June 2018

Investigator® Quantiplex Handbook

For quantification of human and male DNA in forensic samples

Sample to Insight

QIAGEN

Contents

Kit Contents

Investigator Quantiplex Kit	**(200)**
Catalog no.	**387016**
Number of 25 µl reactions	**200**
Reaction Mix YQ	2 x 1.15 ml
Primer Mix IC FQ	2 x 1.15 ml
Male Control DNA M1 (50 ng/µl)	0.2 ml
QuantiTect® Nucleic Acid Dilution Buffer	1 vial
Quick-Start Protocol	1

Storage

Store the reagents between 2–8°C. Avoid freezing these kit components. The QuantiTect Nucleic Acid Dilution Buffer may also be stored at –30°C to –15°C, if desired. Avoid repeated freezing and thawing. Primer Mix IC FQ must be stored protected from the light. DNA samples should be stored separately from the PCR reagents. Under these conditions, the components are stable until the expiration date indicated on the kit.

Intended Use

The Investigator Quantiplex 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 Kits is tested against predetermined specifications to ensure consistent product quality.

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 Kit was developed for the quantification of human genomic 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) and also to establish if a sample contains inhibitors that may interfere with such applications, thus necessitating further sample purification.

Principle and procedure

The Investigator Quantiplex Kit is a ready-to-use system for the detection of human DNA using PCR. The Investigator Quantiplex Kit provides fast and accurate quantification of human DNA in forensic database and casework samples. The assay provides sensitivity down to <1 pg/µl, with accurate quantification below 4.9 pg/µl, where the standard curve shows linearity.

The kit contains reagents and a DNA polymerase for specific amplification of a 146 bp proprietary region present on several autosomes of the human genome (patent pending), and for detection of the specific PCR products on the Rotor-Gene® Q or Applied Biosystems® 7500 Real-Time PCR Systems. The human quantification target region was selected in order to give high sensitivity with high reliability within different individuals and populations. This target region was validated in an external study by a number of forensic laboratories where the conservation of its copy number in the human population was analyzed. A quantitative real-time PCR was run to compare a human specific single-copy and the Investigator Quantiplex multi-copy target. The Delta-C_T for the two targets was comparable in different populations and between male and female DNA samples, demonstrating the conservation of the target copy number across gender and ethnic groups. The human quantification target region is detected using the green channel on the Rotor-Gene Q or the FAM™ dye channel on Applied Biosystems instruments.

In addition, the Investigator Quantiplex Kit contains a balanced internal amplification control that is used to test successful amplification and detects PCR inhibitors. This heterologous amplification system is detected as a 200 bp internal control (IC) in the yellow channel on the Rotor-Gene Q or in the VIC® dye channel on Applied Biosystems instruments.

Detection of amplification is performed using Scorpions® primers and a novel, fast PCR chemistry. Scorpions primers are bifunctional molecules containing a PCR primer covalently linked to a probe (Figure 1). The fluorophore in this probe interacts with a quencher, also incorporated into the probe, which reduces fluorescence. During PCR, when the probe binds to the PCR products, the fluorophore and quencher become separated. This leads to an increase in fluorescence in the reaction tube.

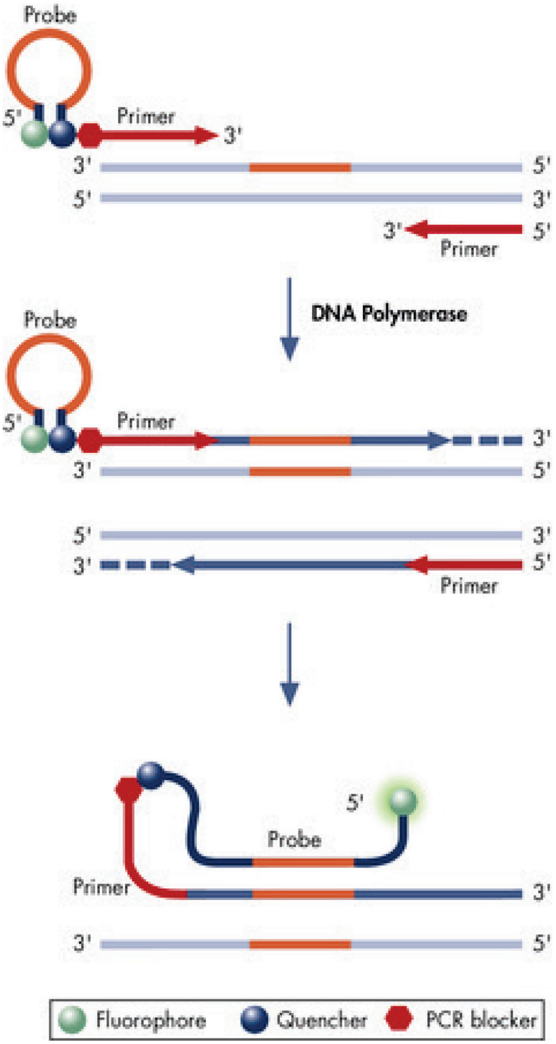


Figure 1. Scorpions primers and their function.

Scorpions primers are well known for their rapid hybridization to the target sequence, via the intramolecular reaction (Whitcombe, 1999). The reaction chemistry was carefully optimized to further support the rapid mechanism.

Internal control

The Internal Control (IC) is amplified and detected in the yellow channel on the Rotor-Gene Q or in the VIC dye channel on Applied Biosystems instruments. The fluorophore can be detected with the existing VIC calibration. The IC is designed to be more sensitive to inhibitors than the human quantification target. The 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 may provide an indication of potential inhibition of the reaction in the unknown samples. Therefore, even in the case that 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.

Positive amplification of the IC system will generate a C_T value of approximately 31*. A variation of ±1 in the C_T values of the IC system for the standard curve samples can be expected. Using large amounts of human DNA (>150 ng/reaction) can give a higher C_T value for the IC system.

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

* This value may vary depending on the instrument used.

Reaction Mix YQ

The Reaction Mix YQ contains a uniquely formulated DNA polymerase and reaction buffer. The novel PCR chemistry and enzyme, which were specifically developed for human identification, enable high sensitivity and speed. The newly formulated enzyme and reaction buffer blend is particularly suited to support the fast Scorpions primer reaction.

Furthermore, the specially developed PCR buffer 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.

The Reaction Mix YQ 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. Four-fold dilution series with 7 concentration points in the standard curve for each assay are strongly recommended. To ensure pipetting accuracy, the minimum input volume of DNA for dilutions should be 10 µl. The standard curve is designed to be easily set up using in a convenient dilution series. If using the unique QuantiTect Nucleic Acid Dilution buffer to dilute the Male Control DNA M1, 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 Kit.

Templates for routine work

In order to streamline the instrument setup and the analysis of the results on the Rotor-Gene Q, Applied Biosystems 7500 Real-Time PCR System, Applied Biosystems 7500 Real-Time PCR

System for Human Identification, and 7500 Fast Real-Time PCR System, QIAGEN has developed a set of template files. These templates can be downloaded from the 'Resources' tab at www.qiagen.com/Quantiplex.

Table 1. Template files available for the Rotor Gene Q

Template file	Purpose
Investigator Quantiplex Kit Cycling	Preset cycling protocol
Investigator Quantiplex Kit Sample	Sample name, sample type and concentration for standard curve and internal control
Investigator Quantiplex Kit Analysis Settings for the Yellow Channel	C_T settings

Description of protocols

Protocols for four different cyclers are provided in this handbook.

- Rotor-Gene Q
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System for Human Identification
- Applied Biosystems 7500 Fast 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.

- Pipets and pipet tips
- 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
- Cooling device or ice
- Real-time thermal cycler (Rotor-Gene Q, Applied Biosystems 7500 Real-Time PCR System, Applied Biosystems 7500 Real-Time PCR System for Human Identification, or Applied Biosystems 7500 Fast Real-Time PCR System)
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your thermal cycler)
- For the Rotor-Gene Q: Strip Tubes and Caps, 0.1 ml (cat. no. 981103 or 981106) are recommended.

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 the ones listed have not been validated by QIAGEN for DNA quantification using the Investigator Quantiplex Kit.

Note: An automated setup is also possible.

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

Real-time thermal cycler	Protocol
Rotor-Gene Q	Page 16
Applied Biosystems 7500 Real-Time PCR System	Page 39
Applied Biosystems 7500 Real-Time PCR System for Human Identification	Page 51
Applied Biosystems 7500 Fast Real-Time PCR System	Page 65

Contamination risks

Do not remove the seal on 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. The quantification using the Investigator Quantiplex 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 in at least duplicate.

Internal positive control

An internal, positive control (detected using a second Scorpions primer with a different label) is used to test for successful amplification and for the presence of PCR inhibitors. Primer, Scorpions primer, and template for the internal control are all contained in the Primer Mix IC FQ.

Protocol: Quantification of DNA Using the Rotor-Gene Q

This protocol is optimized for use of the Investigator Quantiplex Kit on the Rotor-Gene Q.

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.
- Always use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Always use the template volume specified in the protocol. The reaction is optimized for use with 2 µl template DNA. Do not use more or less than 2 µl per 25 µl reaction.
- We recommend using a 72-well rotor.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored at between +2 and +8°C for at least 1 week.
- Optimal analysis settings are a prerequisite for accurate quantification data. Always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- Download the template files “Investigator Quantiplex Kit Cycling file,” “Investigator Quantiplex Kit Sample file,” and “Investigator Quantiplex Kit Analysis Settings for the Yellow Channel file.” Go to the ‘Resources’ tab at www.qiagen.com/Quantiplex to download the files.

Procedure

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 3. Vortex for at least 5 s and centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipet tip for each dilution.

   Take care not to introduce cross-contamination.

Table 3. Serial dilutions of Male Control DNA M1

Standard	Serial dilution of control DNA (ng/µl)	Control DNA (µl)	QuantiTect Nucleic Acid Dilution Buffer (µl)
–	50 (stock conc.)	–	–
Standard -1	20	20 (from stock)	30
Standard -2	5	10	30
Standard -3	1.25	10	30
Standard -4	0.3125	10	30
Standard -5	0.078125	10	30
Standard -6	0.01953125	10	30
Standard -7	0.0048828125	10	30

Note: Alternative standard curves are listed in "Appendix: Alternative Standard Curves," page 86.

3. Thaw template nucleic acids.

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

c. Reaction setup can usually be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

Table 4. Master Mix for DNA quantification

Component	Volume per 25 µl reaction	Final concentration
Reaction Mix YQ	11.5 µl	1x
Primer Mix IC FQ	11.5 µl	1x
Total volume of master mix	**23 µl**	

5. Mix the master mix thoroughly, and dispense 23 µl into Rotor-Gene Q Strip Tubes.
6. Add 2 µl QuantiTect Nucleic Acid Dilution Buffer to the NTC tubes. Ensure that the NTC tubes do not come in contact with human DNA.
7. Add 2 µl control DNA dilutions or 2 µl unknown sample DNA to the individual PCR tubes and mix thoroughly. It is very important to mix carefully in order to avoid localized concentrations of salt and DNA.

   Table 5 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 5. Possible plate setup of reactions on the Rotor-Gene Q

Well contents																	
1	20	**9**	0.0781	**17**	UNK	**25**	UNK	**33**	UNK	**41**	UNK	**49**	UNK	**57**	UNK	**65**	UNK
2	20	**10**	0.0781	**18**	UNK	**26**	UNK	**34**	UNK	**42**	UNK	**50**	UNK	**58**	UNK	**66**	UNK
3	5	**11**	0.0195	**19**	UNK	**27**	UNK	**35**	UNK	**43**	UNK	**51**	UNK	**59**	UNK	**67**	UNK
4	5	**12**	0.0195	**20**	UNK	**28**	UNK	**36**	UNK	**44**	UNK	**52**	UNK	**60**	UNK	**68**	UNK
5	1.25	**13**	0.0049	**21**	UNK	**29**	UNK	**37**	UNK	**45**	UNK	**53**	UNK	**61**	UNK	**69**	UNK
6	1.25	**14**	0.0049	**22**	UNK	**30**	UNK	**38**	UNK	**46**	UNK	**54**	UNK	**62**	UNK	**70**	UNK
7	0.3125	**15**	NTC	**23**	UNK	**31**	UNK	**39**	UNK	**47**	UNK	**55**	UNK	**63**	UNK	**71**	UNK
8	0.3125	**16**	NTC	**24**	UNK	**32**	UNK	**40**	UNK	**48**	UNK	**56**	UNK	**64**	UNK	**72**	UNK

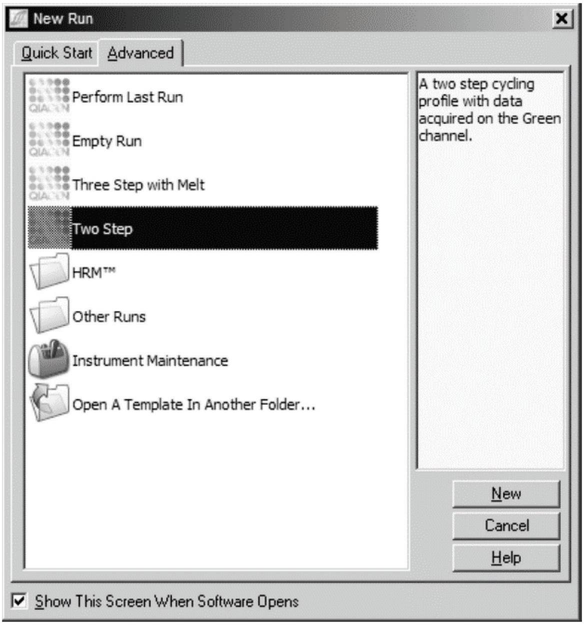
* All amounts in ng/µl. UNK = Unknown sample.

8. Close the PCR tubes, place them in the 72-well rotor in the Rotor-Gene Q cycler, and attach the locking ring.

   If there are fewer than 72 reactions, empty positions in the 72-well rotor should be filled with empty PCR tubes.

9. Open the Rotor-Gene software. In the Advanced Wizard, select "Open A Template In Another Folder…" and load the file "Investigator Quantiplex Kit Cycling file." To setup cycling manually, select "Two Step" cycling profile, and click "New."

   We recommend using the provided template file (see page 16 for download information) to facilitate the reaction setup.

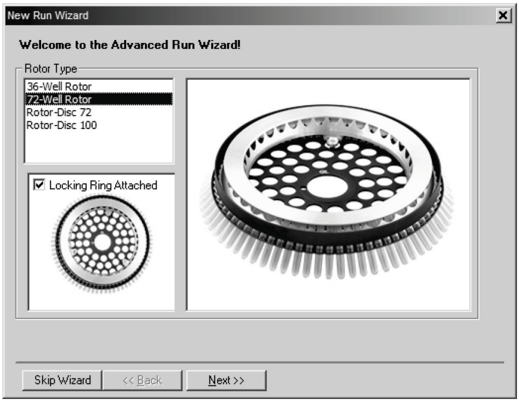


A two step cycling profile with data acquired on the Green channel.

☑ Show This Screen When Software Opens

Advanced Wizard of the Rotor-Gene software.

10. Select "72-Well Rotor," and confirm the locking ring is attached by checking the check box. Click "Next" to continue.



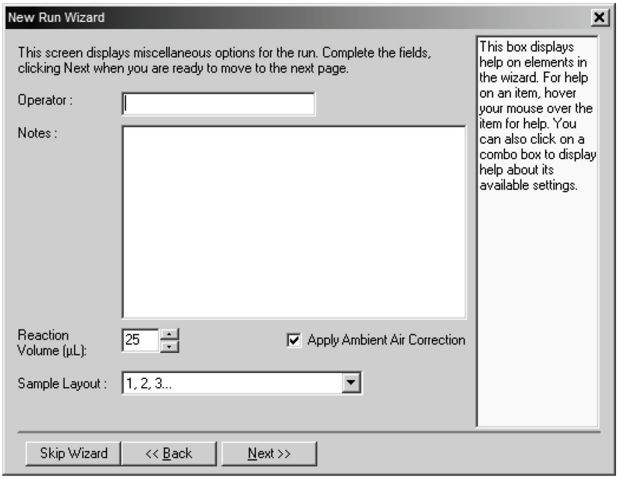
Welcome to the Advanced Run Wizard!

☑ Locking Ring Attached

Confirmation that the locking ring is attached.

11. Ensure that the reaction volume is 25 µl and that the "Apply Ambient Air Correction" check box is checked. Click "Next" to continue.

    Note: The Apply Ambient Air Correction function is not available in all versions of the software. If this does not apply to your software, simply click "Next" to continue.



This screen displays miscellaneous options for the run. Complete the fields, clicking Next when you are ready to move to the next page.

Operator :

Notes :

Reaction Volume (µL): 25

☑ Apply Ambient Air Correction

Sample Layout : 1, 2, 3...

This box displays help on elements in the wizard. For help on an item, hover your mouse over the item for help. You can also click on a combo box to display help about its available settings.

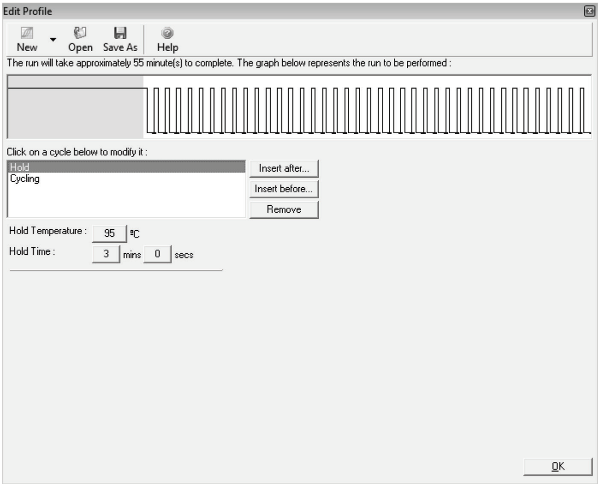
Enter reaction volume and check "Apply Ambient Air Correction," if relevant to your software.

