April 2023

Investigator® Quantiplex® Pro Handbook for QuantStudio™ 5 Real-Time PCR Systems

For quantification of human and male DNA in forensic samples

Sample to Insight

Contents

Kit Contents

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

Shipping and Storage

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

Intended Use

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

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

Safety Information

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

Quality Control

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

Introduction

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

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

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

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

Principle and procedure

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

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

Target regions

The target region for human DNA quantification (4NS1C®) is a 91 bp proprietary region present on several autosomes of the human genome. It was selected to give high sensitivity and is detected using the FAM™ dye channel on QuantStudio 5 Real-Time PCR Systems.

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 QuantStudio 5 Real-Time PCR Systems.

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 QuantStudio 5 Real-Time PCR Systems.

Internal Control

In addition, the Investigator Quantiplex Pro Kit contains a balanced internal amplification control that is used to test successful amplification and identify the presence of PCR inhibitors. This heterologous amplification system is detected as a 434 bp internal control (IC) in the JOE™ dye channel on QuantStudio 5 Real-Time PCR Systems. 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 on QuantStudio 5 Real-Time PCR Systems for Investigator Quantiplex Pro Kit

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

Quantiplex Pro Reaction Mix

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

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

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

Male Control DNA M1 and standard curve

DNA quantification standards are critical for accurate analysis. We strongly recommend a 27-fold dilution series with 4 concentration points in the standard curve for each assay. The Control DNA contains pooled male DNA at a concentration of 50 ng/µL. To ensure pipetting accuracy, the minimum input volume of DNA for dilutions should be 5 µL. The standard curve

is designed to be easily set up using a convenient 1:27 dilution series. If using QuantiTect Nucleic Acid Dilution Buffer to dilute the Control DNA, the dilutions are stable for at least 1 week at 2–8°C.

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

Templates for routine work

In order to streamline the instrument setup and the analysis of the results on the QuantStudio 5 Real-Time PCR Systems, QIAGEN has developed a set of template files. Download the template files from the product resources page at **www.qiagen.com/QPpro-template-files**.

Equipment and Reagents to Be Supplied by User

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

Equipment

- Cooling device or ice
- QuantStudio 5 Real-Time PCR System

Material

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

Reagents

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

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

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

Important Notes

Selecting kits and protocols

This handbook contains protocols and recommendations for DNA quantification using the QuantStudio 5 Real-Time PCR System. Real-time cyclers other than the one listed here or in HB-2335 *Investigator® Quantiplex® Pro Handbook for Applied Biosystems 7500 Real-Time PCR Systems* have not been validated by QIAGEN for DNA quantification using the Investigator Quantiplex Pro Kit.

Contamination risks

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

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

Controls

No-template control (NTC)

Replicates of NTC reactions should be included in each quantification run in order to detect contamination. NTCs should contain all the components of the reaction, except for the template. Quantification using the Investigator Quantiplex Pro Kit is highly sensitive; despite the fact that the reagents contained in the Quantiplex Kit undergo strict quality controls to assess that they are free of human DNA contamination, background DNA may be detected in

rare cases due to the high assay sensitivity. Take great care to avoid contamination when pipetting the NTC.

We recommend performing NTC reactions at least in duplicate.

Internal positive control

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

Calibration of QuantStudio 5 Real-Time PCR System

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 (cat. no. 387416). Refer to the instrument user guide for additional information on correct setup.

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 (v1.4.1 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 regions 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 2.

Table 2. Dilution scheme for Investigator Quantiplex Pro Calibration Kit standards

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

4. Mix each calibration standard by vortexing for at least 5 s.
5. Assign and label one separate optical 96-well reaction plate for each Investigator Quantiplex Pro Calibration Kit standard (5 in total).
6. Dispense 20 µL of diluted Investigator Quantiplex Pro Calibration Kit standard FAM into the assigned dye-specific optical 96-well reaction plate.
7. Close the plate with an optical adhesive film.
8. Repeat these steps for each of the other remaining Investigator Quantiplex Pro Calibration Kit standards (JOE, ATTO 550, ROX, and ATTO 647N).
9. Centrifuge plates briefly. Always protect plates from light.
10. After calibration, store the calibration plates at –30 to –15°C in a constant-temperature freezer, protected from light. Calibration plates can be stored and reused for up to 3 months.

Procedure B: Calibration protocol for 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 3.

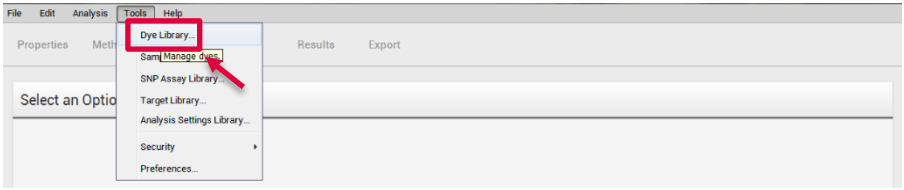
Table 3. Calibrated dye filter signal

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 (v1.4.1 or higher)

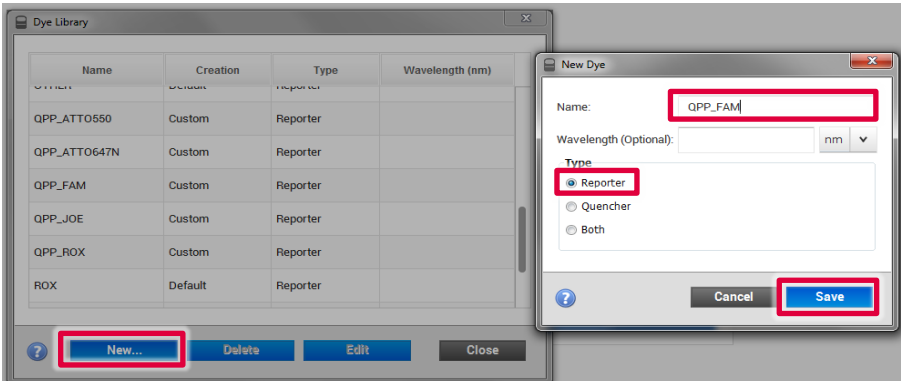
1. Open the QuantStudio Design and Analysis Software and select **Tools** > **Dye Library**.



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 Kit on the QuantStudio 5 Real-Time PCR System using QuantStudio Design and Analysis Software (v1.4.1 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

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- When using the 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.
- Use the template volume specified in the protocol. The reaction is optimized for use with exactly 2 µL template DNA. Do not use more than or less than 2 µL per 20 µL reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored between 2 and 8°C for at least 1 week.
- 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: PCR

1. Mix all solutions thoroughly before use to avoid localized concentrations of salt.
2. Prepare fresh serial dilutions of the Male Control DNA M1 according to Table 4. 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.

   Note: Alternative standard curves are listed in Appendix on page 57.

Table 4. Serial dilutions of Male Control DNA M1

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

3. Thaw the template nucleic acids.

   Mix all solutions thoroughly before use to avoid localized concentrations of salt.
4. Prepare a Master Mix according to Table 5.

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

   Prepare a volume of Master Mix 10% greater than what is required for the total number of PCR assays to be performed. This should include positive and negative control reactions.

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

Table 5. Master Mix for DNA quantification

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

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

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

   Mix carefully to avoid localized concentrations of salt.

   Table 6 shows a possible plate setup. Ensure that the Master Mix and template are thoroughly mixed.

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

Table 6. Possible plate setup of reactions on the QuantStudio 5 Real-Time PCR System

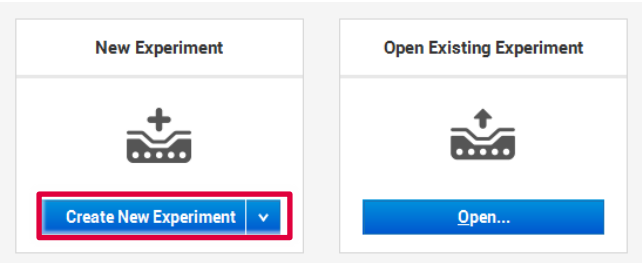
Well contents	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	1.8519	1.8519	0.0686	0.0686	0.0025	0.0025	NTC	NTC	UNK	UNK
B	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK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

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

8. Open the QuantStudio Design and Analysis Software (v1.4.1 or higher).
9. 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 20 to define DNA sample names and assign to the plate layout. Then proceed to step 25 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", page 35.
   - If you are not using a template file, proceed with step 10 below.

