June 2018

# Investigator® Quantiplex® HYres Kit Handbook

For quantification of human and male DNA in forensic samples

Sample to Insight

QIAGEN

# Contents

# Kit Contents

| **Investigator Quantiplex HYres Kit** | **(200)** |
|---|---|
| **Catalog no.** | **387116** |
| **Number of 20 µl reactions** | **200** |
| Reaction Mix YQ | 1 x 1.8 ml |
| Primer Mix IC YQ | 1 x 1.8 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 and +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 YQ must be stored protected from light. DNA samples should be stored separately from PCR reagents. Under these conditions, the components are stable until the expiration date indicated on the kit.

# Intended Use

The Investigator Quantiplex HYres 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 HYres 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 product line consists of the Investigator Quantiplex Kit, which is for total human genomic DNA quantification, and the Investigator Quantiplex HYres Kit, which is for the quantification of human genomic and male DNA in a sample using quantitative real-time PCR. Both kits are designed to confirm whether a sample contains sufficient DNA to enable DNA fingerprinting analysis (such as STR, DIP, or SNP analysis). Furthermore, the kits may help in establishing if a sample contains inhibitors that may interfere with such applications, thus necessitating further sample purification.

## Principle and procedure

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

The kit contains reagents and a DNA polymerase for specific amplification of 4NS1C®, which is 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 real-time PCR cycler 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. They analyzed the conservation of its copy number in the human population. A quantitative real-time PCR was run to compare a human specific single-copy and the Investigator Quantiplex HYres 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.

The target region for male DNA quantification was selected in order to give high sensitivity with high reliability within different individuals and populations and in the presence of mixed DNA samples. This target region was also validated in an external study by a number of forensic laboratories where the conservation of its copy number in the human population was analyzed. Again, a quantitative real-time PCR was run to compare a human specific single-copy and the Investigator Quantiplex HYres male multi-copy target. The $\Delta C_T$ for the two targets was comparable in different populations and no signal could be detected for female DNA samples, demonstrating the conservation of the target copy number across ethnic groups and the specificity for the Y-chromosome. The male quantification target region is detected as a 129 bp fragment using the red channel on the Rotor-Gene Q or the Cy5™ dye channel on Applied Biosystems instruments.

In addition, the Investigator Quantiplex HYres Kit contains a balanced internal amplification control that is used to test successful amplification and identify the presence of PCR inhibitors. This heterologous amplification system is detected as a 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.

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.

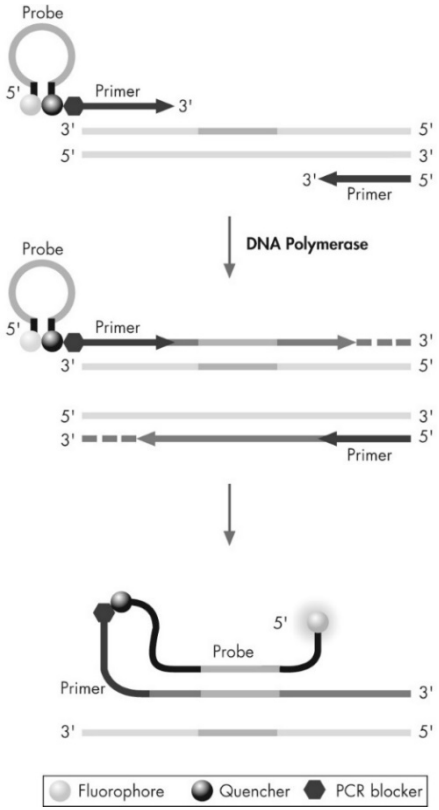



**Figure 1. Scorpions primers and their function.**

## 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 and the male quantification targets. The comparison of the $C_T$ values of the IC system for DNA standards with the $C_T$ values of the IC system for unknown samples may provide an indication of potential inhibition of the reaction in the unknown samples. Therefore, even if the IC system reports the presence of inhibitors in the sample, the DNA quantification will typically provide a reliable result. The presence of inhibitors in the sample may affect the downstream application and must be considered.