12. Click "Edit Profile" and program the Rotor-Gene cycler according to Table 6. See also the figures on pages 22 and 23.

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

Table 6. Cycling conditions for the Rotor-Gene Q

Step	Temperature	Time	Number of cycles	Additional comments
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C for 3 min to activate the DNA polymerase.
Two-step cycling:			40	
Denaturation	95°C	5 s		
Combined annealing/ extension	60°C	10 s		Perform fluorescence data collection using the green and the yellow channels with auto-gain optimization.



The run will take approximately 55 minute(s) to complete. The graph below represents the run to be performed :

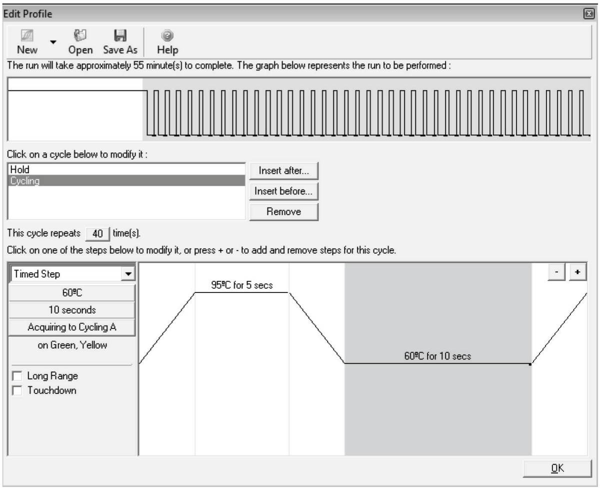
Click on a cycle below to modify it :

Hold
Cycling

Hold Temperature : 95 °C

Hold Time : 3 mins 0 secs

Initial PCR activation step. PCR requires an initial incubation at 95°C for 3 min to activate the DNA polymerase.



The run will take approximately 55 minute(s) to complete. The graph below represents the run to be performed :

Click on a cycle below to modify it :

Hold
Cycling

This cycle repeats 40 time(s).

Click on one of the steps below to modify it, or press + or - to add and remove steps for this cycle.

Timed Step
60ºC
10 seconds
Acquiring to Cycling A
on Green, Yellow

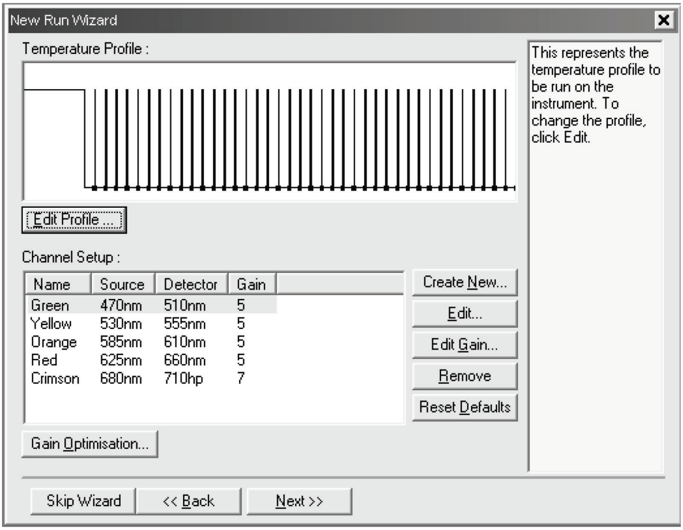
☐ Long Range
☐ Touchdown

95ºC for 5 secs
60ºC for 10 secs

Two-step cycling. PCR requires 40 cycles. Each cycle is comprised of 2 steps: 95°C for 5 s (denaturation step) and 60°C for 10 s (annealing/extension step).

13. To set the gain optimization settings for the green and yellow channels, first select the green channel, and then click “Gain Optimization,” followed by “Optimize Acquiring.” In the dialog box that opens, confirm the standard settings for both channels. Then click “OK.”
14. Check the “Perform Optimization Before 1st Acquisition” box, and click “Close.”

    Make sure that the tube at position 1 is not empty, since the gain optimization will be performed on this tube.

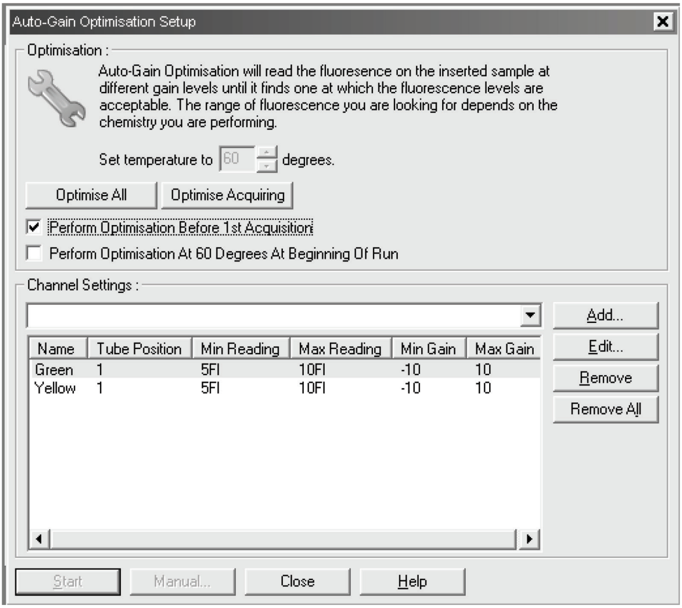


This represents the temperature profile to be run on the instrument. To change the profile, click Edit.

Channel Setup :

Name	Source	Detector	Gain
Green	470nm	510nm	5
Yellow	530nm	555nm	5
Orange	585nm	610nm	5
Red	625nm	660nm	5
Crimson	680nm	710hp	7

Gain optimization.



Auto-Gain Optimisation will read the fluoresence on the inserted sample at different gain levels until it finds one at which the fluorescence levels are acceptable. The range of fluorescence you are looking for depends on the chemistry you are performing.

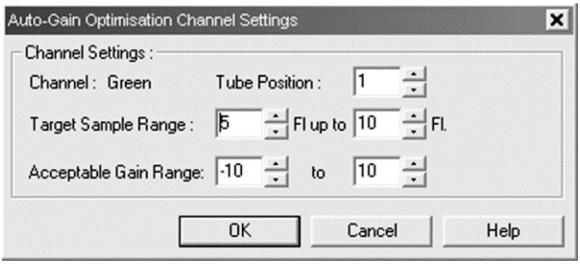
Set temperature to 60 degrees.

☑ Perform Optimisation Before 1st Acquisition

☐ Perform Optimisation At 60 Degrees At Beginning Of Run

Name	Tube Position	Min Reading	Max Reading	Min Gain	Max Gain
Green	1	5FI	10FI	-10	10
Yellow	1	5FI	10FI	-10	10

Optimize acquisition.



Channel Settings :

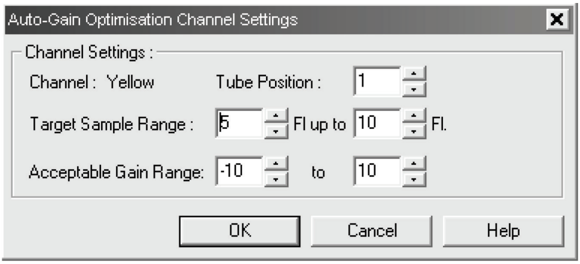
Channel : Green

Tube Position : 1

Target Sample Range : 5 Fl up to 10 Fl.

Acceptable Gain Range: -10 to 10

Confirming Gain Optimization settings of the green channel.



Channel Settings :

Channel : Yellow

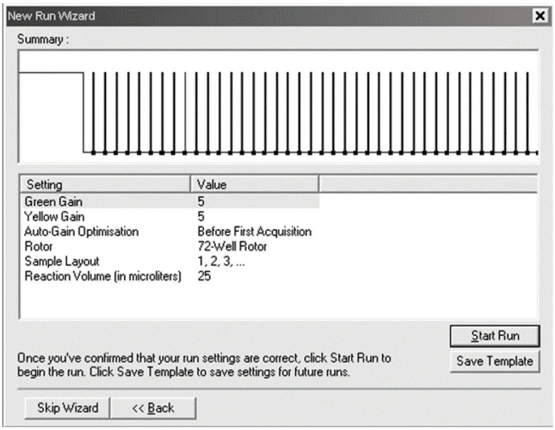
Tube Position : 1

Target Sample Range : 5 Fl up to 10 Fl.

Acceptable Gain Range: -10 to 10

Confirming Gain Optimization settings for the yellow channel.

15. Click "Next" to confirm the temperature profile and channel setup, and check that all parameters are correct.



Setting	Value
Green Gain	5
Yellow Gain	5
Auto-Gain Optimisation	Before First Acquisition
Rotor	72-Well Rotor
Sample Layout	1, 2, 3, ...
Reaction Volume (in microliters)	25

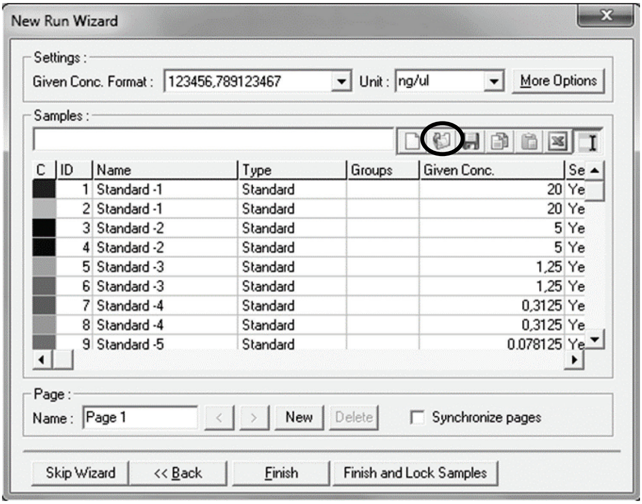
Once you've confirmed that your run settings are correct, click Start Run to begin the run. Click Save Template to save settings for future runs.

Summary of parameters.

16. Start the Rotor-Gene cycler by clicking “Start run.” You will be prompted to enter a file name and to save the run file.

17. After the run has started, you can enter a name and description for each reaction while you wait for the run to end.

    If using the plate setup recommended in Table 5, you can also use the “Investigator Quantiplex Kit Sample file” (see page 16 for download information). To load the template file, go to “Open” and select the template file required. An example template file is shown below.



Given Conc. Format : 123456,789123467

Unit : ng/ul

C	ID	Name	Type	Groups	Given Conc.	Se
■	1	Standard -1	Standard		20	Ye
■	2	Standard -1	Standard		20	Ye
■	3	Standard -2	Standard		5	Ye
■	4	Standard -2	Standard		5	Ye
■	5	Standard -3	Standard		1,25	Ye
■	6	Standard -3	Standard		1,25	Ye
■	7	Standard -4	Standard		0,3125	Ye
■	8	Standard -4	Standard		0,3125	Ye
■	9	Standard -5	Standard		0.078125	Ye

Name : Page 1

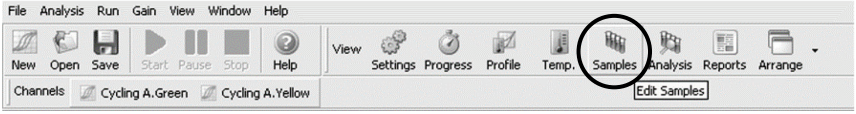
Template file for editing the standard curve.

Data Analysis

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

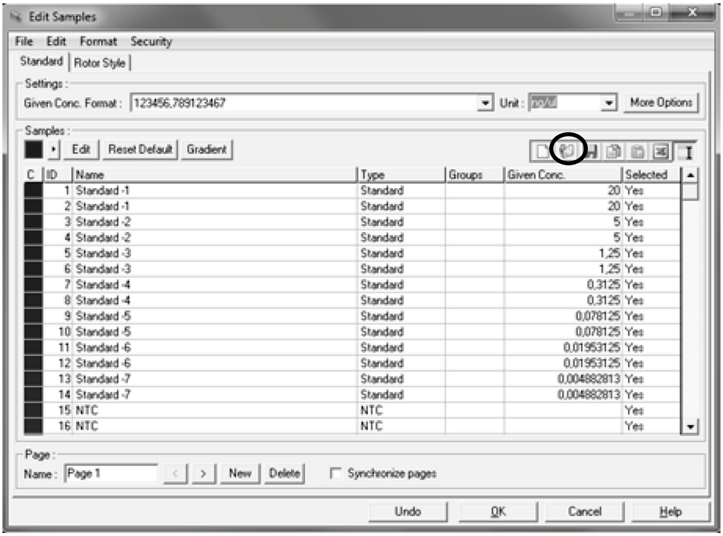
Procedure

1. Open the run file using the Rotor-Gene Q software. Go to "File," followed by "Open" and then "Browse" to locate the saved file.
2. Standards must first be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 10.
   If using the plate setup recommended in Table 5, you can also import the sample name and type, and the concentration of the standards directly using the "Investigator Quantiplex Kit Sample file." We recommend using that file to streamline the reaction setup. See page 16 for download information.
3. Select the Edit Sample tool by clicking "Samples."



Edit Samples tool in the Rotor-Gene Q software.

4. The “Investigator Quantiplex Kit Sample file” can be loaded by going to “Open” and choosing the file.



Given Conc. Format : 123456,789123467

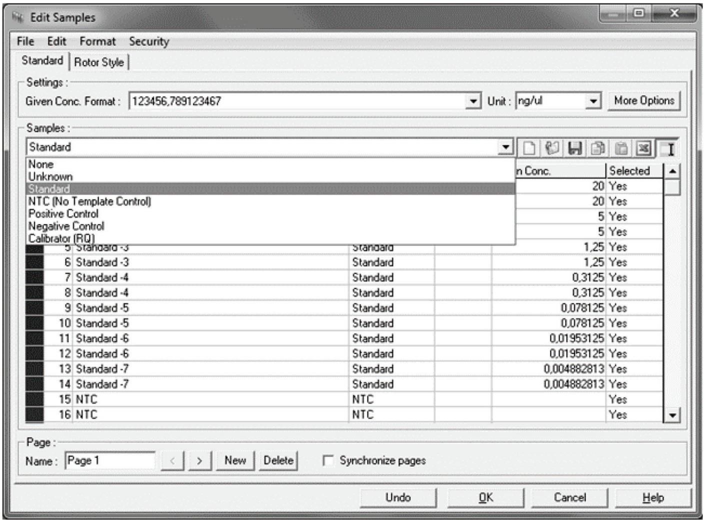
C	ID	Name	Type	Groups	Given Conc.	Selected
■	1	Standard -1	Standard		20	Yes
■	2	Standard -1	Standard		20	Yes
■	3	Standard -2	Standard		5	Yes
■	4	Standard -2	Standard		5	Yes
■	5	Standard -3	Standard		1,25	Yes
■	6	Standard -3	Standard		1,25	Yes
■	7	Standard -4	Standard		0,3125	Yes
■	8	Standard -4	Standard		0,3125	Yes
■	9	Standard -5	Standard		0,078125	Yes
■	10	Standard -5	Standard		0,078125	Yes
■	11	Standard -6	Standard		0,01953125	Yes
■	12	Standard -6	Standard		0,01953125	Yes
■	13	Standard -7	Standard		0,004882813	Yes
■	14	Standard -7	Standard		0,004882813	Yes
■	15	NTC	NTC			Yes
■	16	NTC	NTC			Yes

Name : Page 1

☐ Synchronize pages

Template file for editing the standard curve.

5. If the template file is not loaded, use the dropdown menu to define the sample type in each of the wells, e.g., “Standard” for DNA standards and “NTC” for no template controls.



Given Conc. Format : 123456,789123467

Unit : ng/ul

Samples : Standard

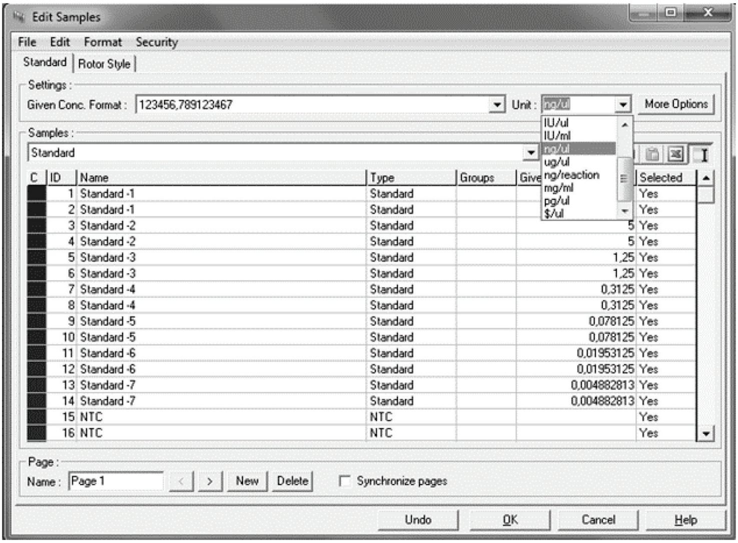
					n Conc.	Selected
					20	Yes
					20	Yes
					5	Yes
					5	Yes
■	5	Standard -3	Standard		1,25	Yes
■	6	Standard -3	Standard		1,25	Yes
■	7	Standard -4	Standard		0,3125	Yes
■	8	Standard -4	Standard		0,3125	Yes
■	9	Standard -5	Standard		0,078125	Yes
■	10	Standard -5	Standard		0,078125	Yes
■	11	Standard -6	Standard		0,01953125	Yes
■	12	Standard -6	Standard		0,01953125	Yes
■	13	Standard -7	Standard		0,004882813	Yes
■	14	Standard -7	Standard		0,004882813	Yes
■	15	NTC	NTC			Yes
■	16	NTC	NTC			Yes

Name : Page 1

☐ Synchronize pages

Editing the samples in the Rotor-Gene Q software.