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

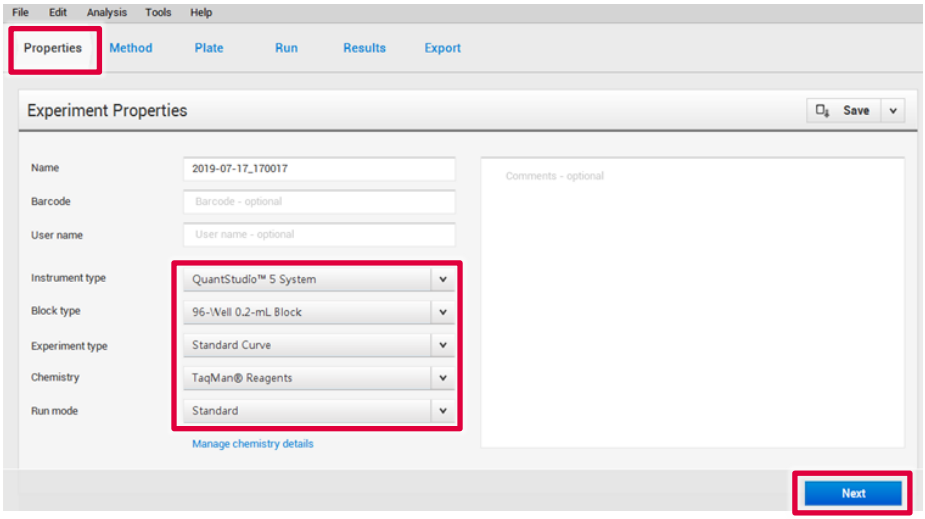
10. If you are not using a template file, select **Create New Experiment**.



11. 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**.



12. In the **Method** tab, adjust thermal profile by changing the holding times to those in Table 7.

    Change **Volume** to **20 µL**.

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

    Click **Next**.

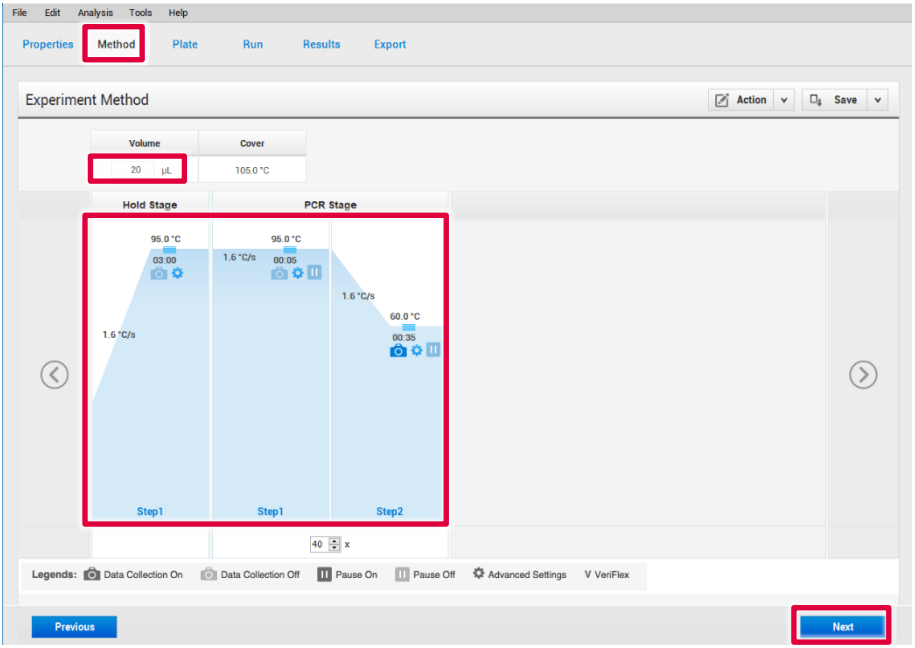
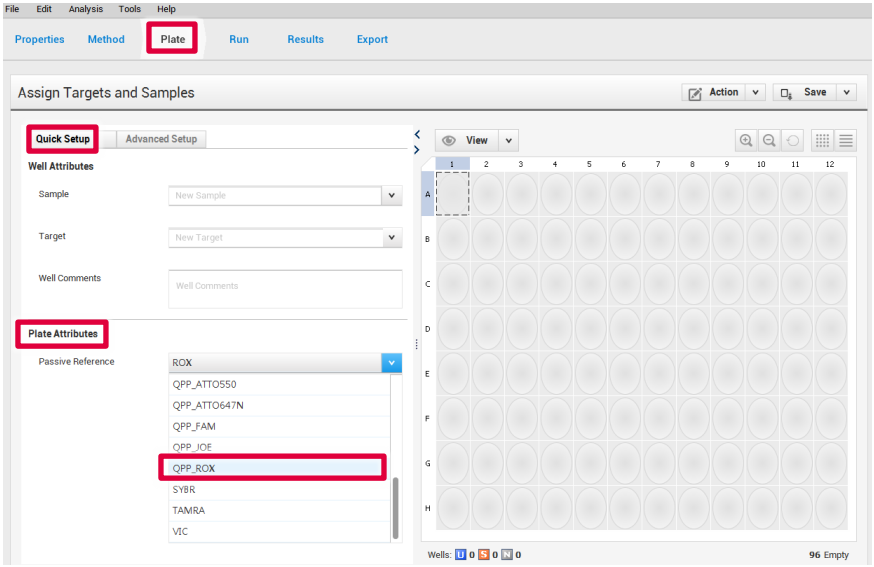


Table 7. Cycling protocol using QuantStudio 5 Real-Time PCR System

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

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



14. Still in the **Plate** tab, select **Advanced Setup**. Click **Add** 3 times, and then add the targets listed in Table 8.

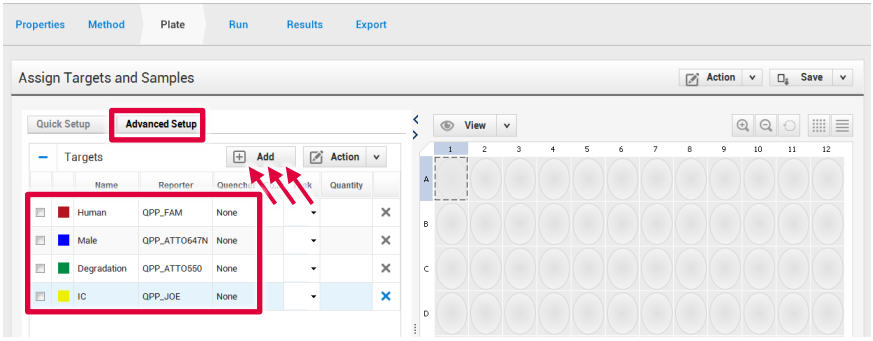
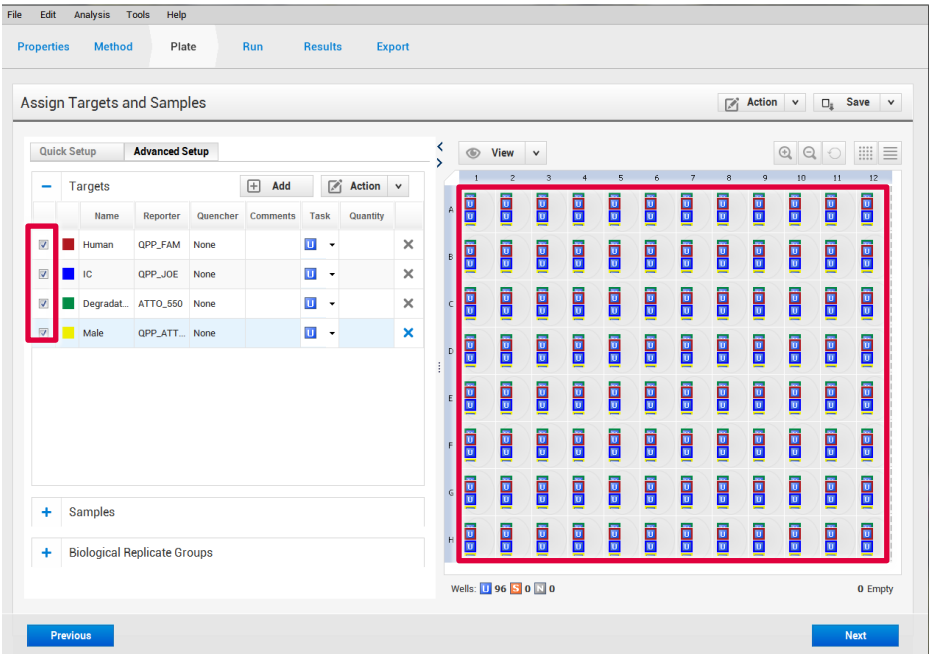