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.

## Reaction Mix YQ

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

* This value may vary depending on the instrument used.

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.

### Control DNA 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 recommended. The Control DNA contains pooled male DNA at a concentration of 50 ng/µl. To ensure pipetting accuracy, the minimum input volume of DNA for dilutions should be 5 µl. The standard curve is designed to be easily set up using a convenient 1:4 dilution series. If using QuantiTect Nucleic Acid Dilution Buffer to dilute the Male Control DNA M1, the dilutions are stable for at least 1 week at +2 to +8°C.

**Important**: Male Control DNA M1 is optimized for use with the Investigator Quantiplex HYres 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 for Human Identification, and Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems, QIAGEN has developed a set of template files (Table 1). These templates can be downloaded from the "Resources" tab at **www.qiagen.com/QuantiplexHYres**.

**Table 1. Template files available for the Rotor-Gene Q**

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

## Description of protocols

Protocols for 4 different cyclers are provided in this handbook.

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

# Equipment and Reagents to Be Supplied by User

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

- 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 for Human Identification, or Applied Biosystems 7500 or 7500 Fast Real-Time PCR Systems)
- 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.
  PCR Tubes, 0.2 ml (cat. no. 981005 or 981008) and the Rotor-Disc 100 can also be used.

# 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 HYres Kit.

**Note**: An automated setup is also possible. Protocols for QIAgility® are available upon request.

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

| Real-time thermal cycler | Protocol |
|---|---|
| Rotor-Gene Q | Page 15 |
| Applied Biosystems 7500 Real-Time PCR System for Human Identification | Page 43 |
| Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems | Page 59 |

## Contamination risks

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

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

## Controls

### No-template control (NTC)

Replicates of NTC reactions should be included in each quantification run in order to detect contamination. NTCs should contain all the components of the reaction, except for the template. The quantification using the Investigator Quantiplex HYres 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 YQ.

# Protocol: Quantification of DNA Using the Rotor-Gene Q

This protocol is optimized for use of the Investigator Quantiplex HYres 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.
- Use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Use the template volume specified in the protocol. The reaction is optimized for use with 2 µl template DNA. Do not use more or less than 2 µl per 20 µl reaction.
- 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. 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 HYres Kit Cycling file," "Investigator Quantiplex HYres Kit Sample file," and "Investigator Quantiplex HYres Kit Analysis Settings for the Yellow Channel file" from the 'Resources' tab at **www.qiagen.com/QuantiplexHYres**.

## 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 81.

3. Thaw the template nucleic acids.

   Mix all solutions thoroughly before use to avoid localized concentrations of salt.
4. Prepare a master mix according to Table 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, we recommend keeping the reagents, samples, and controls on ice or in a cooling device.

**Table 4. Master mix for DNA quantification**

| Component | Volume per 20 µl reaction | Final concentration |
|---|---|---|
| Reaction Mix YQ | 9 µl | 1x |
| Primer Mix IC YQ | 9 µl | 1x |
| **Total volume of master mix** | **18 µl** | - |

5. Mix the master mix thoroughly, and dispense 18 µl into Rotor-Gene Q Tubes or into the Rotor-Disc.
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.

   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 using Strip Tubes**

| 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 |

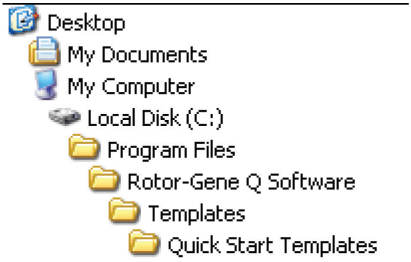
All amounts in ng/µl. UNK: Unknown sample.