6. In the Given Conc. column, enter the DNA concentration of the standard and define the unit (ng/µl) from the dropdown menu. Enter the sample name (e.g., Standard -1).



Given Conc. Format : 123456,789123467

Unit : ng/ul

Standard

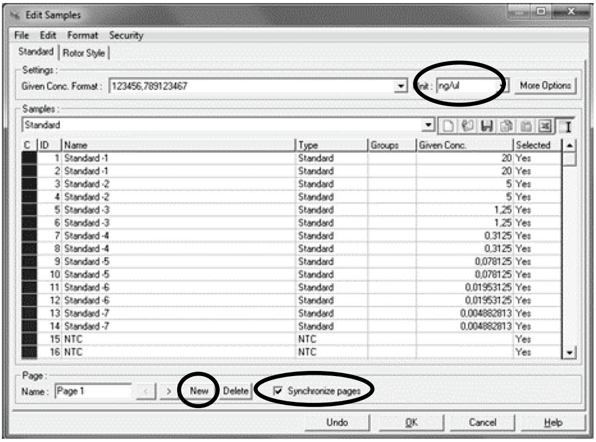
C	ID	Name	Type	Groups	Give	Selected
■	1	Standard -1	Standard			Yes
■	2	Standard -1	Standard			Yes
■	3	Standard -2	Standard		5	Yes
■	4	Standard -2	Standard		5	Yes
■	5	Standard -3	Standard		1,25	Yes
■	6	Standard -3	Standard		1,25	Yes
■	7	Standard -4	Standard		0,3125	Yes
■	8	Standard -4	Standard		0,3125	Yes
■	9	Standard -5	Standard		0,078125	Yes
■	10	Standard -5	Standard		0,078125	Yes
■	11	Standard -6	Standard		0,01953125	Yes
■	12	Standard -6	Standard		0,01953125	Yes
■	13	Standard -7	Standard		0,004882813	Yes
■	14	Standard -7	Standard		0,004882813	Yes
■	15	NTC	NTC			Yes
■	16	NTC	NTC			Yes

Name : Page 1

☐ Synchronize pages

Editing the units in the Rotor-Gene Q software.

7. Create a new page by clicking “New” and selecting “Synchronize pages.” Leave all the samples as “Unknown” in the Type column.



Settings:

Given Conc. Format: 123456,789123467

Unit: ng/ul

Samples: Standard

C	ID	Name	Type	Groups	Given Conc.	Selected
■	1	Standard -1	Standard		20	Yes
■	2	Standard -1	Standard		20	Yes
■	3	Standard -2	Standard		5	Yes
■	4	Standard -2	Standard		5	Yes
■	5	Standard -3	Standard		1,25	Yes
■	6	Standard -3	Standard		1,25	Yes
■	7	Standard -4	Standard		0,3125	Yes
■	8	Standard -4	Standard		0,3125	Yes
■	9	Standard -5	Standard		0,078125	Yes
■	10	Standard -5	Standard		0,078125	Yes
■	11	Standard -6	Standard		0,01953125	Yes
■	12	Standard -6	Standard		0,01953125	Yes
■	13	Standard -7	Standard		0,004882813	Yes
■	14	Standard -7	Standard		0,004882813	Yes
■	15	NTC	NTC			Yes
■	16	NTC	NTC			Yes

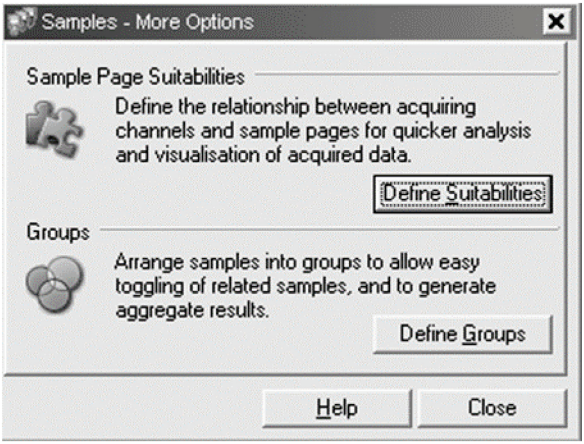
Page:

Name: Page 1 < > New

☑ Synchronize pages

Create a new sample page.

8. Open “More Options” menu and define the Sample Page Suitabilities. Select the option “Only display this sample page when analyzing data.”



Sample Page Suitabilities

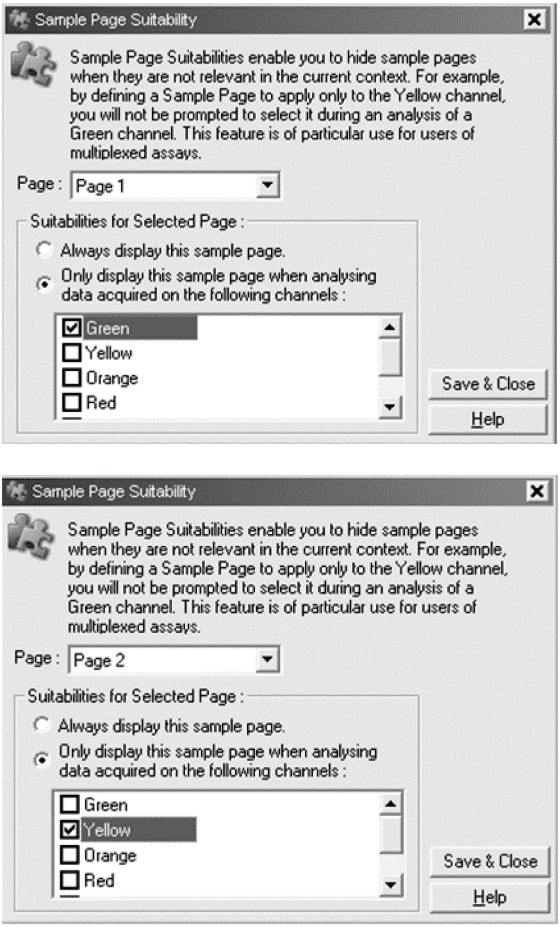
Define the relationship between acquiring channels and sample pages for quicker analysis and visualisation of acquired data.

Groups

Arrange samples into groups to allow easy toggling of related samples, and to generate aggregate results.

Sample Page Suitabilities.

9. Select the green channel for page 1 and the yellow channel for page 2. Click “Save and Close.” Close the Sample Page Suitabilities by clicking “Close.”



Sample Page Suitability

Sample Page Suitabilities enable you to hide sample pages when they are not relevant in the current context. For example, by defining a Sample Page to apply only to the Yellow channel, you will not be prompted to select it during an analysis of a Green channel. This feature is of particular use for users of multiplexed assays.

Page : Page 1

Suitabilities for Selected Page :

○ Always display this sample page.

● Only display this sample page when analysing data acquired on the following channels :

☑ Green
☐ Yellow
☐ Orange
☐ Red

Sample Page Suitability

Sample Page Suitabilities enable you to hide sample pages when they are not relevant in the current context. For example, by defining a Sample Page to apply only to the Yellow channel, you will not be prompted to select it during an analysis of a Green channel. This feature is of particular use for users of multiplexed assays.

Page : Page 2

Suitabilities for Selected Page :

○ Always display this sample page.

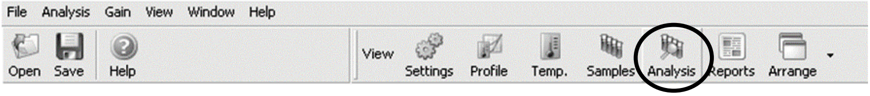
● Only display this sample page when analysing data acquired on the following channels :

☐ Green
☑ Yellow
☐ Orange
☐ Red

Selecting color channels.

10. Click “OK” in the Edit Samples tab.

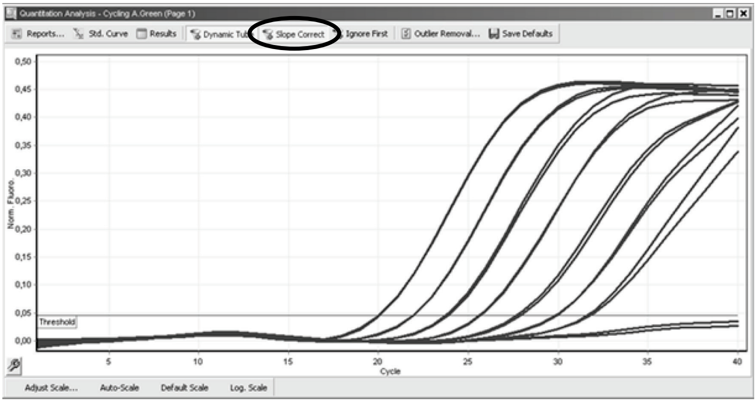
11. To analyze the samples, open the analysis tool by clicking “Analysis.”



12. On the Quantitation tab, select “Page 1” for the green channel and click “Show.” Select “Page 2” for the yellow channel and click “Show.” If the Autofind Threshold window opens automatically, click “Cancel.”
The amplification plots for both the green and yellow channels are displayed in the “Quantitation Analysis” window.

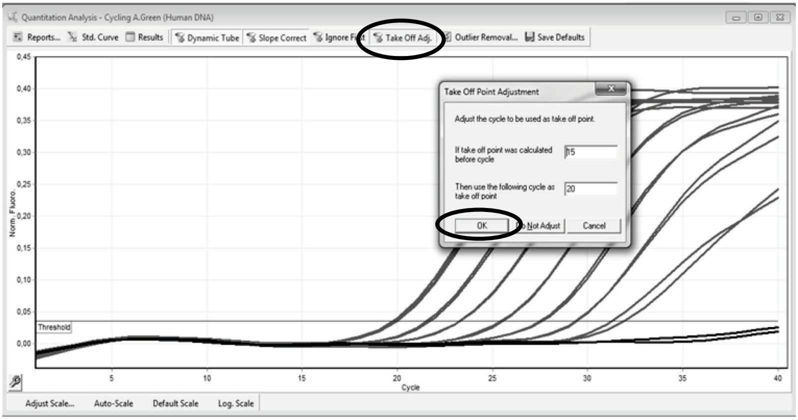
Quantitation tab in the Analysis tool.

13. Activate the Slope Correction tool for the green channel.



Slope correction tool.

14. If available, activate the Take Off Point Adjustment tool for the green channel. Choose the default settings and click "OK."



Take Off Point Adjustment

Adjust the cycle to be used as take off point.

If take off point was calculated before cycle: 15

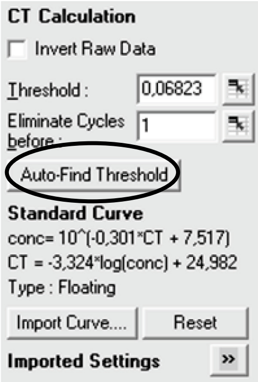
Then use the following cycle as take off point: 20

Take Off Point Adjustment tool.

15. Select the samples in the table on the right. On the right side at the bottom of the panel, select “Auto-Find threshold” for the green channel.

    The C_T values are reported in the “Quantitation Results” window.

    Setting the appropriate threshold value may require further internal validation at your facility.



CT Calculation

☐ Invert Raw Data

Threshold : 0,06823

Eliminate Cycles before : 1

Standard Curve

conc= 10^(-0,301*CT + 7,517)

CT = -3,324*log(conc) + 24,982

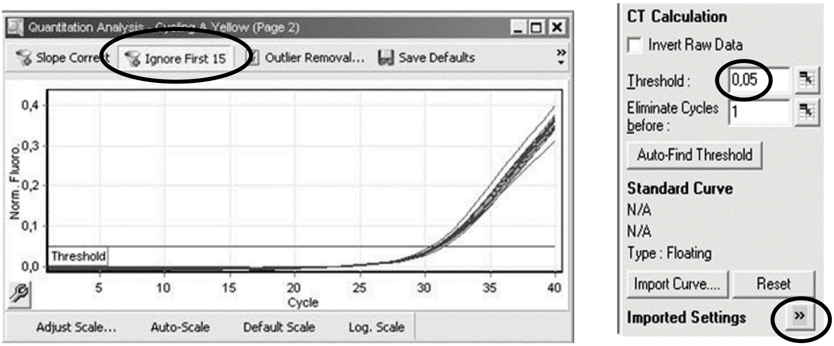
Type : Floating

Setting the C_T value for the Green Channel using the Auto-Find Threshold.

16. For the yellow channel, import the Settings from the “Investigator Quantiplex Kit Analysis Settings for the Yellow Channel file” using the Import tool at the bottom of the panel. See page 16 for information on how to download this file. Alternatively, select a C_T threshold value of 0.05 and ignore the first 15 cycles.

    The C_T values are reported in the “Quantitation Results” window.

Setting the appropriate threshold value may require further internal validation at your facility.



☐ Invert Raw Data

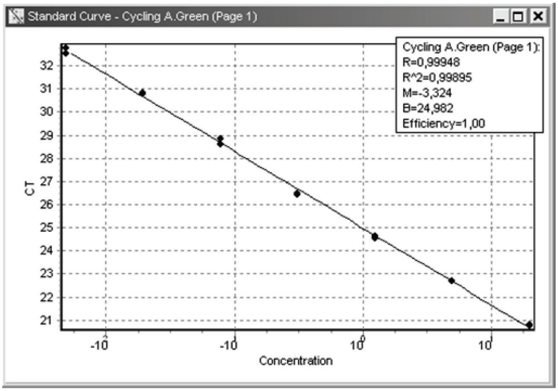
Threshold : 0,05

Eliminate Cycles before : 1

Type : Floating

Setting the C_T value for the Yellow Channel using the Import tool.

17. The Standard Curve is shown in the “Standard Curve” window for the green channel. View the calculated regression line, slope (M), y intercept (B), and R^2 values.

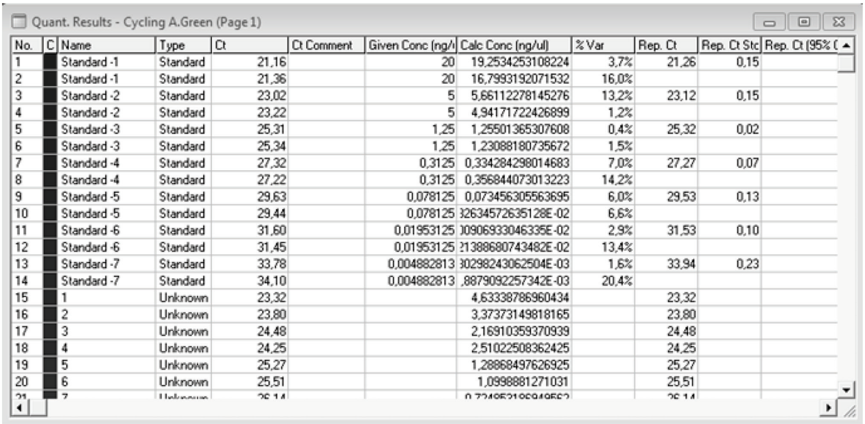


Cycling A.Green (Page 1):
R=0,99948
R^2=0,99895
M=-3,324
B=24,982
Efficiency=1,00

Standard curve.

18. View the concentration of the unknown samples.

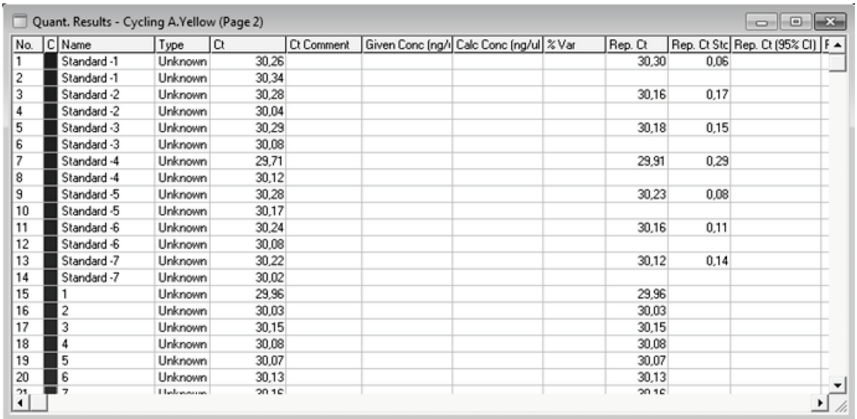
    The “Quantitation Results – Cycling A. Green” window displays data for selected wells, and summarizes the quantity of DNA present in the unknown samples. The unit is shown at the top of the column.



No.	C	Name	Type	Ct	Ct Comment	Given Conc (ng/	Calc Conc (ng/ul)	% Var	Rep. Ct	Rep. Ct Std	Rep. Ct (95% (
1	■	Standard -1	Standard	21,16		20	19,2534253108224	3,7%	21,26	0,15	
2	■	Standard -1	Standard	21,36		20	16,7993192071532	16,0%			
3	■	Standard -2	Standard	23,02		5	5,66112278145276	13,2%	23,12	0,15	
4	■	Standard -2	Standard	23,22		5	4,94171722426899	1,2%			
5	■	Standard -3	Standard	25,31		1,25	1,25501365307608	0,4%	25,32	0,02	
6	■	Standard -3	Standard	25,34		1,25	1,23088180735672	1,5%			
7	■	Standard -4	Standard	27,32		0,3125	0,334284298014683	7,0%	27,27	0,07	
8	■	Standard -4	Standard	27,22		0,3125	0,356844073013223	14,2%			
9	■	Standard -5	Standard	29,63		0,078125	0,073456305563695	6,0%	29,53	0,13	
10	■	Standard -5	Standard	29,44		0,078125	32634572635128E-02	6,6%			
11	■	Standard -6	Standard	31,60		0,01953125	0906933046335E-02	2,9%	31,53	0,10	
12	■	Standard -6	Standard	31,45		0,01953125	21388680743482E-02	13,4%			
13	■	Standard -7	Standard	33,78		0,004882813	30298243062504E-03	1,6%	33,94	0,23	
14	■	Standard -7	Standard	34,10		0,004882813	,8879092257342E-03	20,4%			
15	■	1	Unknown	23,32			4,63338786960434		23,32		
16	■	2	Unknown	23,80			3,37373149818165		23,80		
17	■	3	Unknown	24,48			2,16910359370939		24,48		
18	■	4	Unknown	24,25			2,51022508362425		24,25		
19	■	5	Unknown	25,27			1,28868497626925		25,27		
20	■	6	Unknown	25,51			1,0998881271031		25,51		

The “Quantitation Results – Cycling A. Green” window.