Table 8. Assign targets and samples

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

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

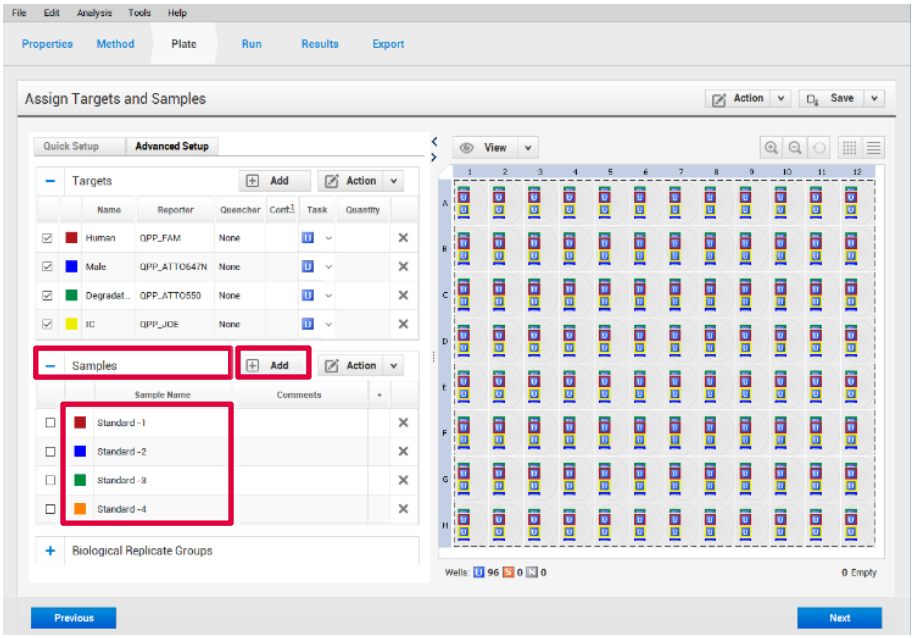
    Important: Do not highlight the wells that are not in use (i.e., those without reaction mix). Including unused wells will significantly impact the scale of the x and y axes when viewing the data.



16. Still 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: Naming of standards is required for proper subsequent analysis with the QIAGEN Quantification Assay Data Handling Tool.

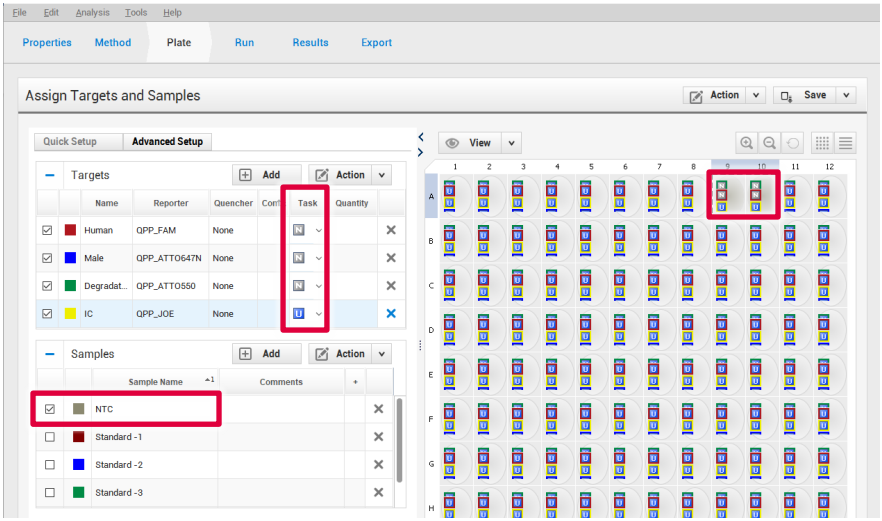
    Important: If replicates are needed, they should be assigned before you proceed to the next step. Define replicates by using the same sample name for 2 or more wells or by using the **Biological Replicate Groups** panel.*



* Instructions for using the Biological Replicate Groups panel are not covered by this handbook

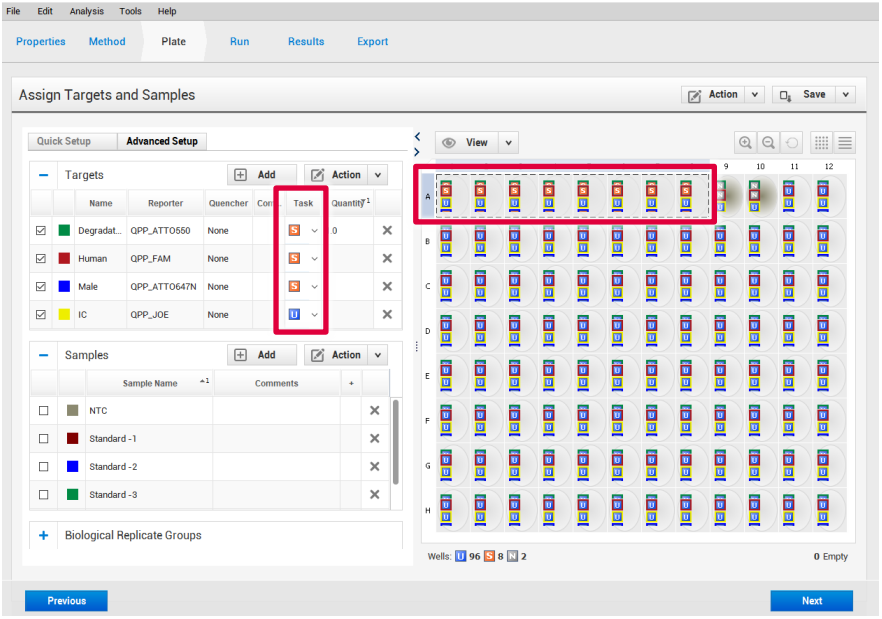
17. Select the wells for the no-template controls (NTC) and flag them as negative control in **Task** by selecting the gray **N** button.

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

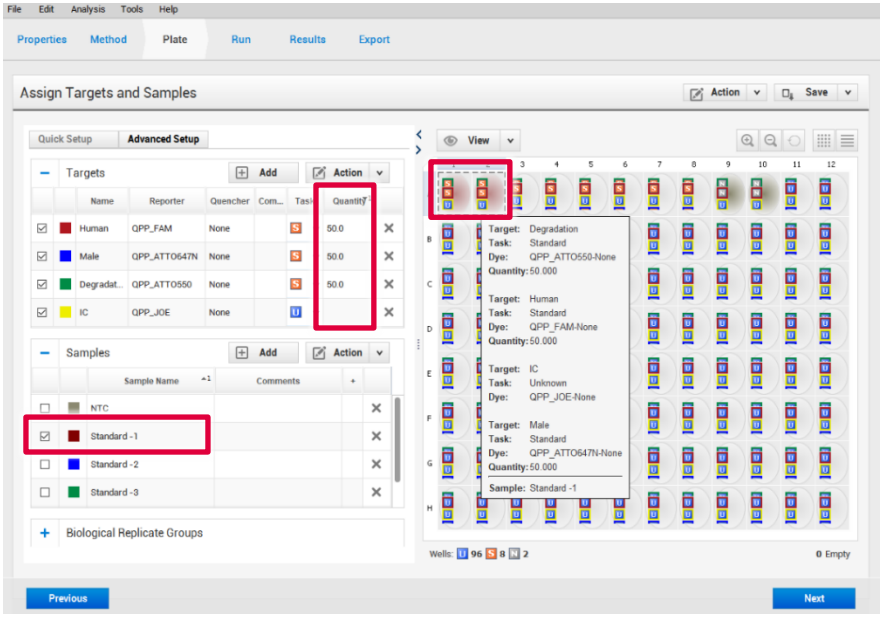


18. Select the wells for the standard curve and flag them in the **Task** column as standard by selecting the orange **S** button.