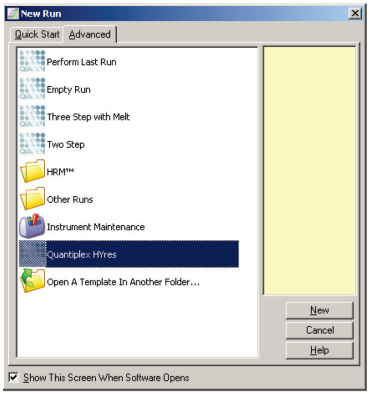
8. Close the PCR tubes, place them in the appropriate rotor in the Rotor-Gene Q cycler, and attach the locking ring.

   If you are using tubes, empty positions in the rotor should be filled with empty PCR tubes.

9. Open the Rotor-Gene Software. We recommend using the template file provided. In the Advanced Wizard, select "Open A Template In Another Folder…" and load the file "Investigator Quantiplex HYres Kit Cycling file."

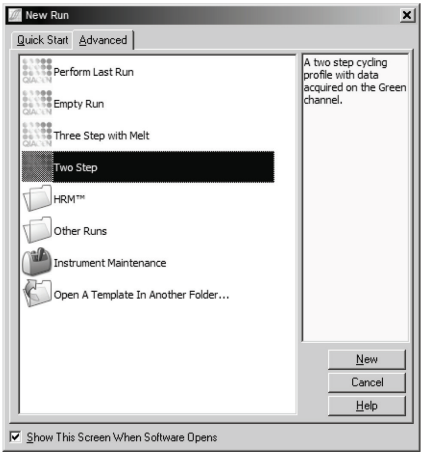
   If you copy the template file "Investigator Quantiplex HYres Kit Cycling file" in the Rotor-Gene Q Templates and in the Quick Start Templates folders, the template will appear directly in the Quick Start and in the Advanced Wizard windows.



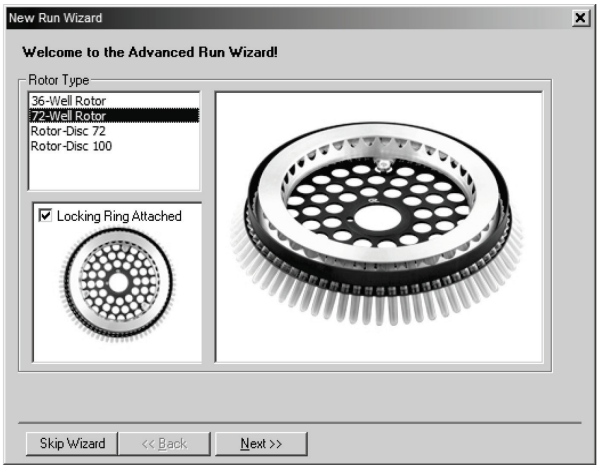

10. To setup cycling manually, select “Two Step” cycling profile, and click “New.”

    We recommend using the provided template file (see page 15 for download information) to facilitate the reaction setup. When using template files, the settings may already be those described in the next step. In this case, click to the next screen.




**Advance Wizard of the Rotor-Gene Software.**

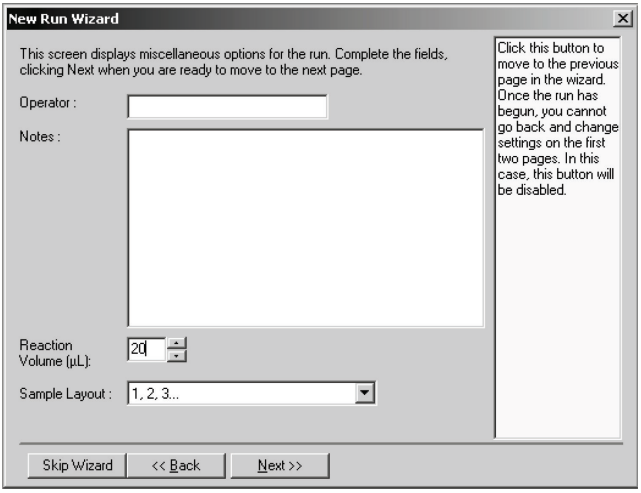
11. Select the correct Rotor, and confirm the locking ring is attached by checking the check box. Click “Next” to continue.