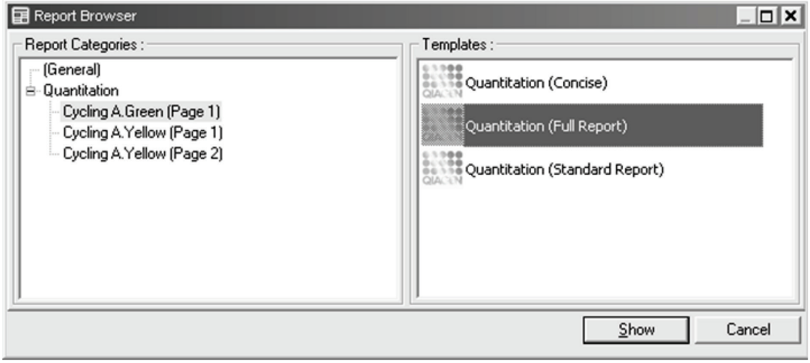
The “Quantitation Results – Cycling A. Yellow” window displays data for selected wells, and summarizes the C_T values for the Internal Control.



No.	C	Name	Type	Ct	Ct Comment	Given Conc (ng/	Calc Conc (ng/ul	% Var	Rep. Ct	Rep. Ct Std	Rep. Ct (95% CI)	F
1	■	Standard -1	Unknown	30,26					30,30	0,06		
2	■	Standard -1	Unknown	30,34								
3	■	Standard -2	Unknown	30,28					30,16	0,17		
4	■	Standard -2	Unknown	30,04								
5	■	Standard -3	Unknown	30,29					30,18	0,15		
6	■	Standard -3	Unknown	30,08								
7	■	Standard -4	Unknown	29,71					29,91	0,29		
8	■	Standard -4	Unknown	30,12								
9	■	Standard -5	Unknown	30,28					30,23	0,08		
10	■	Standard -5	Unknown	30,17								
11	■	Standard -6	Unknown	30,24					30,16	0,11		
12	■	Standard -6	Unknown	30,08								
13	■	Standard -7	Unknown	30,22					30,12	0,14		
14	■	Standard -7	Unknown	30,02								
15	■	1	Unknown	29,96					29,96			
16	■	2	Unknown	30,03					30,03			
17	■	3	Unknown	30,15					30,15			
18	■	4	Unknown	30,08					30,08			
19	■	5	Unknown	30,07					30,07			
20	■	6	Unknown	30,13					30,13			

The “Quantitation Results – Cycling A. Yellow” window.

19. To export the results to Excel, right-click and select "Export to Excel." The results will be saved in *.csv format. To export a full report, go to "File," followed by "Reports" and then "Quantitation."



20. To interpret the results, see "Interpretation of Results," page 78.

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

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

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

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- New dyes do not require calibration when using the Applied Biosystems 7500 Real-Time PCR System.
- Always use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Always use the template volume specified in the protocol. The reaction is optimized for use with 2 µl template DNA. Do not use more or less than 2 µl per 25 µl reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored at between +2 and +8°C for at least 1 week.
- Optimal analysis settings are a prerequisite for accurate quantification data. Always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure

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

2. Prepare fresh serial dilutions of the Male Control DNA M1 according to Table 7. Vortex for at least 5 s and centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipet tip for each dilution.

   Take care not to introduce cross-contamination.

Table 7. Serial dilutions of Male Control DNA M1

Standard	Serial dilution of Control DNA (ng/µl)	Control DNA (µl)	QuantiTect Nucleic Acid Dilution Buffer (µl)
–	50 (stock conc.)	–	–
Standard -1	20	20 (from stock)	30
Standard -2	5	10	30
Standard -3	1.25	10	30
Standard -4	0.3125	10	30
Standard -5	0.078125	10	30
Standard -6	0.01953125	10	30
Standard -7	0.0048828125	10	30

Note: Alternative standard curves are listed in “Appendix: Alternative Standard Curves,” page 86.

3. Thaw template nucleic acids.
4. Mix all solutions thoroughly before use to avoid localized concentrations of salt.
5. Prepare a master mix according to Table 8.
   a. The master mix contains all of the components needed for PCR except the template (sample) DNA and nuclease-free water.
   b. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. This should include positive and negative control reactions.
   c. Reaction setup can usually be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

Table 8. Master mix for DNA quantification

Component	Volume per 25 µl reaction	Final concentration
Reaction Mix YQ	11.5 µl	1x
Primer Mix IC FQ	11.5 µl	1x
Total volume of master mix	**23 µl**	**–**

6. Mix the master mix thoroughly, and dispense 23 µl into PCR tubes or into the wells of a PCR plate.
7. Add 2 µl QuantiTect Nucleic Acid Dilution Buffer to the NTC tubes or wells.

   Make sure that the NTC tubes do not come in contact with human DNA.
8. Add 2 µl control DNA dilutions or 2 µl unknown sample DNA to the individual PCR tubes and mix thoroughly. Close the PCR tubes. It is very important to mix carefully in order to avoid localized concentrations of salt and DNA.

   Table 9 shows a possible plate set-up. 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 9. Possible plate setup of reactions on the Applied Biosystems 7500 Real-Time PCR System

Well contents												
	1	2	3	4	5	6	7	8	9	10	11	12
A	20	20	5	5	1.25	1.25	0.3125	0.3125	0.0781	0.0781	0.0195	0.0195
B	0.0049	0.0049	NTC	NTC	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
F	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
G	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

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

9. Open the Sequence Detection Software and select “Create a New Document.” Click “Next” to continue.

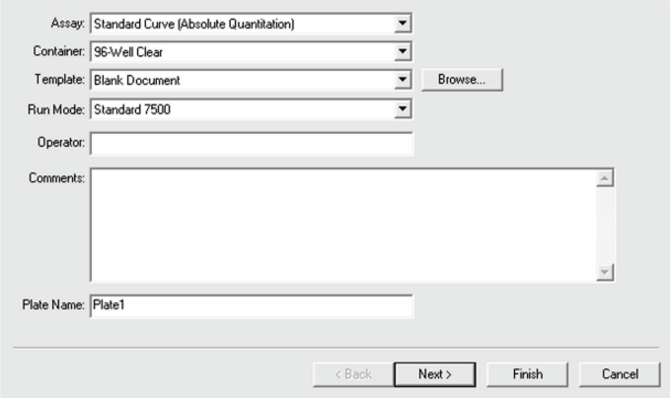
   If you are not using a template file from the Investigator Quantiplex product page, then select the following in the New Document Wizard window.

   Assay: Absolute Quantification (Standard Curve)

   Container: 96-Well Clear Plate

   Template: Blank Document

   Run Mode: Standard 7500



Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: Blank Document

Run Mode: Standard 7500

Operator:

Comments:

Plate Name: Plate1

Creating a new document.

If you are using a template file, then select the following and go to step 12.

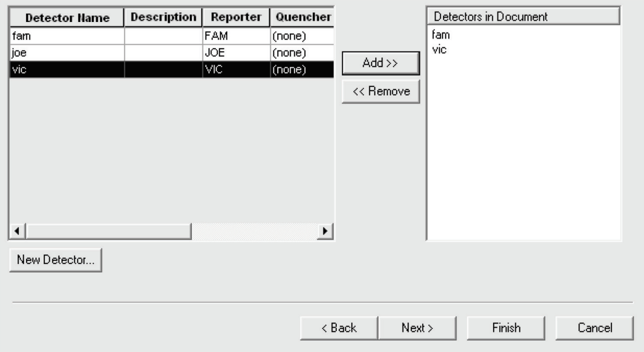
Assay: Absolute Quantification (Standard Curve)

Container: 96-Well Clear Plate

Template: Investigator_Quantiplex_Template_SDS1.4.SDT

Run Mode: Standard 7500

10. Add the detectors FAM and VIC to the document. Click “Next” to continue.



Detector Name	Description	Reporter	Quencher
fam		FAM	(none)
joe		JOE	(none)
vic		VIC	(none)

Detectors in Document
fam
vic

Selecting the detectors.

11. Select the wells in use and check the box for both detectors.

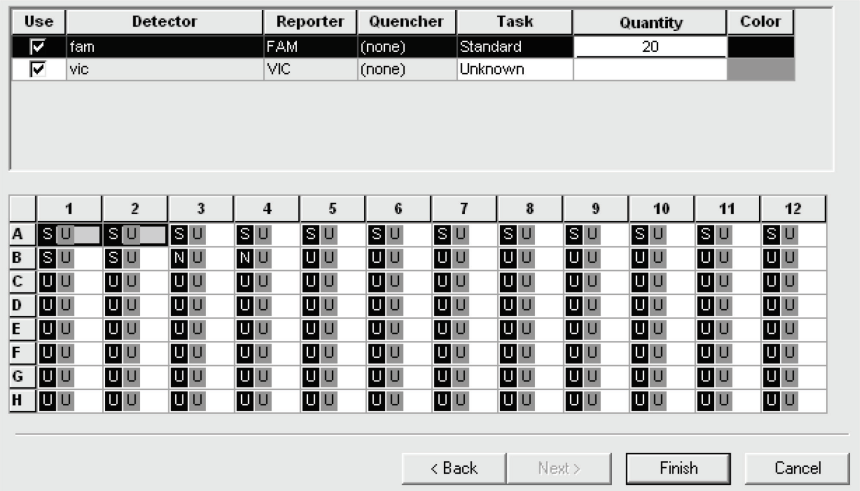
    Important: Do not highlight the wells that are not in use (i.e., those without reaction mix). Including unused wells will significantly impact the scale of the X and Y axes when viewing the data.

    Enter the concentrations of the standard curve to the wells containing the control reactions for the FAM detector.

12. Click “Unknown” in the Task column, and then select “Standard” from the dropdown menu. Select “Quantity” for the appropriate detector and enter the quantity of DNA in the well.

    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 VIC detector Task for standard reactions set to “Unknown.” Enter the sample name (e.g., Standard -1).



Use	Detector	Reporter	Quencher	Task	Quantity	Color
☑	fam	FAM	(none)	Standard	20	■
☑	vic	VIC	(none)	Unknown		■

	1	2	3	4	5	6	7	8	9	10	11	12
A	S U	S U	S U	S U	S U	S U	S U	S U	S U	S U	S U	S U
B	S U	S U	N U	N U	U U	U U	U U	U U	U U	U U	U U	U U
C	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
D	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
E	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
F	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
G	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
H	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U

Setting up the sample plate.

13. Click “Finish.” To program the cycler according to Table 10, click on the “Instrument” tab.

    PCR requires an initial incubation at 95°C for 3 min to activate the DNA polymerase.

    Two-step cycling PCR requires 40 cycles. Each cycle consists of 2 steps:

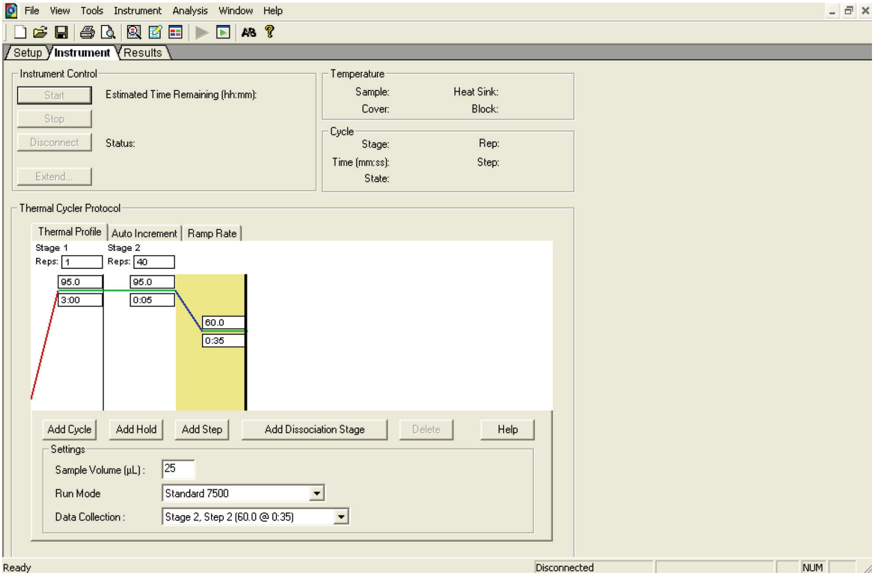
    95°C for 5 s (denaturation step) and 60°C for 35 s (annealing/extension step).

Table 10. Cycling conditions for Applied Biosystems 7500 Real-Time PCR System

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

14. On the thermal profile, delete the first step by pressing “Shift” and clicking within the Stage 1 hold step (50°C for 2 min). After the hold step is selected, press the “Delete” key. Change the holding times to those in Table 10. Be sure to change the Sample volume to 25 µl and select the run mode Standard 7500.

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



Stage 1 Reps: 1

95.0

3:00

Stage 2 Reps: 40

95.0

0:05

60.0

0:35

Sample Volume (µL) : 25

Run Mode: Standard 7500

Data Collection : Stage 2, Step 2 (60.0 @ 0:35)

Ready

Disconnected

NUM

Adjusting the thermal profile (SDS Software Version 1.4).

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

    Ensure that position A1 on the plate is on the top-left side of the tray.
17. Start the reaction by clicking “Start.”

Data analysis

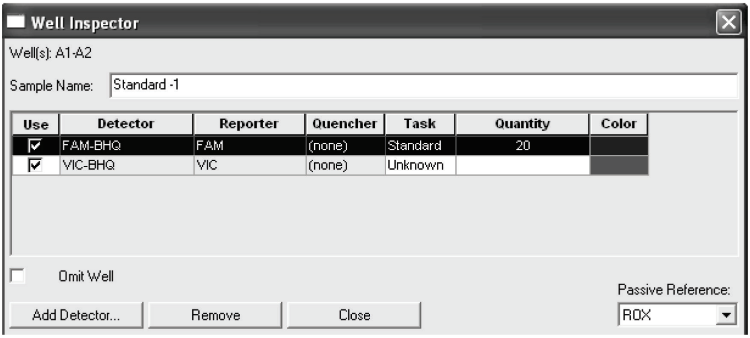
Optimal analysis settings are a prerequisite for accurate quantification data. Always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure

1. Open the run file using the SDS software. Go to "File," followed by "Open" and then "Browse" to locate the saved file.
2. Standards must first be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 4.
3. Go to "View" and select "Well Inspector" from the menu. In the Task column, define the wells that contain DNA standards for the FAM channel as "Standard."

   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 VIC detector Task for standard reactions set to "Unknown." Quantity values are not needed for the internal control. Enter the sample name (e.g., Standard -1).



Well(s): A1-A2

Sample Name: Standard -1

Use	Detector	Reporter	Quencher	Task	Quantity	Color
☑	FAM-BHQ	FAM	(none)	Standard	20	■
☑	VIC-BHQ	VIC	(none)	Unknown		■

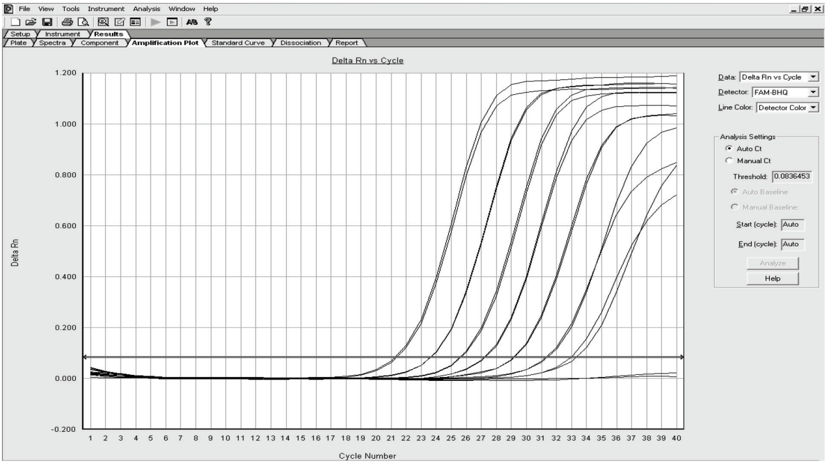
☐ Omit Well

Passive Reference: ROX

Well inspector.

4. In the Amplification Plot tab (found in the Results tab), select the appropriate samples in the table below the amplification plot. Choose "Auto Ct" for both channels and press "Analyze."

   Setting the appropriate threshold value may require further internal validation at your facility.