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

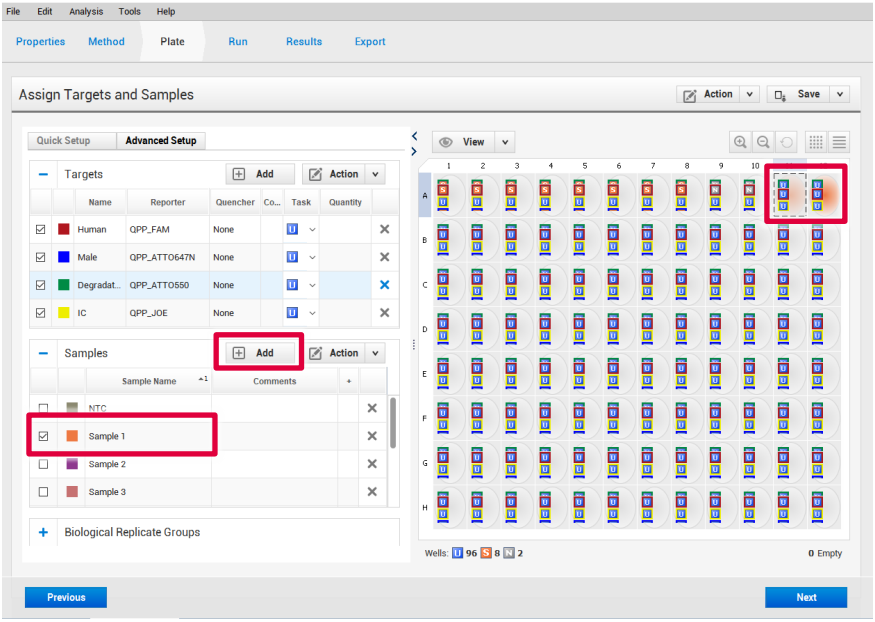


19. Enter the concentration and select a sample name for each standard. Enter the quantity of DNA in the wells according to Table 4, page 20.

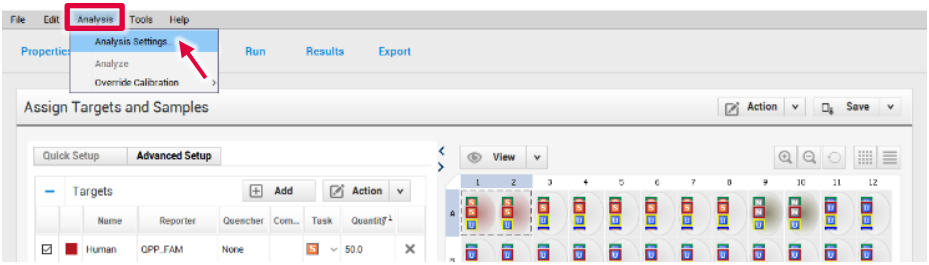


20. In the **Samples** section, click **Add** to enter the names of the DNA samples.

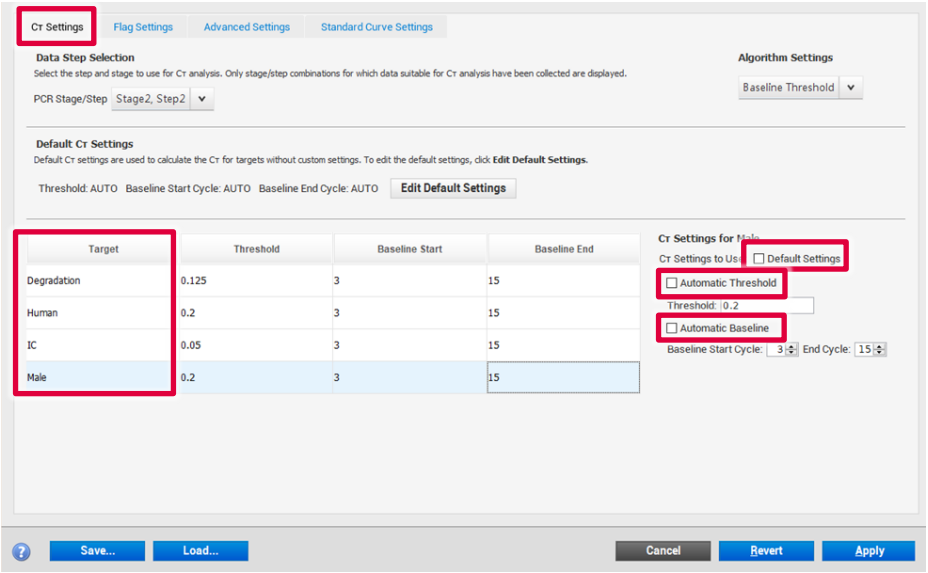
    Assign DNA samples to the plate layout by clicking on the wells and checking the appropriate box on the left **Samples** panel.



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



22. 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**, **Automatic Threshold**, and **Automatic Baseline**. Do the same for the rest of the targets.

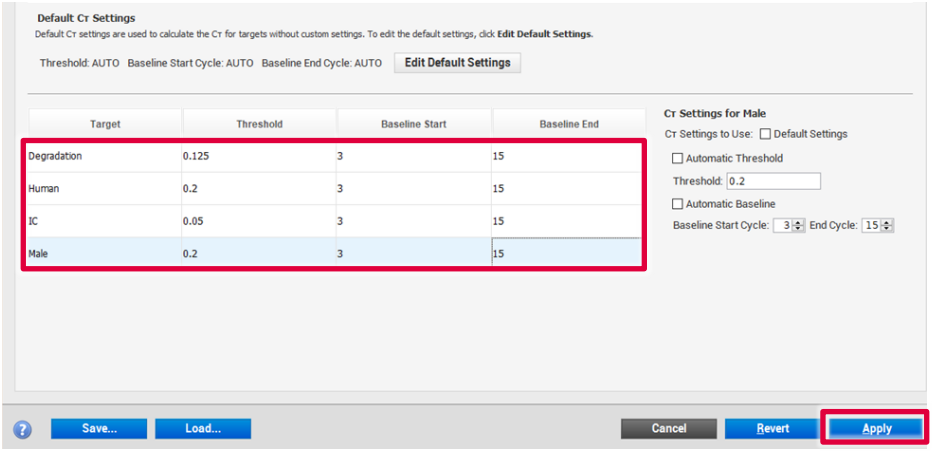


23. Enter the following settings for each target (Table 9), and then click **Apply**:

Table 9. CT Settings

Target	Threshold	Baseline start	Baseline end
Degradation	0.125	3	15
Human	0.2	3	15
IC	0.05	3	15
Male	0.2	3	15

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



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

    24a. Click **File** > **Save as**.

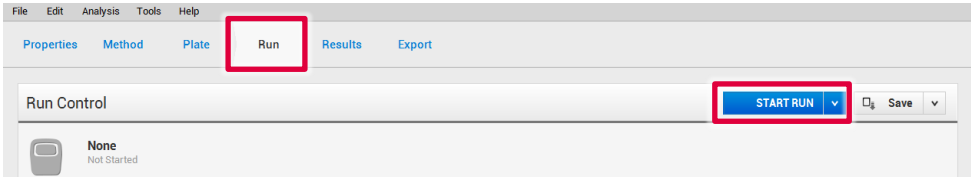
    24b. Enter a name for the template document.

    24c. Click **Save** again.

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

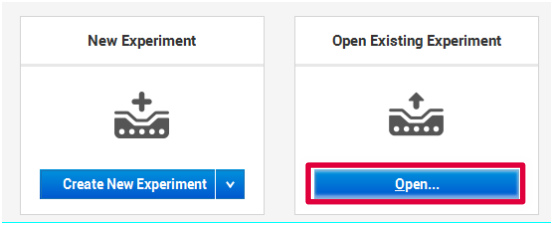
25. Load the plate into the instrument. Ensure that position A1 on the plate is on the top-left side of the tray.

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

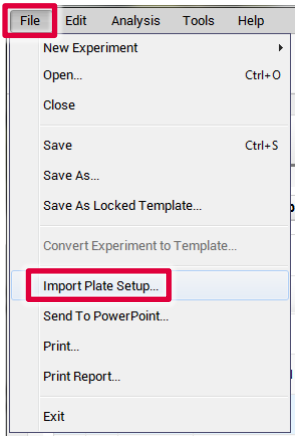


Procedure B: Run setup using a template file and a plate setup file

1. Open the QuantStudio Design and Analysis Software (v1.4.1 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**.



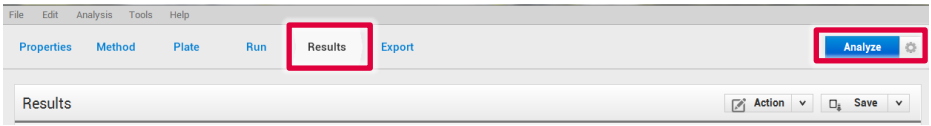
4. After successfully importing the plate setup, load the plate into the instrument. Ensure that position A1 of the plate is on the top-left side of the tray.
5. Select the **Run** tab, and then click **Start Run**.