**Confirmation that the locking ring is attached.**

12. Ensure that the reaction volume is 20 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.

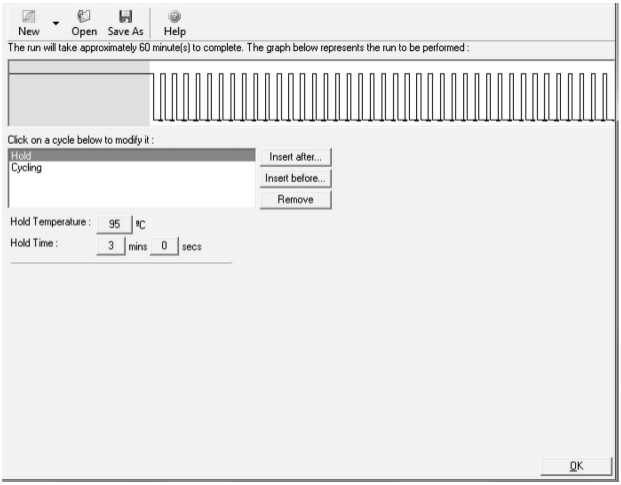



**Enter reaction volume.**

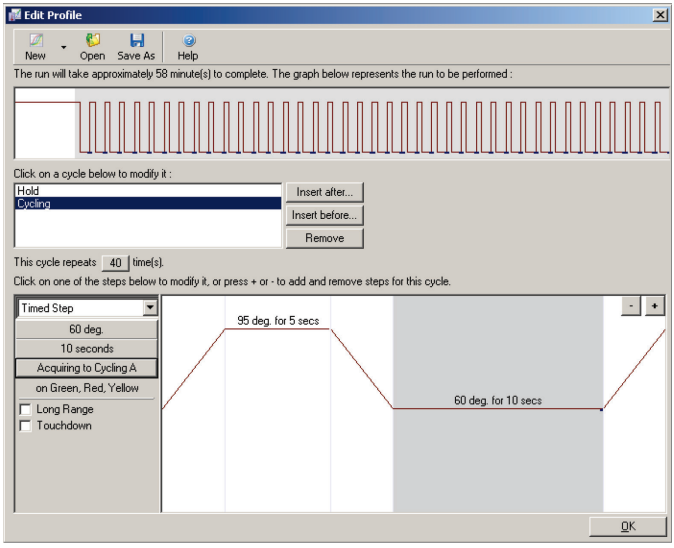
13. Click "Edit Profile" and program the Rotor-Gene Q according to Table 6. See also the figures on pages 22-25.

**Table 6. Cycling conditions for the Rotor-Gene Q**

| Step | Temperature | Time | Number of cycles | Comment |
|---|---|---|---|---|
| **Initial 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 | 10 s | | Perform fluorescence data collection using the green, red, and yellow channels with auto-gain optimization. |