Data: Delta Rn vs Cycle

Detector: FAM-BHQ

Line Color: Detector Color

Analysis Settings

● Auto Ct

○ Manual Ct

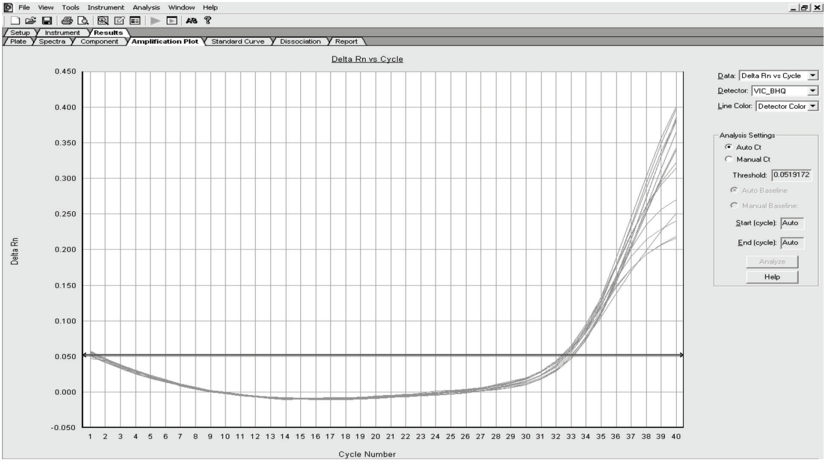
Threshold: 0.0836453

● Auto Baseline

○ Manual Baseline:

Start (cycle): Auto

End (cycle): Auto



Data: Delta Rn vs Cycle

Detector: VIC_BHQ

Line Color: Detector Color

Analysis Settings

● Auto Ct

○ Manual Ct

Threshold: 0.0519172

● Auto Baseline

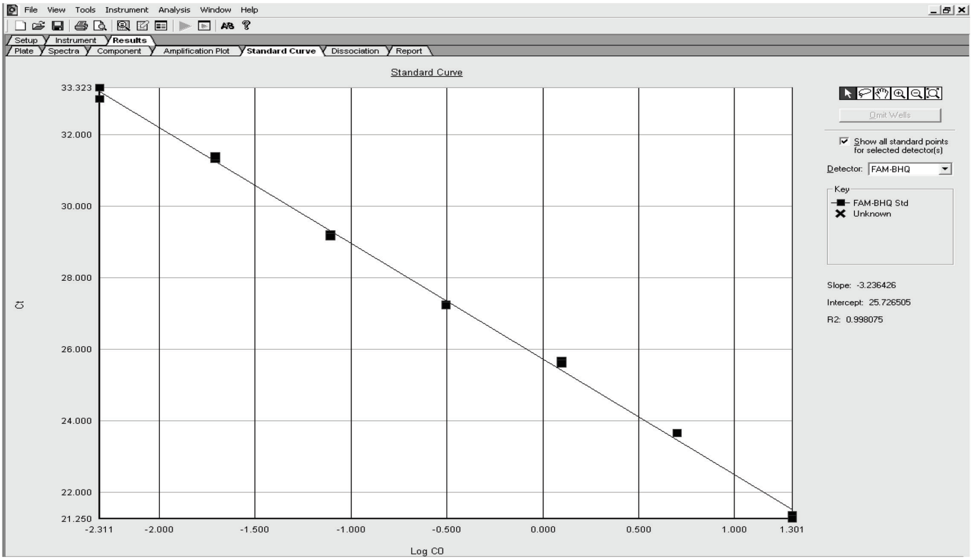
○ Manual Baseline:

Start (cycle): Auto

End (cycle): Auto

Sample analysis for the FAM and the VIC channels.

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



☑ Show all standard points for selected detector(s)

Detector: FAM-BHQ

Slope: -3.236426

Intercept: 25.726505

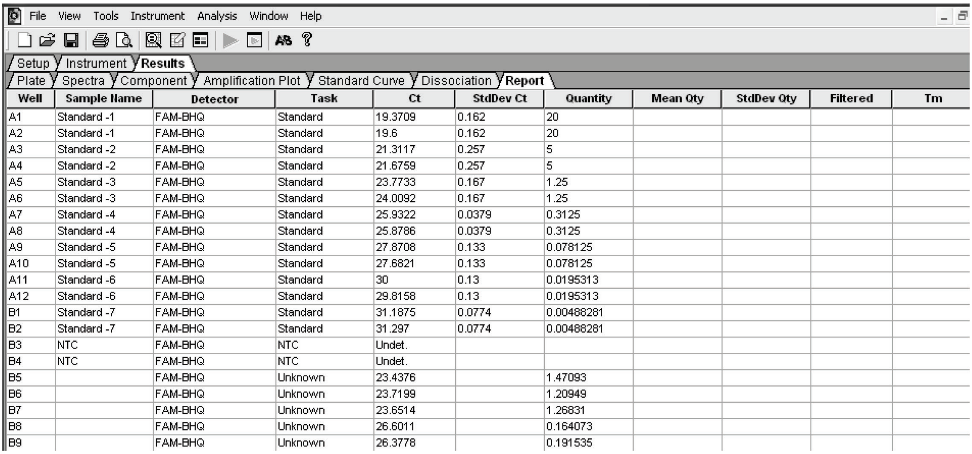
R2: 0.998075

Standard curve.

6. View the concentration of the unknown samples.

   The report displays data for selected wells, and summarizes the quantity of DNA present in the unknown samples. The FAM detector shows the quantity of DNA present in the same unit that was used for the standards. For example, if ng/µl was used for the definition of the standards, then the quantities for the unknowns will be reported in ng/µl.

   The VIC-detector shows the C_T value for the Internal Control.



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A1	Standard -1	FAM-BHQ	Standard	19.3709	0.162	20				
A2	Standard -1	FAM-BHQ	Standard	19.6	0.162	20				
A3	Standard -2	FAM-BHQ	Standard	21.3117	0.257	5				
A4	Standard -2	FAM-BHQ	Standard	21.6759	0.257	5				
A5	Standard -3	FAM-BHQ	Standard	23.7733	0.167	1.25				
A6	Standard -3	FAM-BHQ	Standard	24.0092	0.167	1.25				
A7	Standard -4	FAM-BHQ	Standard	25.9322	0.0379	0.3125				
A8	Standard -4	FAM-BHQ	Standard	25.8786	0.0379	0.3125				
A9	Standard -5	FAM-BHQ	Standard	27.8708	0.133	0.078125				
A10	Standard -5	FAM-BHQ	Standard	27.6821	0.133	0.078125				
A11	Standard -6	FAM-BHQ	Standard	30	0.13	0.0195313				
A12	Standard -6	FAM-BHQ	Standard	29.8158	0.13	0.0195313				
B1	Standard -7	FAM-BHQ	Standard	31.1875	0.0774	0.00488281				
B2	Standard -7	FAM-BHQ	Standard	31.297	0.0774	0.00488281				
B3	NTC	FAM-BHQ	NTC	Undet.						
B4	NTC	FAM-BHQ	NTC	Undet.						
B5		FAM-BHQ	Unknown	23.4376		1.47093				
B6		FAM-BHQ	Unknown	23.7199		1.20949				
B7		FAM-BHQ	Unknown	23.6514		1.26831				
B8		FAM-BHQ	Unknown	26.6011		0.164073				
B9		FAM-BHQ	Unknown	26.3778		0.191535				

Unknown sample concentration.

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 “Interpretation of Results,” page 78.

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

This protocol is optimized for use of the Investigator Quantiplex 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.

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

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- The dyes do not require custom calibration when using the Applied Biosystems 7500 Real-Time PCR System. However, care should be taken that the system is calibrated for the standard dyes VIC and FAM before starting. Please see the instrument's user manual for the correct setup.
- Always use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Always use the template volume specified in the protocol. The reaction is optimized for use with 2 µl template DNA. Do not use more or less than 2 µl per 25 µl reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored at between +2 and +8°C for at least 1 week.

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

Procedure

1. Mix all solutions thoroughly before use to avoid localized concentrations of salt.
2. Prepare fresh serial dilutions of the Male Control DNA M1 according to Table 11. Vortex for at least 5 s and centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipet tip for each dilution.

   Take care not to introduce cross-contamination.

Table 11. Serial dilutions of Male Control DNA M1

Standard	Serial dilution of Control DNA (ng/µl)	Control DNA (µl)	QuantiTect Nucleic Acid Dilution Buffer (µl)
–	50 (stock conc.)	–	–
Standard -1	20	20 (from stock)	30
Standard -2	5	10	30
Standard -3	1.25	10	30
Standard -4	0.3125	10	30
Standard -5	0.078125	10	30
Standard -6	0.01953125	10	30
Standard -7	0.0048828125	10	30

Note: Alternative standard curves are listed in "Appendix: Alternative Standard Curves," page 86.

3. Thaw template nucleic acids.

   Mix all solutions thoroughly before use to avoid localized concentrations of salt.
4. Prepare a master mix according to Table 12.
   a. The master mix contains all of the components needed for PCR except the template (sample) DNA and nuclease-free water.

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

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

Table 12. Master mix for DNA quantification

Component	Volume per 25 µl reaction	Final concentration
Reaction Mix YQ	11.5 µl	1x
Primer Mix IC FQ	11.5 µl	1x
Total volume of master mix	**23 µl**	–

5. Mix the master mix thoroughly, and dispense 23 µl into the wells of a PCR plate.
6. Add 2 µl QuantiTect Nucleic Acid Dilution Buffer to the NTC wells.

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

   Mix carefully in order to avoid localized concentrations of salt.

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

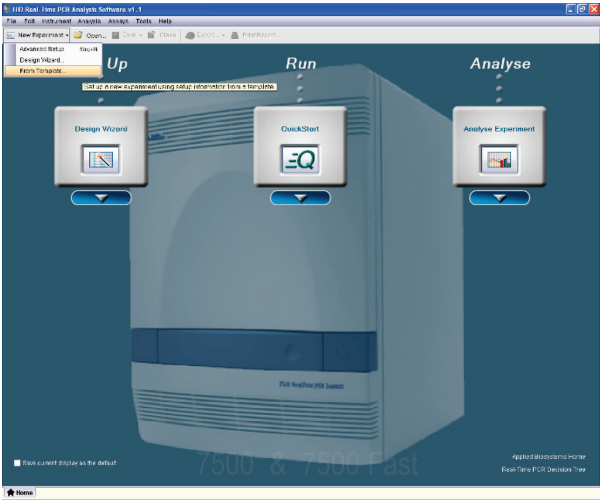
Table 13. Possible plate setup of reactions on the Applied Biosystems 7500 Real-Time PCR System for Human Identification

Well contents												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	20	20	5	5	1.25	1.25	0.3125	0.3125	0.0781	0.0781	0.0195	0.0195
B	0.0049	0.0049	NTC	NTC	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
F	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
G	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

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

8. Open the HID Real-Time PCR Analysis Software v1.1 or v.1.2 in the Custom Assays mode.
9. If you are using a template file, select "New Experiment From Template" and proceed to step 13 to assign the Targets to the Plate Layout. Then proceed to step 17 to save and start the run.

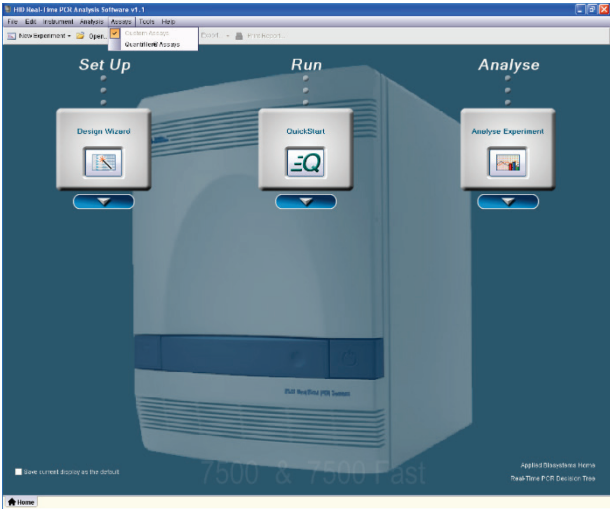
   The template file loads all of the settings needed to start an Investigator Quantiplex run, including the standard curve settings, the cycling profile, and the targets needed for fluorescence acquisition.



Set up a new experiment using setup information from a template

Starting a new experiment from a template.

10. If you are not using template files, select "Advanced Setup" by clicking the arrow below the "Design Wizard."



Starting a new experiment using Advanced Setup.

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

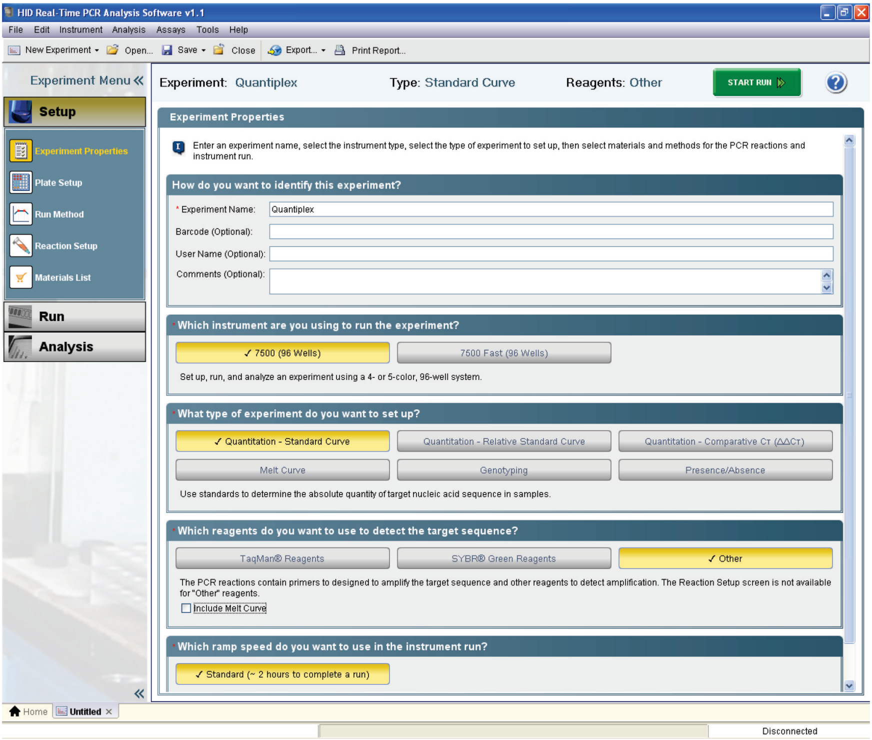
    Instrument: 7500 (96 Wells)

    Experiment Type: Quantitation — Standard curve

    Reagents: Other

    Ramp Speed: Standard

    Deselect the "Include Melt Curve" option.



Experiment: Quantiplex Type: Standard Curve Reagents: Other

Experiment Properties

Enter an experiment name, select the instrument type, select the type of experiment to set up, then select materials and methods for the PCR reactions and instrument run.

How do you want to identify this experiment?

* Experiment Name: Quantiplex
Barcode (Optional):
User Name (Optional):
Comments (Optional):

* Which instrument are you using to run the experiment?

✓ 7500 (96 Wells)

Set up, run, and analyze an experiment using a 4- or 5-color, 96-well system.

* What type of experiment do you want to set up?

✓ Quantitation - Standard Curve

Use standards to determine the absolute quantity of target nucleic acid sequence in samples.

* Which reagents do you want to use to detect the target sequence?

✓ Other

The PCR reactions contain primers to designed to amplify the target sequence and other reagents to detect amplification. The Reaction Setup screen is not available for "Other" reagents.

☐ Include Melt Curve

* Which ramp speed do you want to use in the instrument run?

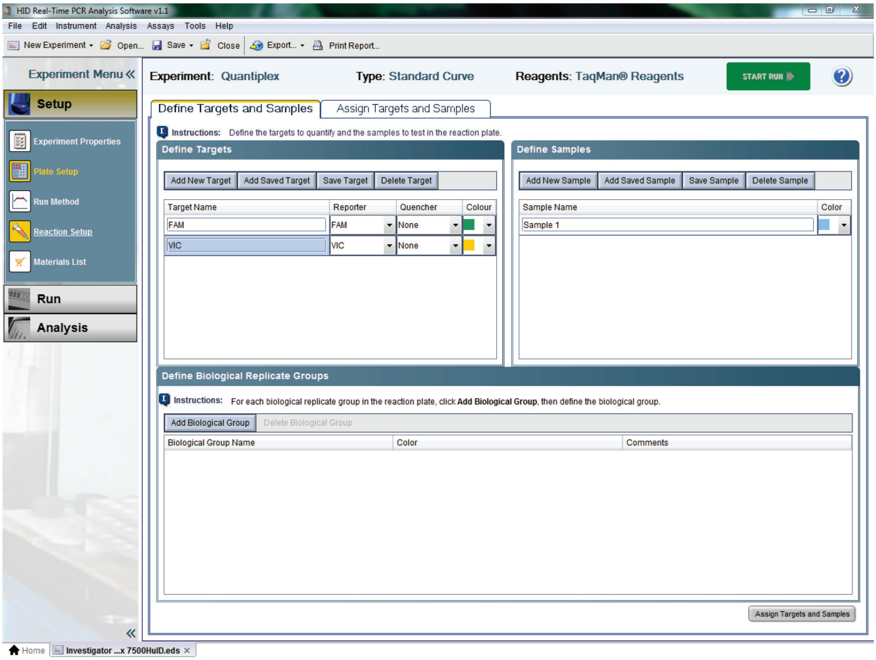
✓ Standard (~ 2 hours to complete a run)

Instrument settings.

12. Click “Plate Setup” and define three Targets by double-clicking “Add New Target.” Select the following settings:

    Human Target: Reporter FAM, **Quencher**: None

    IC: Reporter VIC, **Quencher**: None



Experiment: Quantiplex **Type**: Standard Curve **Reagents**: TaqMan® Reagents

Instructions: Define the targets to quantify and the samples to test in the reaction plate.

Target Name	Reporter	Quencher	Colour
FAM	FAM	None	🟩
VIC	VIC	None	🟨

Sample Name	Color
Sample 1	🟦

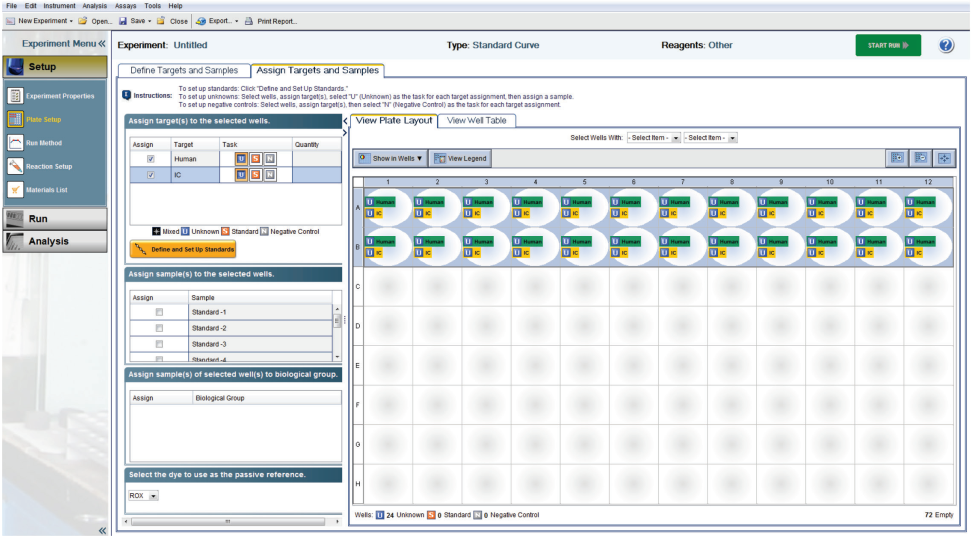
Instructions: For each biological replicate group in the reaction plate, click **Add Biological Group**, then define the biological group.