Procedure C: Data analysis

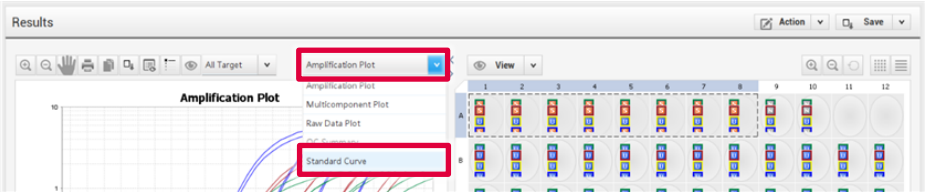
Optimal analysis settings are a prerequisite for accurate quantification data. Check and readjust the analysis settings (i.e., baseline settings and threshold values) if needed for analysis of every reporter dye channel in every run.

1. Open the run file using the QuantStudio Design and Analysis Software (v1.4.1 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 **Setup** and select **Plate Setup**. Define the wells that contain DNA standards as explained in steps 16–19 of "Procedure A: PCR" (starting on page 27).
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**.

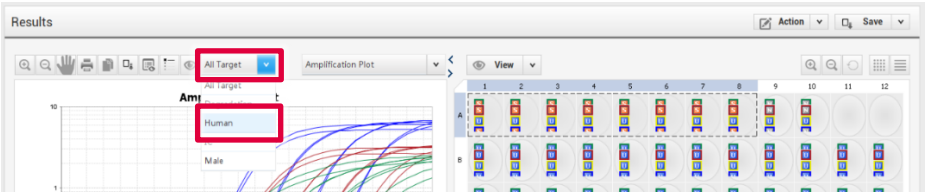


6. To view the standard curve, select **Amplification Plot** > **Standard Curve** from the drop-down menu.



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

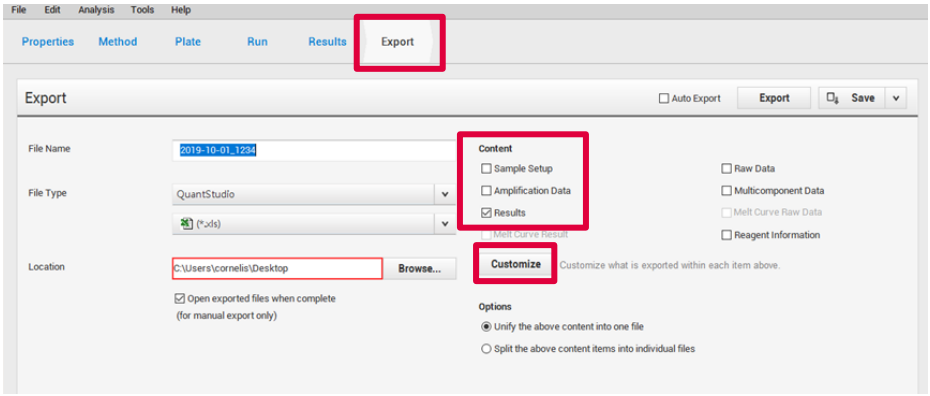
   View the C_T values for the quantification standard reactions, and the calculated regression line, slope, y-intercept, and R^2 values.



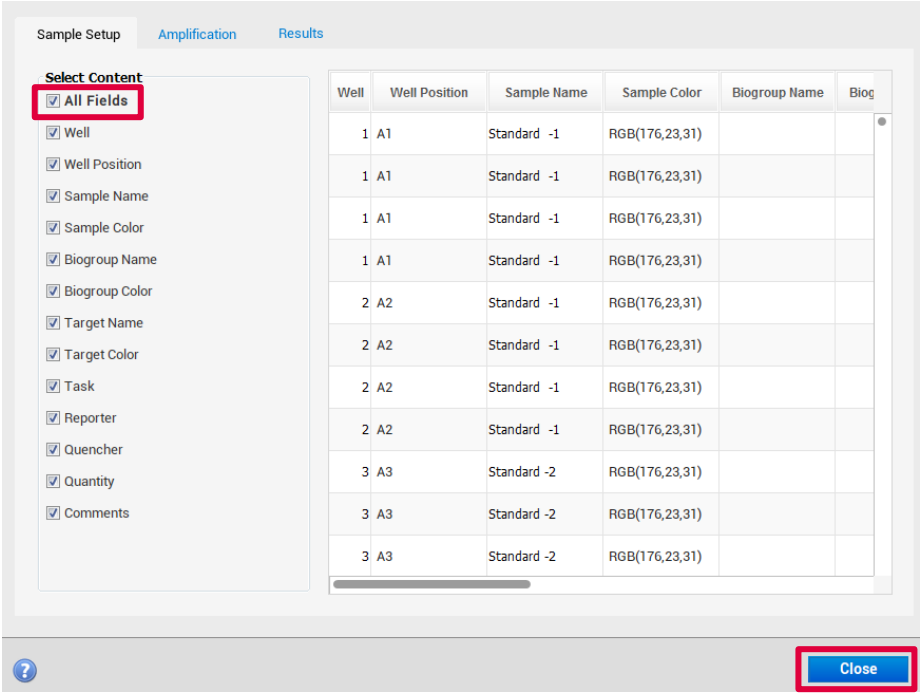
8. To export and save the results report, go to **Export** in the top bar.

   Ensure only the **Results** box is checked under **Content**.

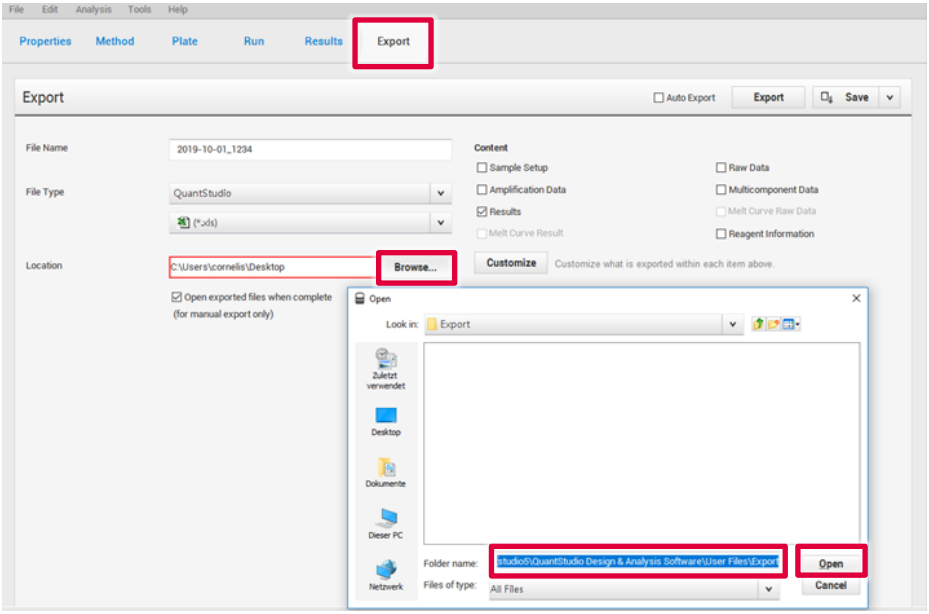
   Click **Customize**.



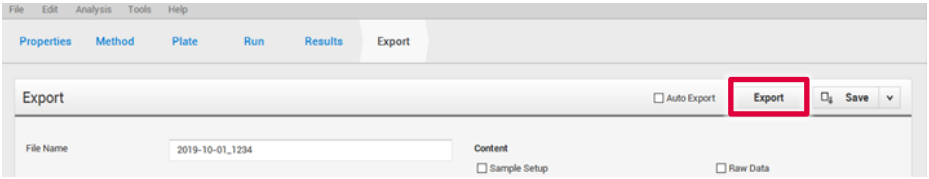
9. Ensure **All Fields** are selected for export. Click **Close**.