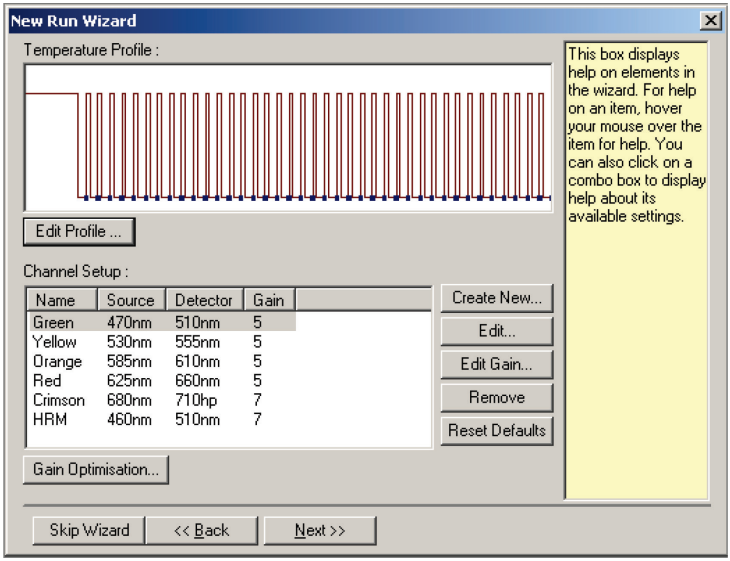

**Initial PCR activation step. PCR requires an initial incubation at 95°C for 3 min to activate the DNA polymerase.**



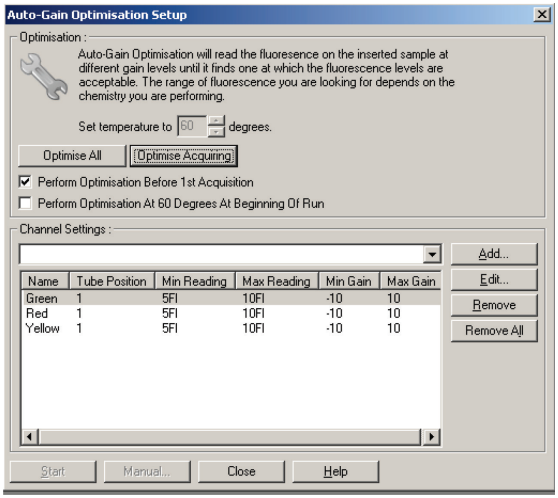

**Two-step cycling. PCR requires 40 cycles. Each cycle consists of 2 steps: 95°C for 5 s (denaturation step) and 60°C for 10 s (annealing/extension step).**

14. Click OK to close the window and return to the Wizard.
15. To set the gain optimization settings for the green, yellow, and red channels, click "Gain Optimization," followed by "Optimize Acquiring." In the dialog box that opens, confirm the standard settings for all three channels. Then click "OK."
16. Check the "Perform Optimization Before 1st Acquisition" box, and click "Close."

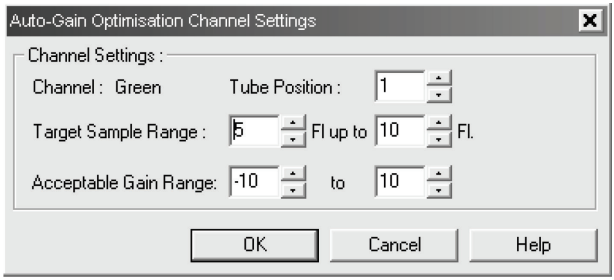
    Make sure that the tube at position 1 is not empty, since the gain optimization will be performed on this tube.



