y is the threshold cycle 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" on page 106 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" on page 106 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 FLX 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. 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, increased template input volumes, and/or running replicates may give more reliable results.

Quantification of female/male mixtures

The Investigator Quantiplex Pro FLX 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 FLX 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 or QPP_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. 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.

	Comments and suggestions
Handling of Quantiplex Pro FLX Plates	
a) Lyophilized cake disturbed by pipetting	Do avoid to touch the cake while pipetting sample or water. With automated setup, check if z-height settings accommodate for cakes. Also consider z-height settings for loosened and/or upside down cakes.
b) No lyophilized cake, or part of it lost	Ensure no lyophilized product sticks to the seal before removing it. Tap the plate to bring all cakes down. Do not use wells from which product was lost.
c) Lyophilized product at the side of the well after setup	Do vortex the re-sealed plate after setup. If only pipette mixing is performed, some lyophilized product may stay at the side of the well.
d) Lyophilized cake shrunk, hard to redissolve	The cake may have become rehydrated. Avoid prolonged open times of the plate before setup and setup under conditions of more than 60% humidity. Rehydrated cakes require more time to dissolve. In general, once completely dissolved, reactions can still be used.
e) Evaporation of chemistry during qPCR run	Always use a compression pad for qPCR runs of full plates.
f) Lyophilized cake shrunk, hard to redissolve after storage	Always store unused part of the sealed PCR plate in the aluminum bag with desiccant and use up within 4 weeks.
g) Issues to remove seals from strips	Use a scalpel to cut between the strips before breaking the plate.

Appendix: Alternative Standard Curves

Table 17. 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 18. 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 19. 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 FLX Kit (576)	6x single blistered 96-well optical plates with Master Mix, Control DNA M1, and QuantiTect Nucleic Acid Dilution Buffer	387516
Investigator Quantiplex Pro Calibration Kit	Calibration Standard FAM, JOE, ATTO 550, ROX, ATTO 647N, and Quantiplex Pro Calibration Buffer	387416
Related products		
Investigator Casework GO! Kit	Casework GO! Lysis Buffer, Proteinase K Solution, and Nuclease-free water	386546
qPCR adhesive plate foil, perforated (100)	Clear adhesive foils for qPCR plates, perforated for tearing into 8-well strips	386055
qPCR adhesive plate foil (100)	Clear adhesive foils for qPCR plates	209003
PCR Water (10 x 1 mL)	Water certified to be free of DNA, DNase and RNase contamination	17000-10
Investigator Human Identification PCR Kits		
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
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

* Larger kit sizes available; please inquire.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
02/2024	Initial release

Limited License Agreement for Investigator Quantiplex Pro FLX Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this Instructions for Use and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this Instructions for Use, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.
3. This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the panel and/or its components.

For updated license terms, see **www.qiagen.com**.

This page intentionally left blank