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



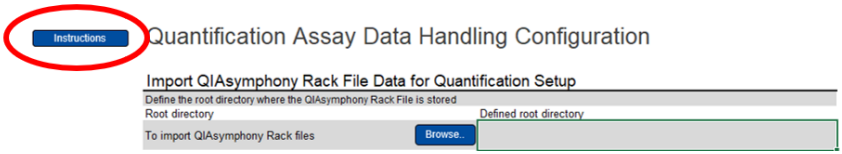
11. Click **Export**. Save the file in XLS format.

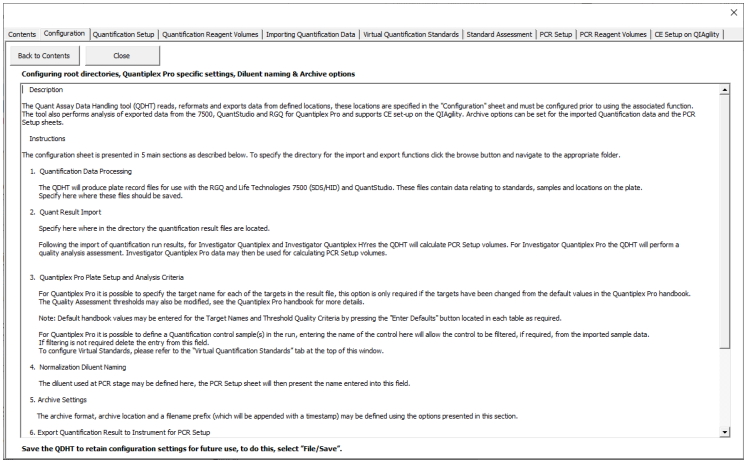


12. To interpret results, see next section “Interpreting Data Using the QIAGEN Quantification Assay Data Handling Tool”.

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.

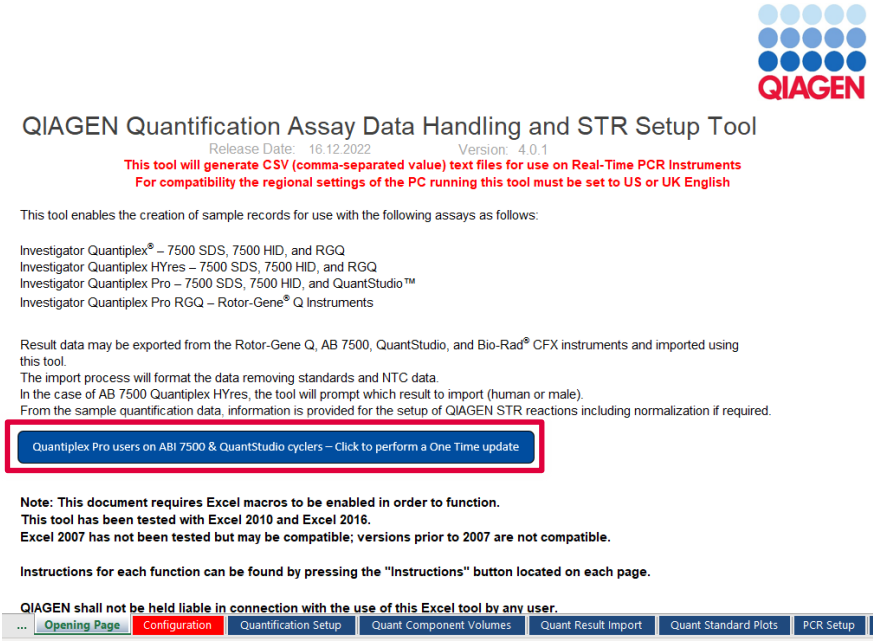




Procedure

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.

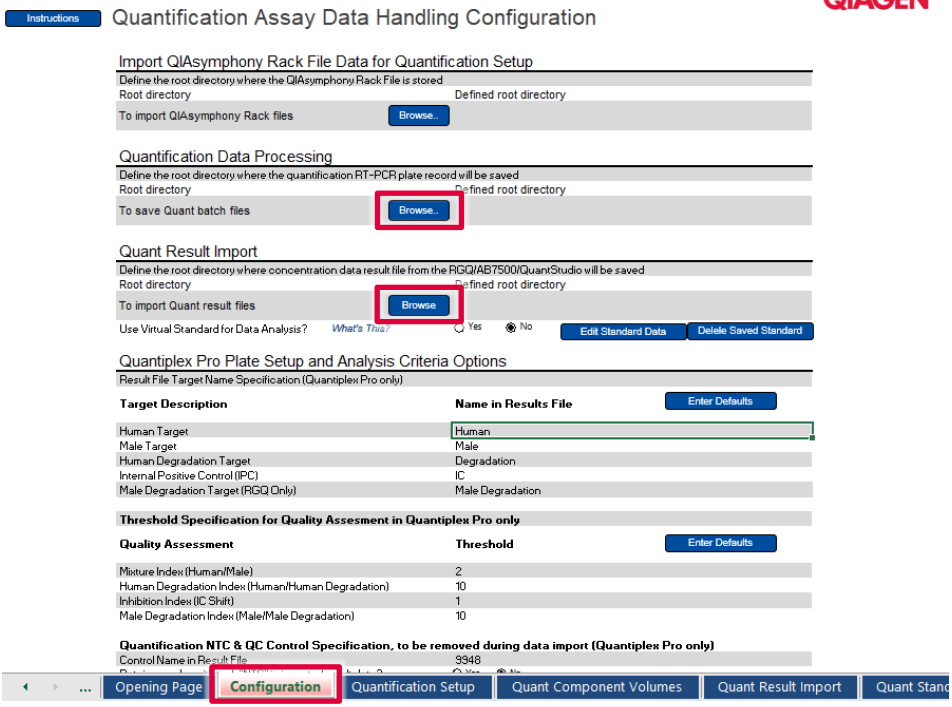
3. Click on the blue **One Time update (ABI 7500 & QuantStudio cyclers)** button.



4. Click the **Configuration** worksheet tab and set:

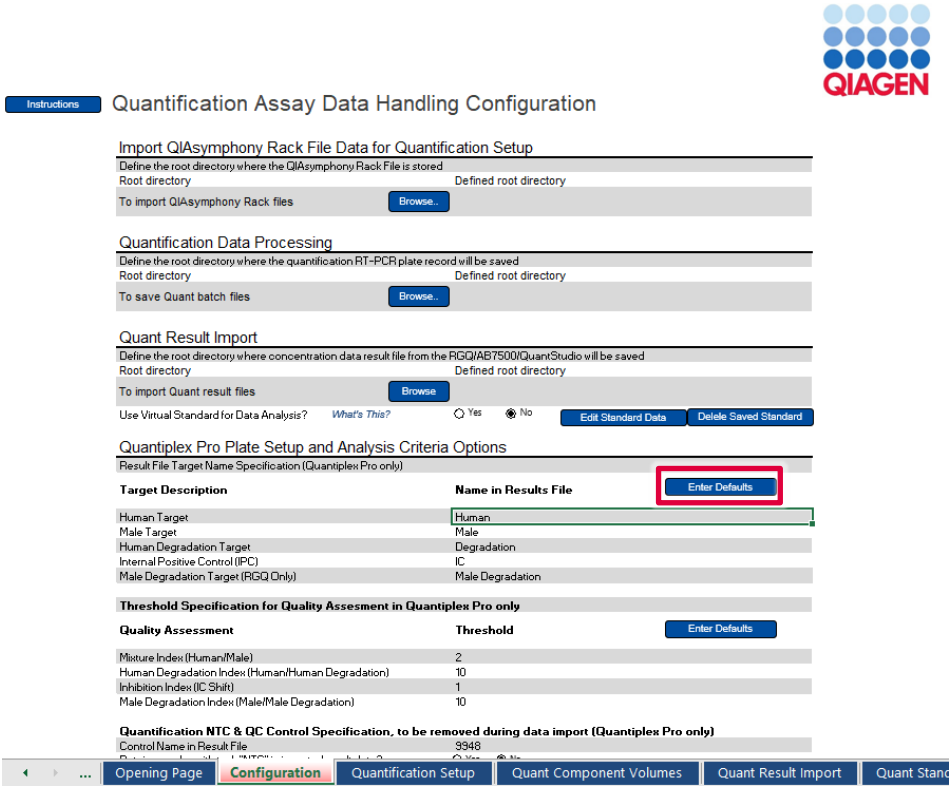
   4a. root/home directory to save Quant batch files