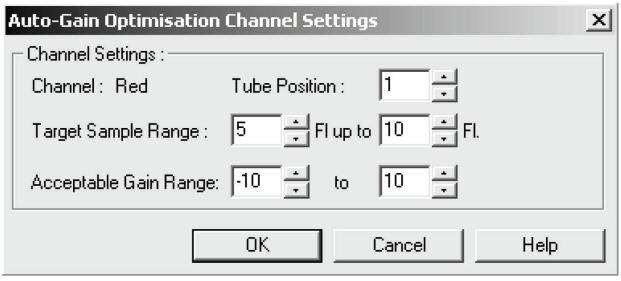

**Gain optimization.**



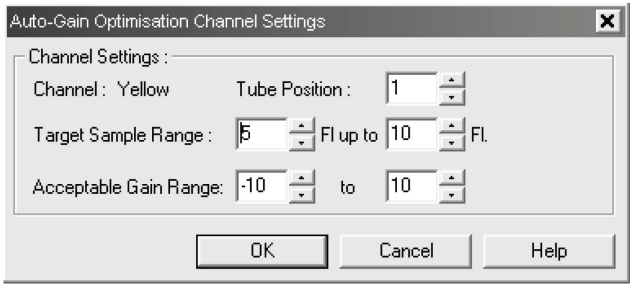

**Optimize acquisition.**




**Confirming Gain Optimization settings for the green channel.**

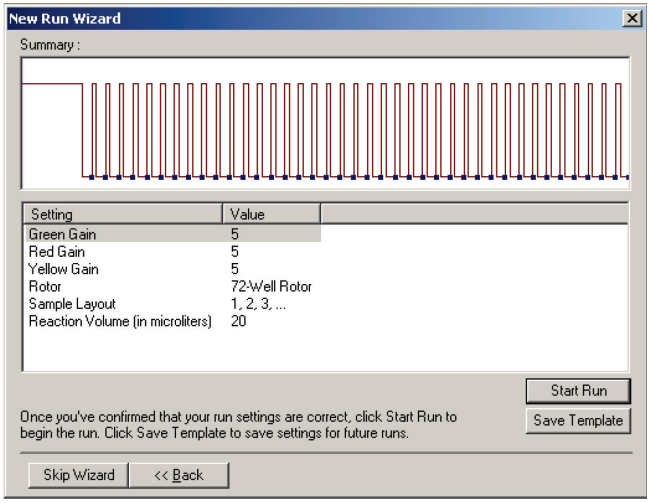



**Confirming Gain Optimization settings for the red channel.**




**Confirming Gain Optimization settings for the yellow channel.**

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

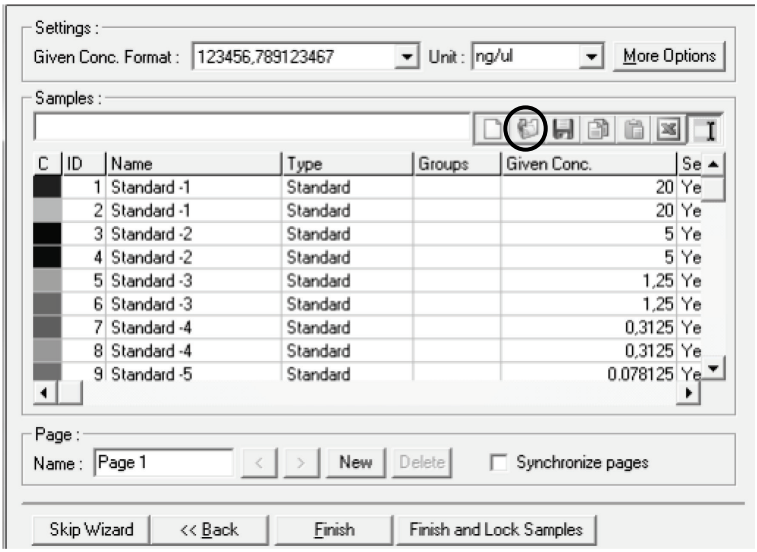



**Summary of parameters.**

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

19. 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 HYres Kit Sample file" (see page 15 for download information). To load the template file, go to "Open" and select the template file required. An example template file is shown below.