Defining the targets.

13. Define the Sample names using the “Define Samples” tool on the right panel.

14. Switch to the “Assign Targets and Samples” tab. In the Plate Layout, select the wells in use and assign all three targets by checking the boxes.

    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.



Experiment: Untitled Type: Standard Curve Reagents: Other

Instructions:
To set up standards: Click "Define and Set Up Standards."
To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for each target assignment, then assign a sample.
To set up negative controls: Select wells, assign target(s), then select "N" (Negative Control) as the task for each target assignment.

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
☑	Human	U S N	
☑	IC	U S N	

Assign sample(s) to the selected wells.

Assign	Sample
☐	Standard -1
☐	Standard -2
☐	Standard -3
☐	Standard -4

Assign sample(s) of selected well(s) to biological group.

Assign	Biological Group

Select the dye to use as the passive reference.
ROX

Select Wells With: - Select Item - - Select Item -

Wells: 24 Unknown 0 Standard 0 Negative Control 72 Empty

Assigning the targets.

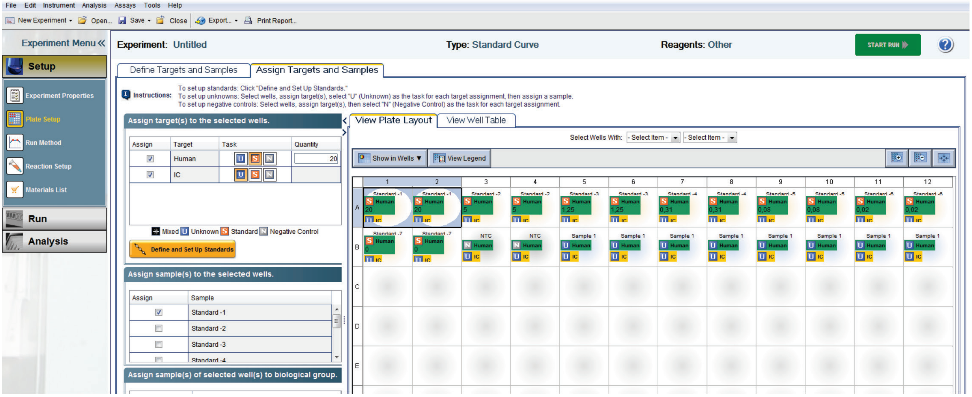
15. Select the wells for the No-Template Controls and flag them as "Negative Control" using the red "N" button.

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

16. Select the wells for the standard curve and flag them as "Standard" using the red "S" button. Select "Quantity" for the appropriate detector and enter the quantity of DNA in the well according to Table 11.

    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 (VIC) Task for standard reactions set to "Unknown." Enter the sample name.



Experiment: Untitled

Type: Standard Curve

Reagents: Other

Instructions: To set up standards: Click "Define and Set Up Standards."
To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for each target assignment, then assign a sample.
To set up negative controls: Select wells, assign target(s), then select "N" (Negative Control) as the task for each target assignment.

Assign target(s) to the selected wells.

Assign	Target	Task	Quantity
☑	Human	U S N	20
☑	IC	U S N	

Assign sample(s) to the selected wells.

Assign	Sample
☑	Standard -1
☐	Standard -2
☐	Standard -3
☐	Standard -4

Assign sample(s) of selected well(s) to biological group.

Setting up the standard curve and assigning the samples to the plate layout.

17. Assign the samples to the plate layout by clicking on the wells and checking the appropriate box on the left panel.
18. Click on "Run Method." Program the cycler according to Table 14.

    PCR requires an initial incubation at 95°C for 3 min to activate the DNA polymerase.

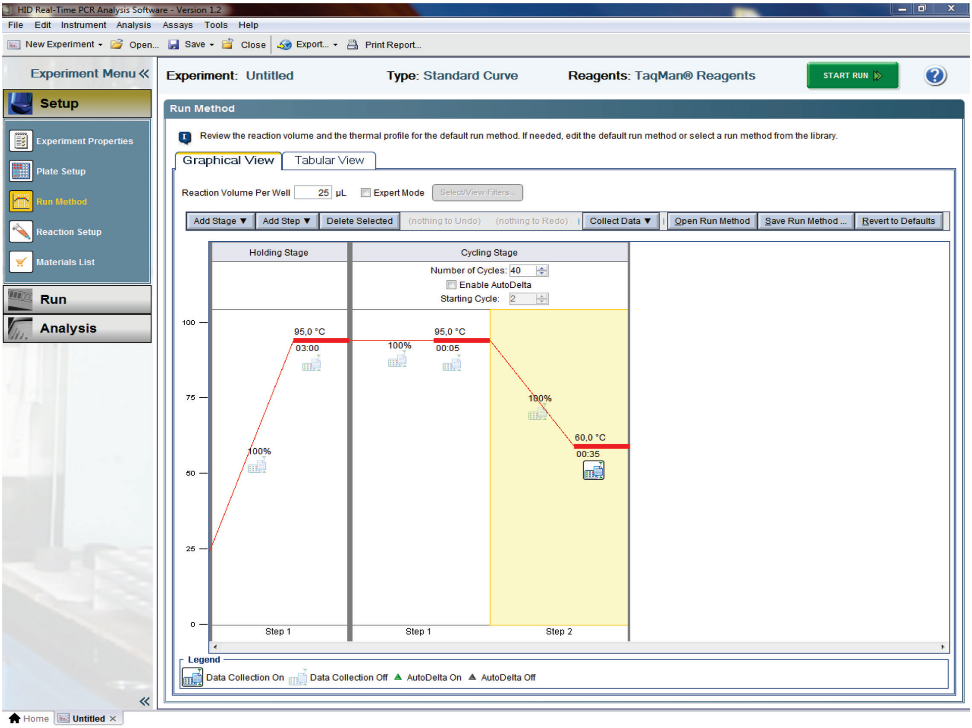
    Two-step cycling PCR requires 40 cycles. Each cycle comprises 2 steps: 95°C for 5 s (denaturation step) and 60°C for 35 s (annealing/extension step).

Table 14. Cycling conditions for the Applied Biosystems 7500 Real-Time PCR System for Human Identification

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

19. On the thermal profile, change the holding times to those in Table 14. Change the Sample volume to 25 µl.

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



Experiment: Untitled

Type: Standard Curve

Reagents: TaqMan® Reagents

Review the reaction volume and the thermal profile for the default run method. If needed, edit the default run method or select a run method from the library.

Reaction Volume Per Well 25 µL

Number of Cycles: 40

☐ Enable AutoDelta

Starting Cycle: 2

Adjusting the thermal profile (HID Real-Time PCR Analysis Software v1.1 or v1.2).

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

21. Load the plate into the instrument.

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

22. Start the reaction by clicking "Start."

Data analysis

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

Procedure

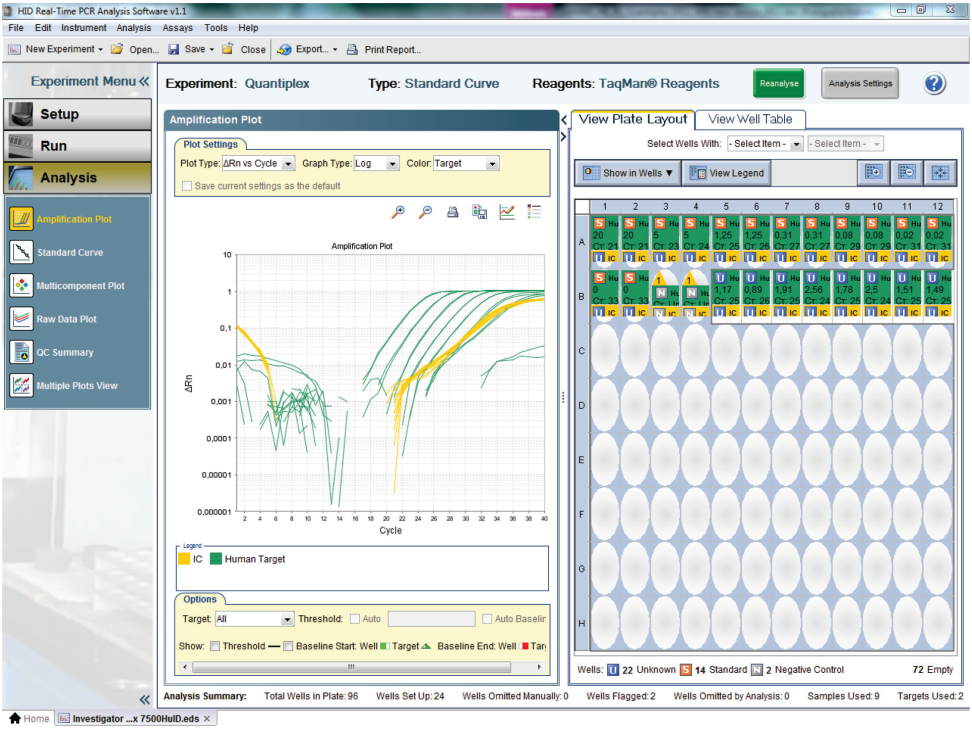
1. Open the run file using the HID Real-Time PCR Analysis Software v1.1 or v1.2. First you have to open the software in the Custom Assays Mode. Then go to “Open” and then “Browse” to locate the saved file.
2. Standards must first be defined before a standard curve can be created. If the standards were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

   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 (VIC) Task for standard reactions set to “Unknown.” Enter the sample name (e.g., Standard -1).

4. In the “Amplification Plot” tab (found in the “Analysis” tab), select the appropriate samples in the table below the amplification plot. Choose “Auto Ct” for both channels and click “Analyze.”

   Setting the appropriate threshold value may require further internal validation at your facility.



Experiment: Quantiplex **Type:** Standard Curve **Reagents:** TaqMan® Reagents

Plot Type: ΔRn vs Cycle Graph Type: Log Color: Target

☐ Save current settings as the default

🟨 IC 🟩 Human Target

Target: All Threshold: ☐ Auto ☐ Auto Baselin

Show: ☐ Threshold — ☐ Baseline Start: Well 🟩 Target △ Baseline End: Well 🟥 Tar

Select Wells With: - Select Item - - Select Item -

	1	2	3	4	5	6	7	8	9	10	11	12
A	S Hu 20 CT: 21 U IC	S Hu 20 CT: 21 U IC	S Hu 5 CT: 23 U IC	S Hu 5 CT: 24 U IC	S Hu 1,25 CT: 25 U IC	S Hu 1,25 CT: 26 U IC	S Hu 0,31 CT: 27 U IC	S Hu 0,31 CT: 27 U IC	S Hu 0,08 CT: 29 U IC	S Hu 0,08 CT: 29 U IC	S Hu 0,02 CT: 31 U IC	S Hu 0,02 CT: 31 U IC
B	S Hu 0 CT: 33 U IC	S Hu 0 CT: 33 U IC	1 N Hu CT: U N IC	1 N Hu CT: U N IC	U Hu 1,17 CT: 25 U IC	U Hu 0,89 CT: 26 U IC	U Hu 1,91 CT: 25 U IC	U Hu 2,56 CT: 24 U IC	U Hu 1,78 CT: 25 U IC	U Hu 2,5 CT: 24 U IC	U Hu 1,51 CT: 25 U IC	U Hu 1,49 CT: 25 U IC
C												
D												
E												
F												
G												
H												

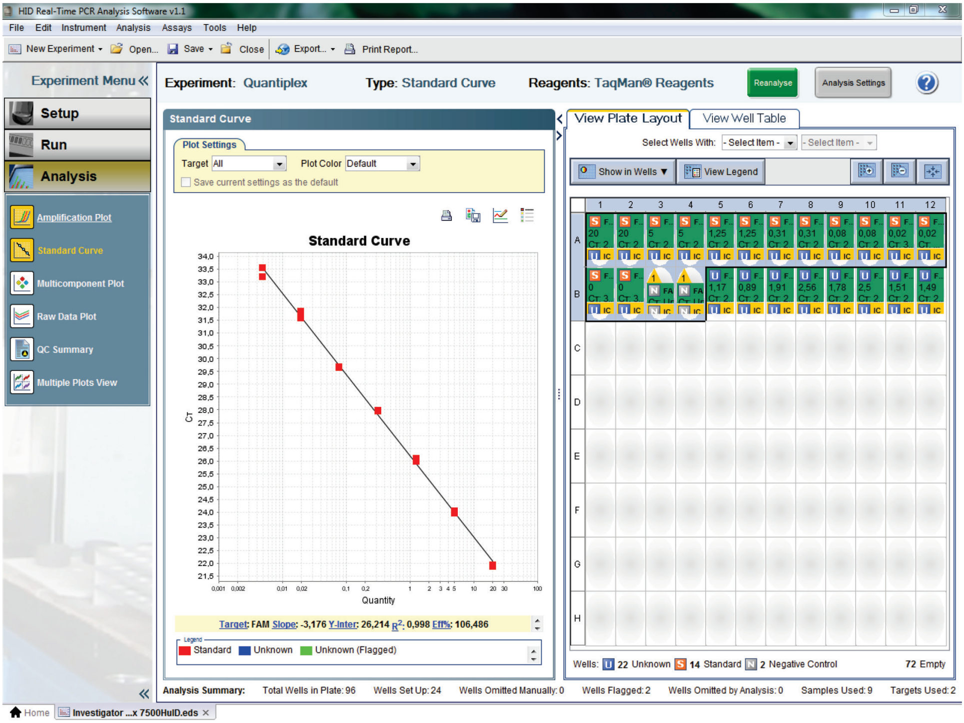
Wells: U 22 Unknown S 14 Standard N 2 Negative Control 72 Empty

Analysis Summary: Total Wells in Plate: 96 Wells Set Up: 24 Wells Omitted Manually: 0 Wells Flagged: 2 Wells Omitted by Analysis: 0 Samples Used: 9 Targets Used: 2

Investigator ...x 7500HuID.eds

Sample analysis for the FAM and VIC channels.

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



Experiment: Quantiplex Type: Standard Curve Reagents: TaqMan® Reagents

Target: All Plot Color: Default

Target: FAM Slope: -3,176 Y-Inter: 26,214 R^2: 0,998 Eff%: 106,486

Legend: 🟥 Standard 🟦 Unknown 🟩 Unknown (Flagged)

Wells: U 22 Unknown S 14 Standard N 2 Negative Control 72 Empty

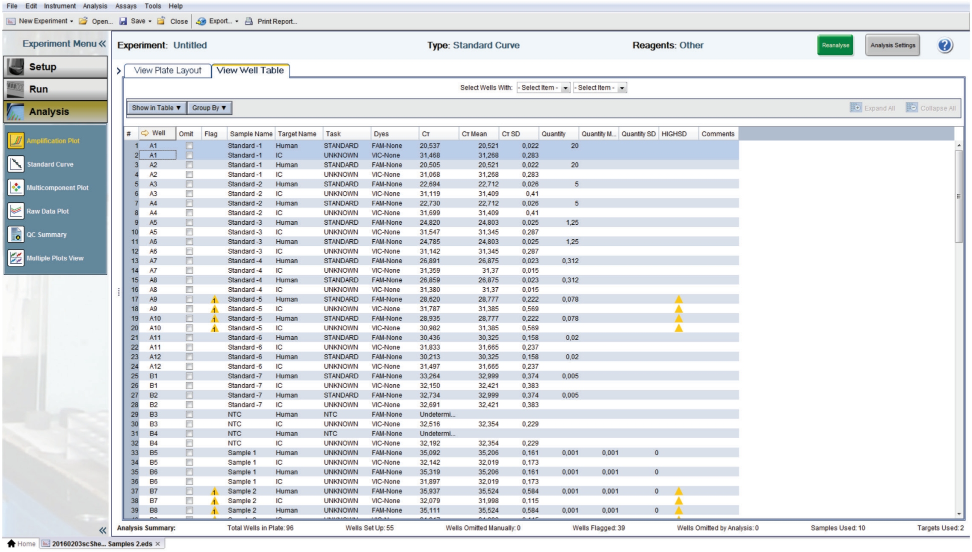
Analysis Summary: Total Wells in Plate: 96 Wells Set Up: 24 Wells Omitted Manually: 0 Wells Flagged: 2 Wells Omitted by Analysis: 0 Samples Used: 9 Targets Used: 2

Standard curve.

6. View the concentration 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 IC Target shows the C_T value for the Internal Control.