   4b. root/home directory to import Quant result files



5. Target names should be assigned for the QuantStudio 5 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.

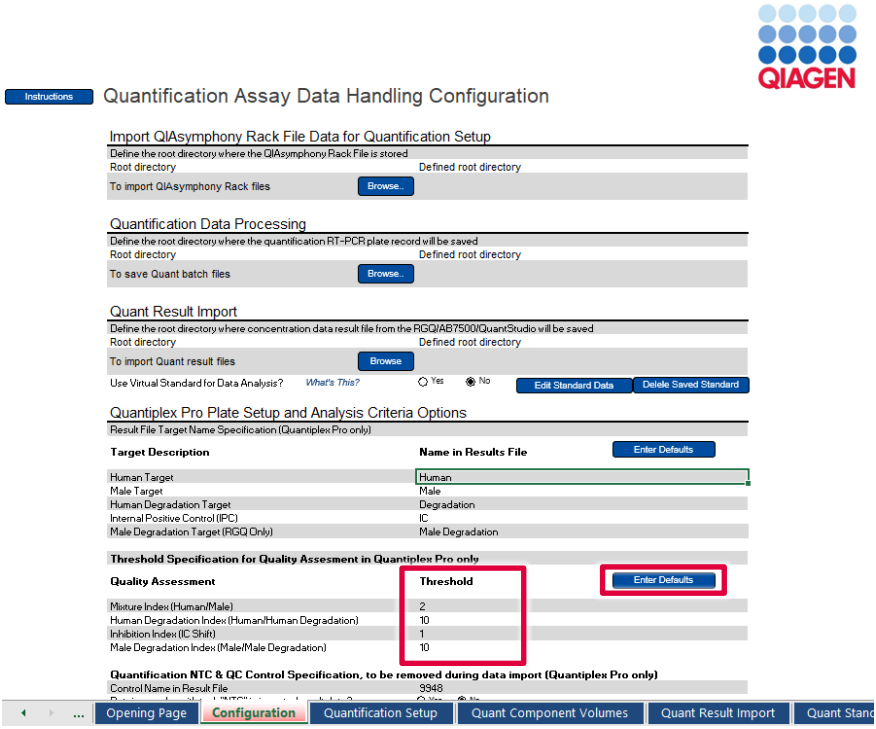


6. Threshold setting for the Quality Assessment can be changed/adjusted as needed. The default threshold settings are

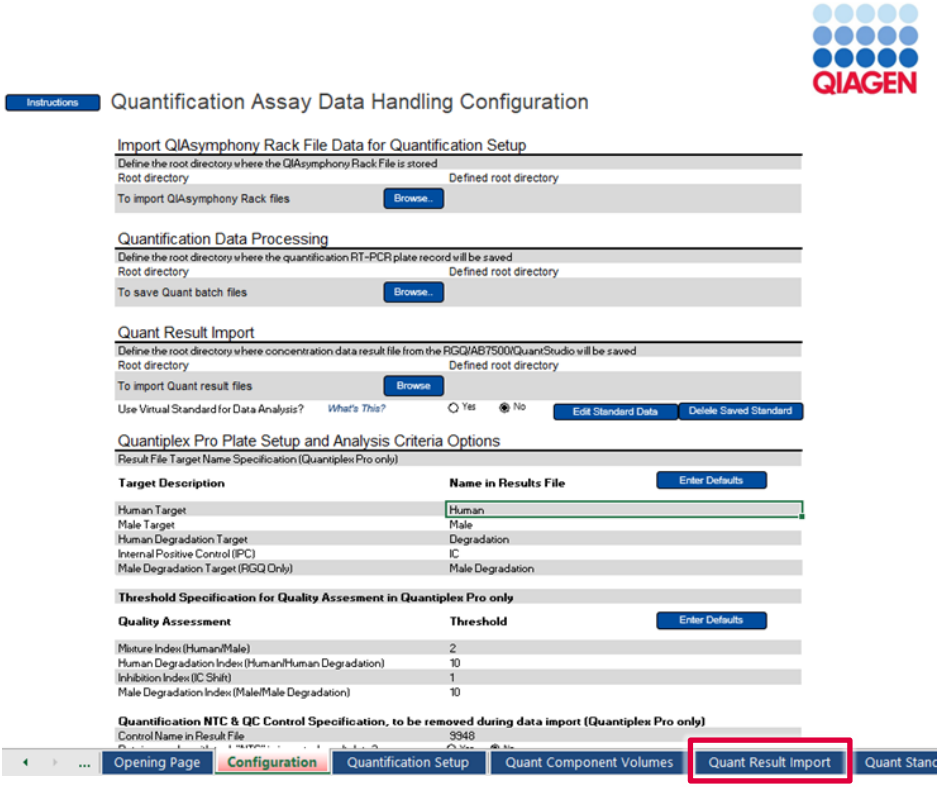
   - Mixture index (Human/Male): **2**
   - Degradation Index (Human/Degradation): **10**
   - Inhibition Index (IC Shift): **1**

   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.

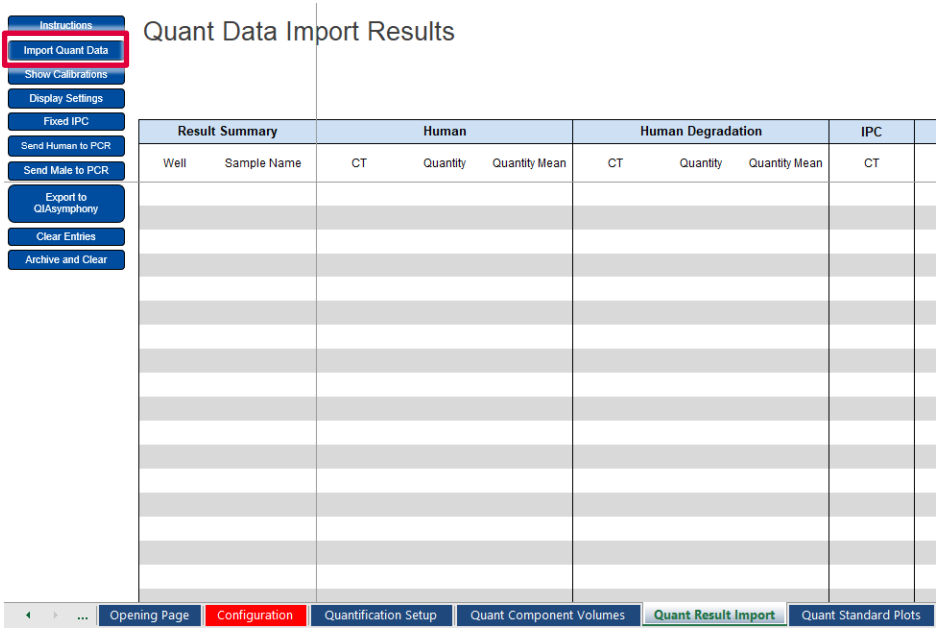
   Defaults can be restored by clicking the **Enter Defaults** button.



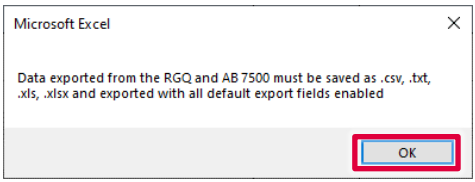
7. To import quantification results click the **Quant Result Import** worksheet tab.



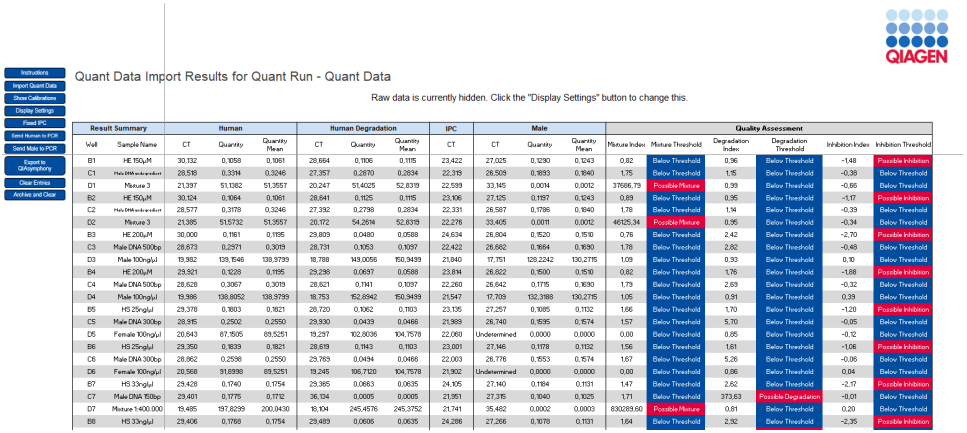
8. Click the **Import Quant Data** button.