**Template for editing the standard curve.**

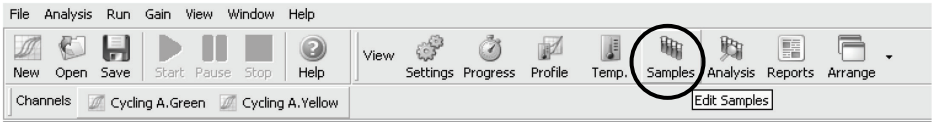
## Data analysis

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

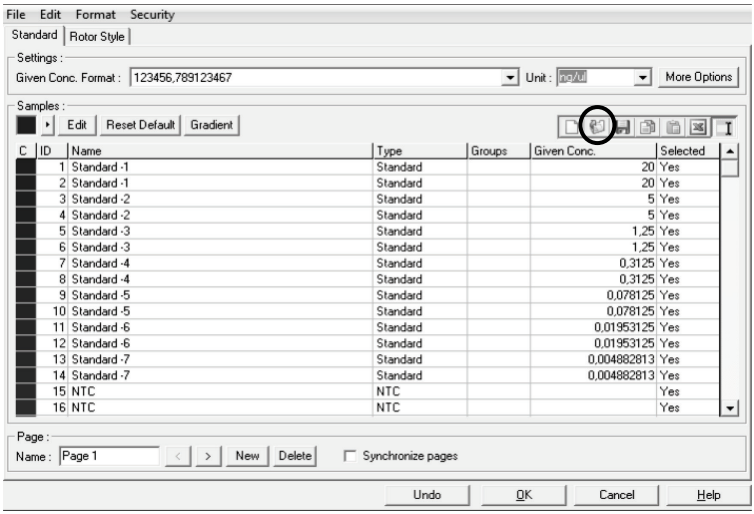
1. Open the run file using the Rotor-Gene Q Software. Go to "File," followed by "Open" and then "Browse" to locate the saved file.
2. Standards must be defined before a standard curve can be created. If the standards were defined before the run was started, proceed to step 13.

   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 HYres Kit Sample file." We recommend using that file to streamline the reaction setup. See page 15 for download information.
3. Select the Edit Sample tool by clicking "Samples."



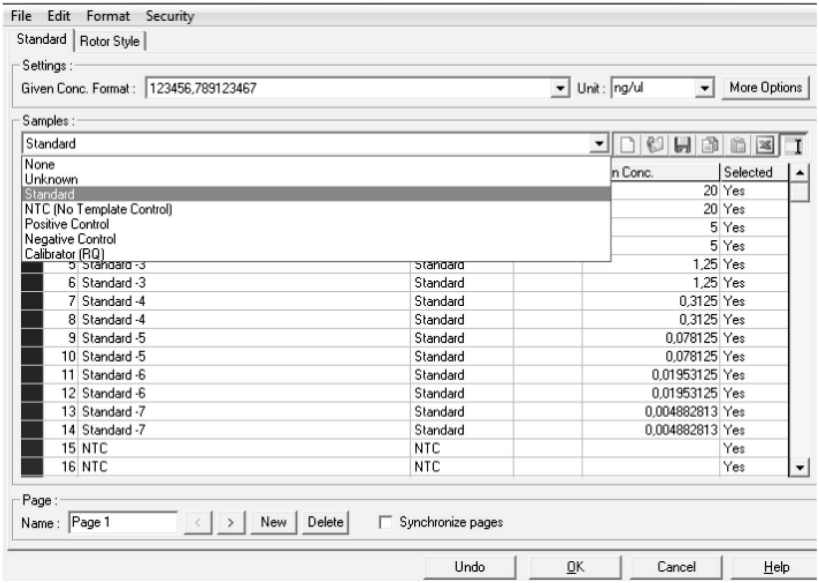

**Edit Samples tool in the Rotor-Gene Q Software.**

4. The “Investigator Quantiplex HYres Kit Sample file” can be loaded by going to “Open” and choosing the file. Proceed to step 14.