Experiment: Untitled **Type:** Standard Curve **Reagents:** Other

#	Well	Omit	Flag	Sample Name	Target Name	Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity M...	Quantity SD	HIGHSD	Comments
1	A1	☐		Standard -1	Human	STANDARD	FAM-None	20,537	20,521	0,022	20				
2	A1	☐		Standard -1	IC	UNKNOWN	VIC-None	31,468	31,268	0,283					
3	A2	☐		Standard -1	Human	STANDARD	FAM-None	20,505	20,521	0,022	20				
4	A2	☐		Standard -1	IC	UNKNOWN	VIC-None	31,068	31,268	0,283					
5	A3	☐		Standard -2	Human	STANDARD	FAM-None	22,694	22,712	0,026	5				
6	A3	☐		Standard -2	IC	UNKNOWN	VIC-None	31,119	31,409	0,41					
7	A4	☐		Standard -2	Human	STANDARD	FAM-None	22,730	22,712	0,026	5				
8	A4	☐		Standard -2	IC	UNKNOWN	VIC-None	31,699	31,409	0,41					
9	A5	☐		Standard -3	Human	STANDARD	FAM-None	24,820	24,803	0,025	1,25				
10	A5	☐		Standard -3	IC	UNKNOWN	VIC-None	31,547	31,345	0,287					
11	A6	☐		Standard -3	Human	STANDARD	FAM-None	24,785	24,803	0,025	1,25				
12	A6	☐		Standard -3	IC	UNKNOWN	VIC-None	31,142	31,345	0,287					
13	A7	☐		Standard -4	Human	STANDARD	FAM-None	26,891	26,875	0,023	0,312				
14	A7	☐		Standard -4	IC	UNKNOWN	VIC-None	31,359	31,37	0,015					
15	A8	☐		Standard -4	Human	STANDARD	FAM-None	26,859	26,875	0,023	0,312				
16	A8	☐		Standard -4	IC	UNKNOWN	VIC-None	31,380	31,37	0,015					
17	A9	☐	⚠1	Standard -5	Human	STANDARD	FAM-None	28,620	28,777	0,222	0,078			▲	
18	A9	☐	⚠1	Standard -5	IC	UNKNOWN	VIC-None	31,787	31,385	0,569				▲	
19	A10	☐	⚠1	Standard -5	Human	STANDARD	FAM-None	28,935	28,777	0,222	0,078			▲	
20	A10	☐	⚠1	Standard -5	IC	UNKNOWN	VIC-None	30,982	31,385	0,569				▲	
21	A11	☐		Standard -6	Human	STANDARD	FAM-None	30,436	30,325	0,158	0,02				
22	A11	☐		Standard -6	IC	UNKNOWN	VIC-None	31,833	31,665	0,237					
23	A12	☐		Standard -6	Human	STANDARD	FAM-None	30,213	30,325	0,158	0,02				
24	A12	☐		Standard -6	IC	UNKNOWN	VIC-None	31,497	31,665	0,237					
25	B1	☐		Standard -7	Human	STANDARD	FAM-None	33,264	32,999	0,374	0,005				
26	B1	☐		Standard -7	IC	UNKNOWN	VIC-None	32,150	32,421	0,383					
27	B2	☐		Standard -7	Human	STANDARD	FAM-None	32,734	32,999	0,374	0,005				
28	B2	☐		Standard -7	IC	UNKNOWN	VIC-None	32,691	32,421	0,383					
29	B3	☐		NTC	Human	NTC	FAM-None	Undetermi...							
30	B3	☐		NTC	IC	UNKNOWN	VIC-None	32,516	32,354	0,229					
31	B4	☐		NTC	Human	NTC	FAM-None	Undetermi...							
32	B4	☐		NTC	IC	UNKNOWN	VIC-None	32,192	32,354	0,229					
33	B5	☐		Sample 1	Human	UNKNOWN	FAM-None	35,092	35,206	0,161	0,001	0,001	0		
34	B5	☐		Sample 1	IC	UNKNOWN	VIC-None	32,142	32,019	0,173					
35	B6	☐		Sample 1	Human	UNKNOWN	FAM-None	35,319	35,206	0,161	0,001	0,001	0		
36	B6	☐		Sample 1	IC	UNKNOWN	VIC-None	31,897	32,019	0,173					
37	B7	☐	⚠1	Sample 2	Human	UNKNOWN	FAM-None	35,937	35,524	0,584	0,001	0,001	0	▲	
38	B7	☐	⚠1	Sample 2	IC	UNKNOWN	VIC-None	32,079	31,998	0,115				▲	
39	B8	☐	⚠1	Sample 2	Human	UNKNOWN	FAM-None	35,111	35,524	0,584	0,001	0,001	0	▲	

Analysis Summary: Total Wells in Plate: 96 Wells Set Up: 55 Wells Omitted Manually: 0 Wells Flagged: 39 Wells Omitted by Analysis: 0 Samples Used: 10 Targets Used: 2

Unknown sample concentration.

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 “Interpretation of Results,” page 78.

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

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

For general instructions on instrument setup, and other software versions, refer to the Applied Biosystems 7500 Fast Real-Time PCR System user manual.

Important points before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross contamination.
- New dyes do not require calibration when using the Applied Biosystems 7500 Real-Time PCR System.
- Always use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Always use the template volume specified in the protocol. The reaction is optimized for use with 2 µl template DNA. Do not use more or less than 2 µl per 25 µl reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored at between +2 and +8°C for at least 1 week.
- Optimal analysis settings are a prerequisite for accurate quantification data. Always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure

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 15. Vortex for at least 5 s and centrifuge each dilution briefly before removing an aliquot for the next dilution. Use a new pipet tip for each dilution.

   Take care not to introduce cross-contamination.

Table 15. Serial dilutions of Male Control DNA M1

Standard	Serial dilution of Control DNA (ng/µl)	Control DNA (µl)	QuantiTect Nucleic Acid Dilution Buffer (µl)
–	50 (stock conc.)	–	–
Standard -1	20	20 (from stock)	30
Standard -2	5	10	30
Standard -3	1.25	10	30
Standard -4	0.3125	10	30
Standard -5	0.078125	10	30
Standard -6	0.01953125	10	30
Standard -7	0.0048828125	10	30

Note: Alternative standard curves are listed in "Appendix: Alternative Standard Curves," page 86.

3. Thaw template nucleic acids.

   Mix all solutions thoroughly before use to avoid localized concentrations of salt.
4. Prepare a master mix according to Table 16.
   a. The master mix contains all of the components needed for PCR except the template (sample) DNA and nuclease-free water.
   b. Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. This should include positive and negative control reactions.

c. Reaction setup can usually be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

Table 16. Master mix for DNA quantification

Component	Volume per 25 µl reaction	Final concentration
Reaction Mix YQ	11.5 µl	1x
Primer Mix IC FQ	11.5 µl	1x
Total volume of master mix	**23 µl**	–

5. Mix the master mix thoroughly, and dispense 23 µl into PCR tubes or into the wells of a PCR plate.
6. Add 2 µl QuantiTect Nucleic Acid Dilution Buffer to the NTC tubes or wells.

   Make sure that the NTC tubes do not come in contact with human DNA.
7. Add 2 µl control DNA dilutions or 2 µl unknown sample DNA to the individual PCR tubes and mix thoroughly. Close the PCR tubes. It is very important to mix carefully in order to avoid localized concentrations of salt.

   Table 17 shows a possible plate set-up. Make sure that the master mix and template are thoroughly mixed. It is required to run duplicates of the control DNA dilutions for each assay and on each reaction plate.

Table 17. Possible plate setup of reactions on the Applied Biosystems 7500 Fast Real-Time PCR System

Well contents												
	1	2	3	4	5	6	7	8	9	10	11	12
A	20	20	5	5	1.25	1.25	0.3125	0.3125	0.0781	0.0781	0.0195	0.0195
B	0.0049	0.0049	NTC	NTC	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
F	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
G	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

All amounts in ng/µl. UNK: Unknown sample.

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

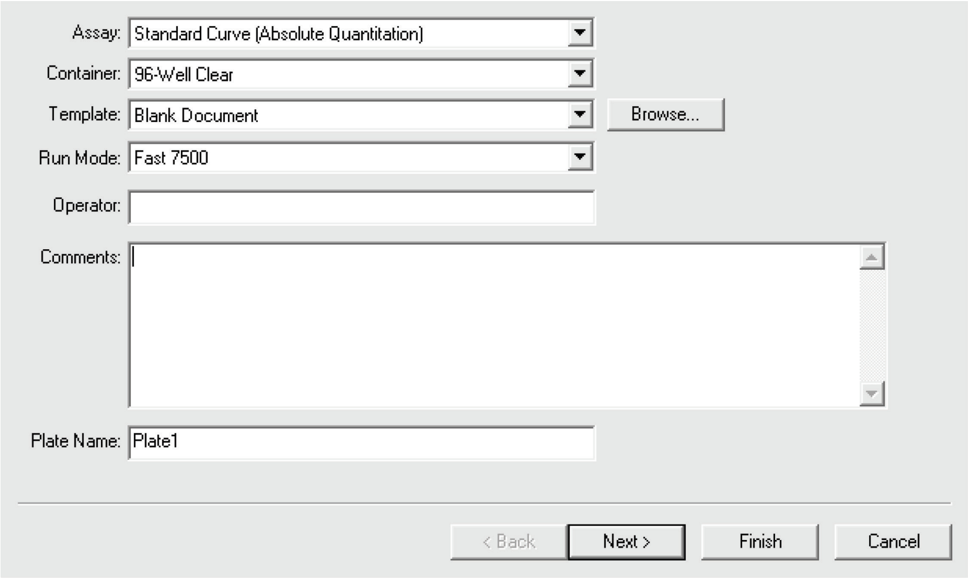
   If you are not using a template file from the Investigator Quantiplex product page, select the following in the New Document Wizard window that opens.

   Assay: Absolute Quantification (Standard Curve)

   Container: 96-Well Clear Plate

   Template: Blank Document

   Run Mode: Fast 7500



Assay: Standard Curve (Absolute Quantitation)

Container: 96-Well Clear

Template: Blank Document

Run Mode: Fast 7500

Operator:

Comments:

Plate Name: Plate1

Creating a new document.

If you are using a template file, then select the following and go to step 12.

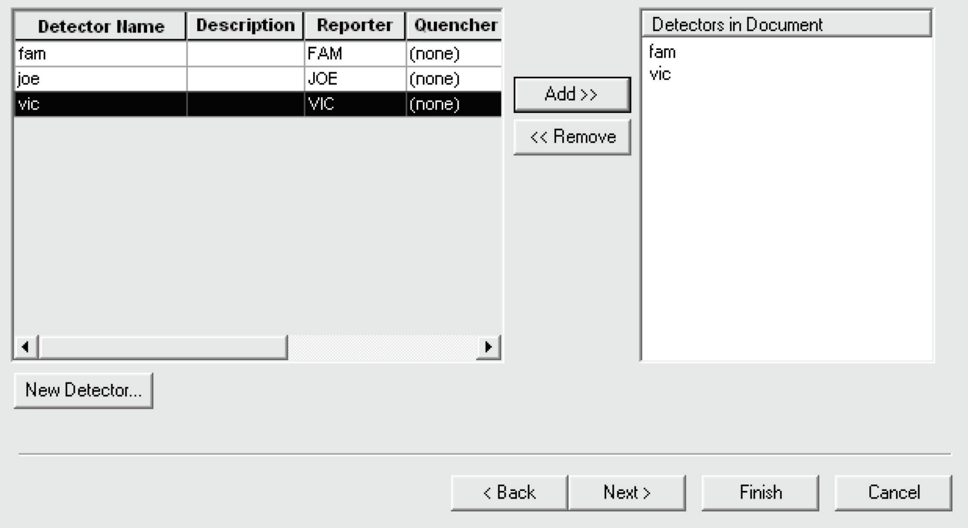
Assay: Absolute Quantification (Standard Curve)

Container: 96-Well Clear Plate

Template: Investigator_Quantiplex_Template_SDS1.4_Fast.SDT

Run Mode: Fast 7500

9. Add the detectors FAM and VIC to the document. Click “Next” to continue.



Detector Name	Description	Reporter	Quencher
fam		FAM	(none)
joe		JOE	(none)
vic		VIC	(none)

Detectors in Document
fam
vic

Selecting the detectors.

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

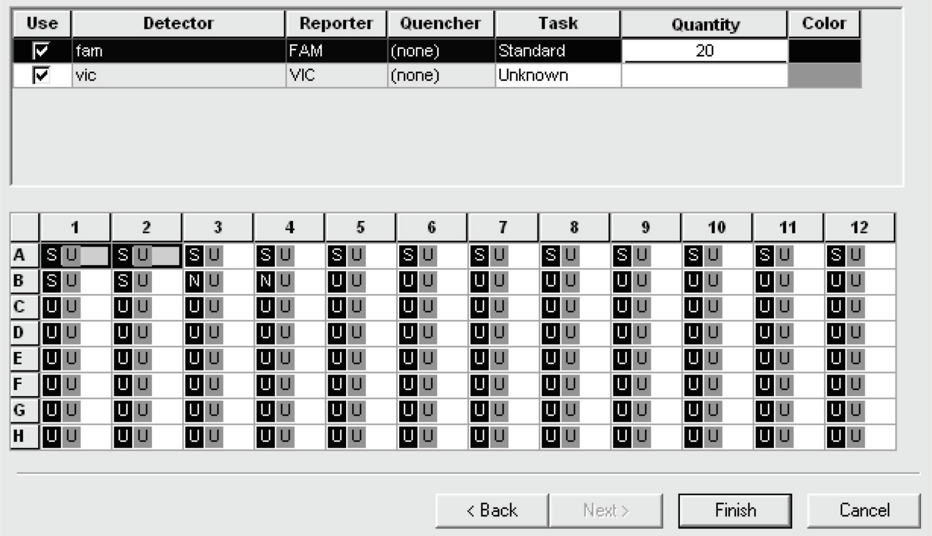
    Important: Do not highlight the wells that are not in use (i.e., those without reaction mix). Including unused wells will significantly impact the scale of the X and Y axes when viewing the data.

    Enter the concentrations of the standard curve to the wells containing the control reactions for the FAM detector.

11. Click “Unknown” in the Task column, and then select “Standard” from the dropdown menu. Select “Quantity” for the appropriate detector and enter the quantity of DNA in the well.

    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 VIC detector Task for standard reactions set to “Unknown.” Enter the sample name (e.g., Standard -1).



Use	Detector	Reporter	Quencher	Task	Quantity	Color
☑	fam	FAM	(none)	Standard	20	■
☑	vic	VIC	(none)	Unknown		▨

	1	2	3	4	5	6	7	8	9	10	11	12
A	S U	S U	S U	S U	S U	S U	S U	S U	S U	S U	S U	S U
B	S U	S U	N U	N U	U U	U U	U U	U U	U U	U U	U U	U U
C	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
D	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
E	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
F	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
G	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U
H	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U	U U

Setting up the sample plate.

12. Click “Finish.” To program the cycler according to Table 18, click on the “Instrument” tab.

    PCR requires an initial incubation at 95°C for 3 min to activate the DNA polymerase.

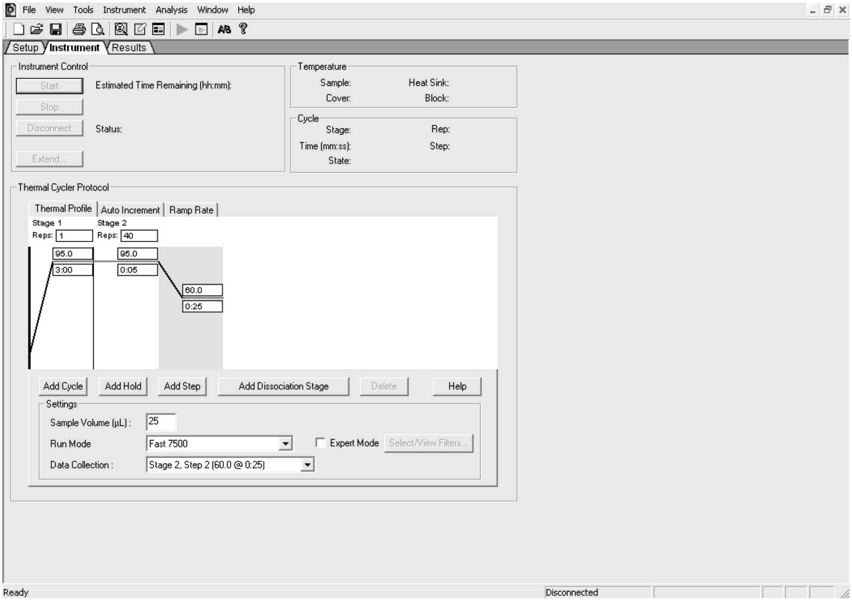
    Two-step cycling PCR requires 40 cycles. Each cycle is comprised of 2 steps: 95°C for 5 s (denaturation step) and 60°C for 25 s (annealing/extension step).

Table 18. Possible plate setup of reactions on the Applied Biosystems 7500 Fast Real-Time PCR System

Step	Temperature	Time	Number of cycles	Comment
Initial PCR activation step	95°C	3 min	–	PCR requires an initial incubation at 95°C to activate the DNA polymerase.
Two-step cycling:			40	
Denaturation	95°C	5 s		
Combined annealing/ extension	60°C	25 s		Perform fluorescence data collection.

13. On the thermal profile, change the holding times to those in Table 18. Be sure to change the Sample volume to 25 µl and select the run mode Fast 7500.

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



Stage 1 Reps: 1 — 95.0, 3:00

Stage 2 Reps: 40 — 95.0, 0:05; 60.0, 0:25

Sample Volume (µL): 25

Run Mode: Fast 7500

Data Collection: Stage 2, Step 2 (60.0 @ 0:25)

Adjusting the thermal profile (SDS Software Version 1.4).

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

    Ensure that position A1 on the plate is on the top-left side of the tray.
16. Start the reaction by clicking "Start."