9. Confirm that your data are in the necessary 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".



Result Summary		Human			Human Degradation			IPC	Male			Quality Assessment				
Well	Sample Name	CT	Quantity	Quantity Mean	CT	Quantity	Quantity Mean	CT	CT	Quantity	Quantity Mean	Mixture Index	Mixture Threshold	Degradation Index	Degradation Threshold	Inhibition Index	Inhibition Threshold
B1	HE 150µM	30,132	0,1058	0,1061	28,664	0,1106	0,1115	23,422	27,025	0,1230	0,1243	0,82	Below Threshold	0,96	Below Threshold	-1,48	Possible Inhibition
C1	[illegible]	28,516	0,3314	0,3246	27,357	0,2870	0,2834	22,319	26,928	0,1833	0,1840	1,75	Below Threshold	1,15	Below Threshold	-0,38	Below Threshold
D1	Mixture 3	21,397	51,1382	51,3557	20,247	51,4025	52,8319	22,599	33,145	0,0014	0,0012	37696,79	Possible Mixture	0,99	Below Threshold	-0,66	Below Threshold
B2	HE 150µM	30,124	0,1064	0,1061	28,641	0,1125	0,1115	23,106	27,125	0,1197	0,1243	0,89	Below Threshold	0,95	Below Threshold	-1,17	Possible Inhibition
C2	[illegible]	28,577	0,3178	0,3246	27,392	0,2798	0,2834	22,331	26,587	0,1786	0,1840	1,78	Below Threshold	1,14	Below Threshold	-0,39	Below Threshold
D2	Mixture 3	21,385	51,5732	51,3557	20,172	54,2614	52,8319	22,276	33,405	0,0011	0,0012	46125,34	Possible Mixture	0,95	Below Threshold	-0,34	Below Threshold
B3	HE 200µM	30,000	0,1161	0,1195	29,809	0,0480	0,0588	24,634	26,804	0,1520	0,1510	0,76	Below Threshold	2,42	Below Threshold	-2,70	Possible Inhibition
C3	Male DNA 500bp	28,673	0,2971	0,3019	28,731	0,1053	0,1097	22,422	26,682	0,1664	0,1690	1,78	Below Threshold	2,82	Below Threshold	-0,48	Below Threshold
D3	Male 100ng/µl	19,982	139,1546	138,3799	18,788	149,0056	150,9439	21,840	17,751	128,2242	130,2715	1,09	Below Threshold	0,93	Below Threshold	0,10	Below Threshold
B4	HE 200µM	29,921	0,1228	0,1195	29,296	0,0637	0,0588	23,814	26,822	0,1500	0,1510	0,82	Below Threshold	1,76	Below Threshold	-1,68	Possible Inhibition
C4	Male DNA 500bp	28,628	0,3067	0,3019	28,621	0,1141	0,1097	22,260	26,642	0,1715	0,1690	1,79	Below Threshold	2,69	Below Threshold	-0,32	Below Threshold
D4	Male 100ng/µl	19,986	138,8052	138,3799	18,753	152,8942	150,9439	21,547	17,709	132,3188	130,2715	1,05	Below Threshold	0,91	Below Threshold	0,33	Below Threshold
B5	HS 25ng/µl	29,378	0,1803	0,1821	28,720	0,1062	0,1103	23,135	27,257	0,1085	0,1132	1,66	Below Threshold	1,70	Below Threshold	-1,20	Possible Inhibition
C5	Male DNA 300bp	28,915	0,2502	0,2550	29,930	0,0439	0,0468	21,989	26,740	0,1535	0,1574	1,57	Below Threshold	5,70	Below Threshold	-0,05	Below Threshold
D5	Female 100ng/µl	20,643	67,1505	69,5251	19,297	102,8036	104,7578	22,060	Undetermined	0,0000	0,0000	0,00	Below Threshold	0,85	Below Threshold	-0,12	Below Threshold
B6	HS 25ng/µl	29,350	0,1839	0,1821	28,619	0,1143	0,1103	23,001	27,146	0,1178	0,1132	1,56	Below Threshold	1,61	Below Threshold	-1,06	Possible Inhibition
C6	Male DNA 300bp	28,862	0,2598	0,2550	29,769	0,0494	0,0468	22,003	26,776	0,1553	0,1574	1,67	Below Threshold	5,26	Below Threshold	-0,06	Below Threshold
D6	Female 100ng/µl	20,568	91,6998	69,5251	19,245	106,7120	104,7578	21,902	Undetermined	0,0000	0,0000	0,00	Below Threshold	0,86	Below Threshold	0,04	Below Threshold
B7	HS 33ng/µl	29,428	0,1740	0,1754	29,365	0,0663	0,0635	24,105	27,140	0,1184	0,1131	1,47	Below Threshold	2,62	Below Threshold	-2,17	Possible Inhibition
C7	Male DNA 150bp	29,401	0,1775	0,1712	36,134	0,0005	0,0005	21,951	27,315	0,1040	0,1025	1,71	Below Threshold	373,63	Possible Degradation	-0,01	Below Threshold
D7	Mixture 1:400,000	19,495	197,8239	200,0430	18,104	245,4576	245,3752	21,741	35,462	0,0002	0,0003	830283,60	Possible Mixture	0,81	Below Threshold	0,20	Below Threshold
B8	HS 33ng/µl	29,406	0,1768	0,1754	29,493	0,0636	0,0635	24,286	27,266	0,1078	0,1131	1,64	Below Threshold	2,92	Below Threshold	-2,35	Possible Inhibition

10. 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.

General Interpretation of Results

General considerations for data analysis

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

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

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

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

Standard curve

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

$$y = mx + b$$

where x = log concentration and y = C_T.

The slope

The slope (m) describes the PCR efficiency. A slope of –3.3 indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). Typically, the slope ranges between –3.0 and –3.6. If the values fall outside of this range, see the Troubleshooting Guide, page 54, for more information.

The Y-intercept

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

The R^2 value

The R^2 value is a measure of the fit of the data points to the regressed line. In general, the standard curve has an R^2 value ≥0.990. Low R^2 values (R^2 ≤0.98) may occur for many different reasons. In case of low R^2 values, see the Troubleshooting Guide, page 54, for more information.

Internal control

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

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

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

Quantification of unknowns

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

Quantification of female/male mixtures

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

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

Degradation status assessment

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

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

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

Troubleshooting Guide

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

		Comments and suggestions
No signal or one or more signals detected late in PCR		
a)	Incorrect cycling conditions	Always use the optimized cycling conditions specified in the protocols. Make sure to select ROX as the passive dye on QuantStudio 5 Real-Time PCR System.
b)	Pipetting error, missing or degraded reagent	Check the storage conditions of the reagents. Repeat the assay.
c)	Incorrect or no detection step	Make sure that fluorescence detection takes place during the combined annealing/extension step.
d)	Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the template DNA are present in the sample.
e)	Problems with starting template	Check the storage conditions of the starting template DNA. Efficient removal of PCR inhibitors is essential for optimal results. Purify nucleic acids from your sample using an appropriate purification method. Ensure that all reagents, buffers and solutions used for isolating and diluting template nucleic acids are free from nucleases.
f)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Ensure that the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.
g)	Degraded control DNA	Make new serial dilutions of the control DNA from the stock solution. Repeat the assay using the new dilutions.

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

		Comments and suggestions
c)	Wavy curve at high template amounts for highly concentrated targets	In the analysis settings, reduce the number of cycles used for background calculation (if the real-time cycler allows this) or reduce the amount of template.

Slope for the standard curve differs significantly from –3.33 or R^2 value is significantly less than 0.98–0.99

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

Appendix: Alternative Standard Curves

Table 10. 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 11. 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 12. Alternative 7-point standard curve (5x dilution)

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

Ordering Information

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

* Larger kit sizes available; please inquire

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

* Larger kit sizes available; please inquire.

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Document Revision History

Date	Changes
04/2023	Initial release as handbook HB-3355, expanded from supplementary protocol HB-2700.

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