Data analysis

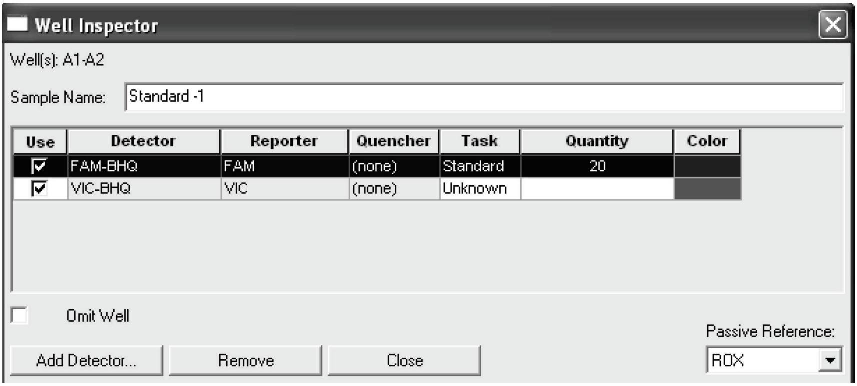
Optimal analysis settings are a prerequisite for accurate quantification data. Always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Procedure

1. Open the run file using the SDS software. Go to "File" followed by "Open" and then "Browse" to locate the saved file.
2. Standards must first be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 4.
3. Go to "View" and select "Well Inspector" from the menu. In the Task column, define the wells that contain DNA standards for the FAM channel as "Standard."

   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 VIC detector Task for standard reactions set to "Unknown." Quantity values are not needed for the internal control. Enter the sample name (e.g., Standard -1).



Well(s): A1-A2

Sample Name: Standard -1

Use	Detector	Reporter	Quencher	Task	Quantity	Color
☑	FAM-BHQ	FAM	(none)	Standard	20	■
☑	VIC-BHQ	VIC	(none)	Unknown		■

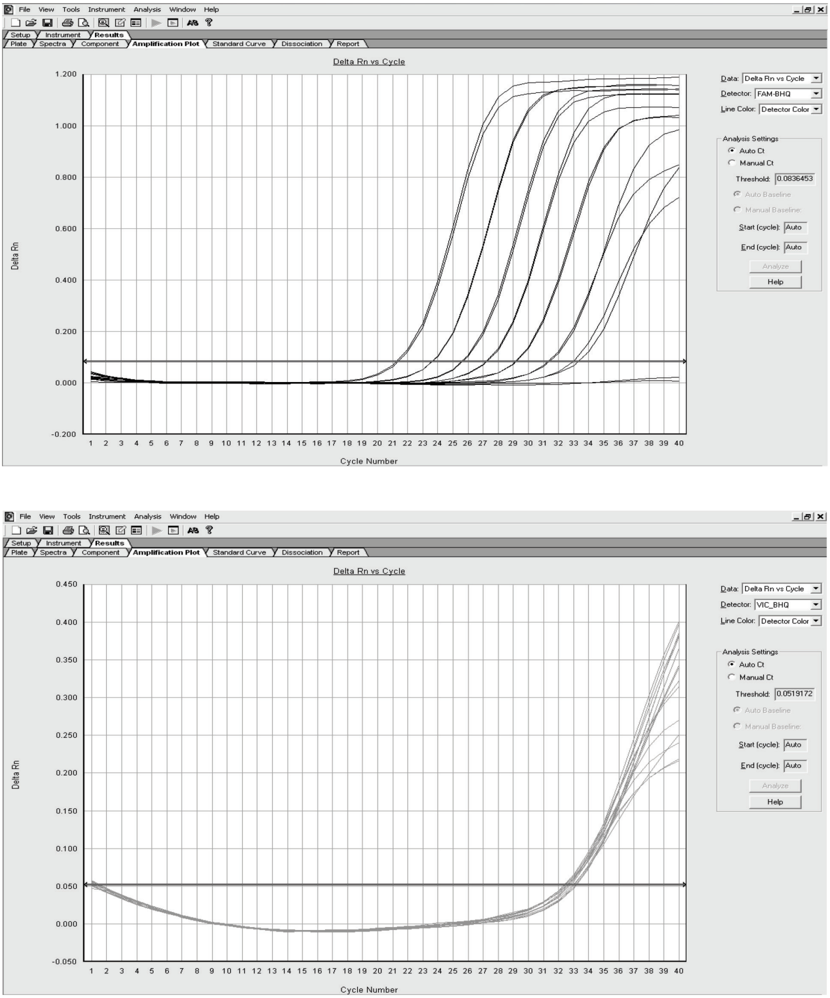
☐ Omit Well

Passive Reference: ROX

Well inspector.

4. In the Amplification Plot tab (found in the Results tab), select the appropriate samples in the table below the amplification plot. Choose “Auto Ct” for both channels and click “Analyze.”

   Setting the appropriate threshold value may require further internal validation at your facility.

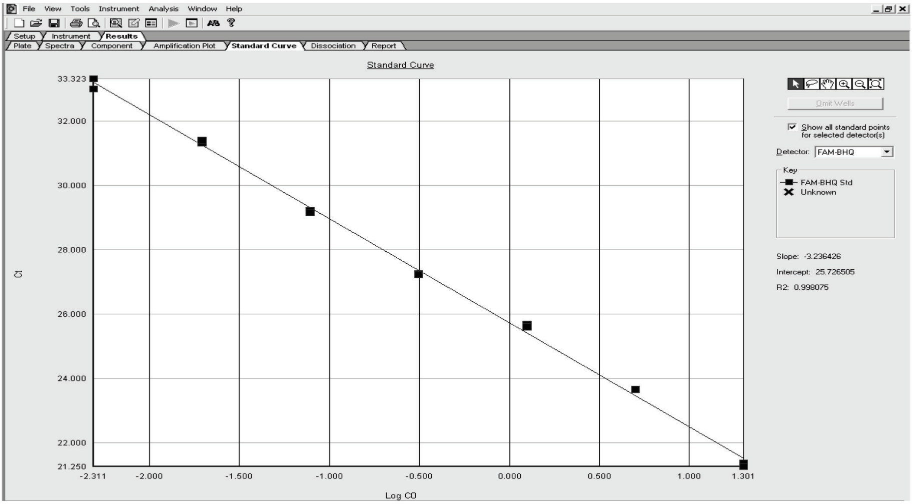


Data: Delta Rn vs Cycle
Detector: FAM-BHQ
Line Color: Detector Color
● Auto Ct
○ Manual Ct
Threshold: 0.0836453
● Auto Baseline
○ Manual Baseline:
Start (cycle): Auto
End (cycle): Auto

Data: Delta Rn vs Cycle
Detector: VIC_BHQ
Line Color: Detector Color
● Auto Ct
○ Manual Ct
Threshold: 0.0519172
● Auto Baseline
○ Manual Baseline:
Start (cycle): Auto
End (cycle): Auto

Sample analysis for the FAM and the VIC channels.

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



☑ Show all standard points for selected detector(s)

Detector: FAM-BHQ

Slope: -3.236426

Intercept: 25.726505

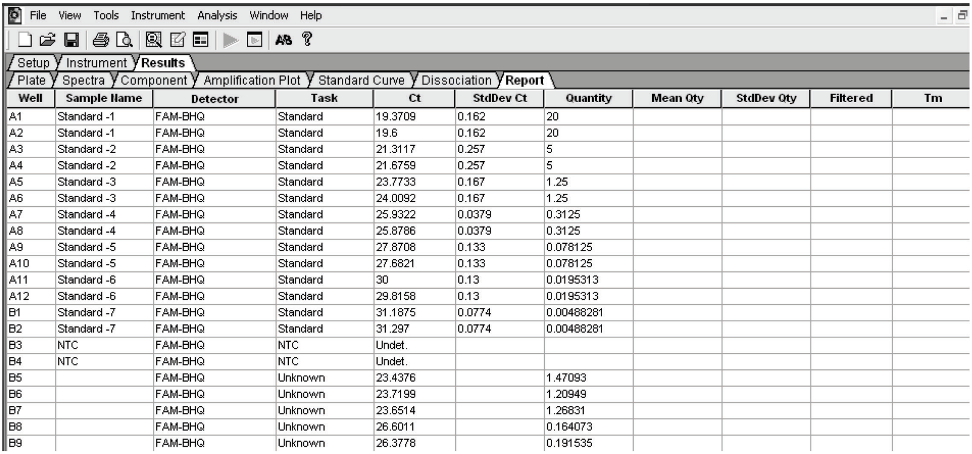
R2: 0.998075

Standard curve.

6. View the concentration of the unknown samples.

   The report displays data for the selected wells and summarizes the quantity of DNA present in the unknown samples. The FAM detector shows the quantity of DNA present in the same unit that was used for the standards. For example, if ng/µl was used for the definition of the standards, then the quantities for the unknowns will be reported in ng/µl.

   The VIC detector shows the C_T value for the Internal Control.



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity	Mean Qty	StdDev Qty	Filtered	Tm
A1	Standard -1	FAM-BHQ	Standard	19.3709	0.162	20				
A2	Standard -1	FAM-BHQ	Standard	19.6	0.162	20				
A3	Standard -2	FAM-BHQ	Standard	21.3117	0.257	5				
A4	Standard -2	FAM-BHQ	Standard	21.6759	0.257	5				
A5	Standard -3	FAM-BHQ	Standard	23.7733	0.167	1.25				
A6	Standard -3	FAM-BHQ	Standard	24.0092	0.167	1.25				
A7	Standard -4	FAM-BHQ	Standard	25.9322	0.0379	0.3125				
A8	Standard -4	FAM-BHQ	Standard	25.8786	0.0379	0.3125				
A9	Standard -5	FAM-BHQ	Standard	27.8708	0.133	0.078125				
A10	Standard -5	FAM-BHQ	Standard	27.6821	0.133	0.078125				
A11	Standard -6	FAM-BHQ	Standard	30	0.13	0.0195313				
A12	Standard -6	FAM-BHQ	Standard	29.8158	0.13	0.0195313				
B1	Standard -7	FAM-BHQ	Standard	31.1875	0.0774	0.00488281				
B2	Standard -7	FAM-BHQ	Standard	31.297	0.0774	0.00488281				
B3	NTC	FAM-BHQ	NTC	Undet.						
B4	NTC	FAM-BHQ	NTC	Undet.						
B5		FAM-BHQ	Unknown	23.4376		1.47093				
B6		FAM-BHQ	Unknown	23.7199		1.20949				
B7		FAM-BHQ	Unknown	23.6514		1.26831				
B8		FAM-BHQ	Unknown	26.6011		0.164073				
B9		FAM-BHQ	Unknown	26.3778		0.191535				

Unknown sample concentration.

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 “Interpretation of Results,” page 78.

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 established for another kit may not be suitable for Investigator Quantiplex Kit, and may need to be adjusted.

For DNA quantification using Investigator Quantiplex 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 to –3.6. When values fall outside of this range, see the Troubleshooting Guide, page 81, for more information.

The Y-intercept

The Y-intercept (b) indicates the expected C_T value for a sample with Qty = 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 (R2 ≤ 0.98) may occur for many different reasons. In the case of low R^2 values, see the Troubleshooting Guide, page 81, for more information.

Internal control

The internal control is intended to report chemistry or instrument failure, errors in assay setup and the presence of inhibition in the sample. The 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 inhibitor is present in the sample. In this case, the operator will get both the information about the concentration of DNA in the sample and the presence of inhibitors. The comparison of the C_T value of the IC system for DNA standards to 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.

Positive amplification of the IC system will generate a C_T value of approximately 31. The internal control system is a very sensitive system to permit indication of inhibitors; therefore a variation of 1–2 C_T across the standard curve samples can be expected. Using high levels of human DNA (>150 ng/reaction) can give a higher C_T value for the IC system.

The result of the IC must be taken in context of the result of the specific target for human DNA. The situations shown in Table 12 may arise.

Table 19. Potential amplification results and their interpretation

Specific human target	Internal Control	Interpretation
No amplification	Positive amplification	No human DNA detected
No amplification	No amplification	Invalid result
Positive amplification with low C_T and high fluorescence signal	No amplification or C_T higher than 32	IC result inconclusive
Positive amplification with high C_T and low fluorescence signal	No amplification or C_T higher than 32	PCR inhibition present

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

Quantification of unknowns

The Investigator Quantiplex Kit can quantify a broad range of DNA amounts in a sample — from 75 ng/µl to about 0.5 pg/µl with a highly linear range between 20 ng/µl and 4.9 pg/µl of human genomic DNA. When 2 µl of a sample at very low concentrations is loaded in a reaction, the well contains approximately 1–1.5 diploid human genome equivalents. In the low DNA concentration range, statistical effects known as stochastic variations can significantly affect the assay result. When using samples containing low concentrations of DNA, make sure that as many replicates as possible are assayed, in order to confirm the result.

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/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
No signal, or one or more signals detected late in PCR	
Incorrect cycling conditions	Always use the optimized cycling conditions specified in the protocols. Be sure to select ROX as the passive dye on Applied Biosystems instruments.
Pipetting error, missing or degraded reagent	Check the storage conditions of the reagents. Repeat the assay.
Incorrect or no detection step	Make sure that fluorescence detection takes place during the combined annealing/extension step.
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.
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.

	Comments and suggestions
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.
Degraded control DNA	Make new serial dilutions of the control DNA from the stock solution. Repeat the assay using the new dilutions.
Differences in C_T values or in PCR efficiencies between runs	
Incorrect handling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of the DNA Polymerase (95°C for 1 min), and the specified times for denaturation and annealing/extension.
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. In some cases, manually changing the threshold enables the optimal reaction efficiency and R^2 value to be obtained.
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. When signals are appearing at very early C_T values, dilute the sample and repeat the reaction.
Increased fluorescence or C_T value for no template control	
Contamination of reagents	Discard all the components of the assay (e.g., master mix). Repeat the assay using new components.
Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.

	Comments and suggestions
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	
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.
Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
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	
Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
Real-time cycler and/or pipets no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions. Calibrate pipets to minimize pipetting variability.
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.

	Comments and suggestions
Problem with dilution of standards	Be sure that the DNA standard is completely thawed and mixed thoroughly before use. Be sure dilutions of the DNA standard are mixed thoroughly before removing each aliquot for the serial dilution. Do not use a sample volume other than 2 µl. Change pipet tips between each dilution step.
The plate was not sealed.	Carefully seal the plates to avoid evaporation.
An error was made during dilution of the DNA standard.	Verify all calculations, and repeat dilution of the DNA standard.
Incorrect concentration values were entered in the software.	Verify the concentrations for all samples used to generate the standard curve.
Abnormal fluorescence	Do not write on the plate. Use caution when handling plates. Wear gloves.
Statistical variation	Some variation in the reaction is normal, particularly with the DNA target present at low copy number. Perform at least duplicates for the standard curve to minimize the effect of this variation. Remove the 0.0048828125 ng/µl dilution of the DNA standard from the standard curve by changing the sample type to “Unknown.”

References

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Cited reference

Whitcombe, D., Theaker, J., Guy, S.P., Brown, T. and Little, S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat. Biotechnol. 17, 804.

Appendix: Alternative Standard Curves

Table 20. 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 21. Alternative 4-point standard curve (27x dilution)

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

Ordering Information

Product	Contents	Cat. no.
Investigator Quantiplex Kit (200)	Reaction Mix YQ, Primer Mix IC FQ, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387016
Related Products		
Investigator Quantiplex HYres Kit (200)	Reaction Mix YQ, Primer Mix IC YQ, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387116
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 RGQ Kit (200)	Quantiplex Pro RGQ Reaction Mix, Quantiplex Pro RGQ Primer Mix, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer	387316
Investigator Human Identification PCR Kits		
Investigator 24plex QS Kit (100)*	Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex QS, DNA size standard 550 (BTO), and nuclease free water	382415
Investigator 24plex GO! Kit (200)*	Primer mix, Fast Reaction Mix 2.0, Control DNA, allelic ladder 24plex GO!, DNA size standard 550 (BTO)	382426

Investigator ESSplex Plus Kit*	Primer mix, Fast Reaction Mix including HotStarTaq Plus DNA Polymerase, Control DNA, allelic ladder ESSplex Plus, DNA size standard 550 (BTO), and nuclease-free water	381535
Investigator ESSplex SE Plus Kit*	Primer mix, Fast Reaction Mix including HotStarTaq Plus DNA Polymerase, Control DNA, allelic ladder ESSplex SE Plus, DNA size standard 550 (BTO), and nuclease-free water	381545
Investigator IDplex Plus Kit*	Primer mix, Fast Reaction Mix including HotStarTaq Plus DNA Polymerase, Control DNA, allelic ladder IDplex Plus, DNA size standard 550 (BTO), and nuclease-free water	381625
Investigator HDplex Kit (100)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	381215
Investigator Triplex AFS QS Kit (400)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	380317
Investigator Triplex DSF Kit (400)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	380327
Investigator Argus X-12 Kit (25)*	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder,	383213

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[*] Larger kit sizes available; please inquire.

	DNA size standard, and nuclease-free water	
Investigator Argus Y-12 QS Kit (100)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	383615
Investigator DIPplex Kit (100)	Primer mix, reaction mix, DNA Polymerase, Control DNA, allelic ladder, DNA size standard, and nuclease-free water	384015
DNA extraction and purification		
QIAamp® DNA Investigator Kit (50)	50 QIAamp MinElute® Columns, Proteinase K, Carrier RNA, Buffers, Collection Tubes (2 ml)	56504
MinElute Reaction Cleanup Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28004
Rotor-Gene Q		
Rotor-Gene Q 2plex	Real-time PCR cycler with 2 channels, laptop computer, software, accessories, 1-year warranty on parts and labor	Inquire
Rotor-Gene Q 2plex HRM	Real-time PCR cycler and High Resolution Melt analyzer with 2 channels, plus HRM channel, laptop, software, accessories, 1 year warranty on parts and labor	Inquire
Rotor-Gene Q 5plex	Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories, 1 year warranty on parts and labor	Inquire

Rotor-Gene Q 5plex HRM	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels, plus HRM channel, laptop, software, accessories, 1 year warranty on parts and labor	Inquire
Rotor-Gene Q 6plex	Real-time PCR cycler with 6 channels, including laptop, software, accessories, 1 year warranty on parts and labor	Inquire

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Document Revision History	
R6 06/2018	Replacement of Control DNA1 Z1 with Male Control DNA M1. Replacement of Reaction Mix FQ with Reaction Mix YQ. Updated tables for standard DNA dilution series and adjusted PCR cycling parameters.
R5 11/2014	Content updates for no template control (NTC) section and quantification protocols.

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