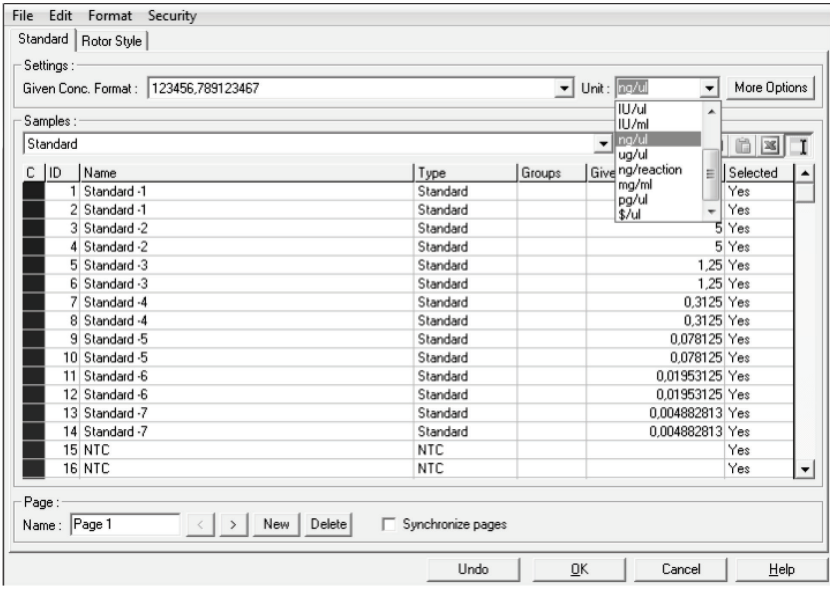

**Template file for editing the standard curve.**

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.




**Editing the samples in the Rotor-Gene Q Software.**

6. In the "Given Conc." column, enter the DNA concentration of the human standards according to Table 7 and define the unit (ng/µl) using the dropdown menu. Enter the sample name (e.g., Standard -1).




**Editing the units in the Rotor-Gene Q Software.**

**Table 7. DNA concentrations of the human and male standards**

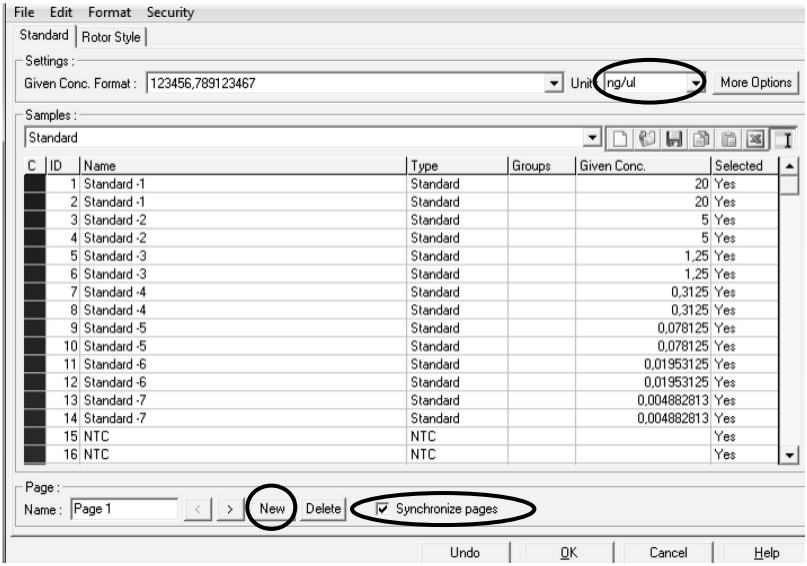
| Standard | Human Standards<br>Green Channel<br>Page 1/Human DNA | Male Standards<br>Red Channel<br>Page 2/Male DNA |
|---|---|---|
| Standard -1 | 20 | 20 |
| Standard -2 | 5 | 5 |
| Standard -3 | 1.25 | 1.25 |
| Standard -4 | 0.3125 | 0.3125 |
| Standard -5 | 0.078125 | 0.078125 |
| Standard -6 | 0.01953125 | 0.01953125 |
| Standard -7 | 0.0048828125 | 0.0048828125 |

**Note**: Alternative standard curves are listed in "Appendix: Alternative Standard Curves," page 81.

7. Create two new pages by clicking “New” twice and selecting “Synchronize pages.”

   **Important**: If you do not select the “Synchronize pages” check box, the names of the wells will not be adopted for all channels.

8. On Page 2/Male DNA, leave the sample type as “Standard” for DNA standards and “NTC” for no template controls.



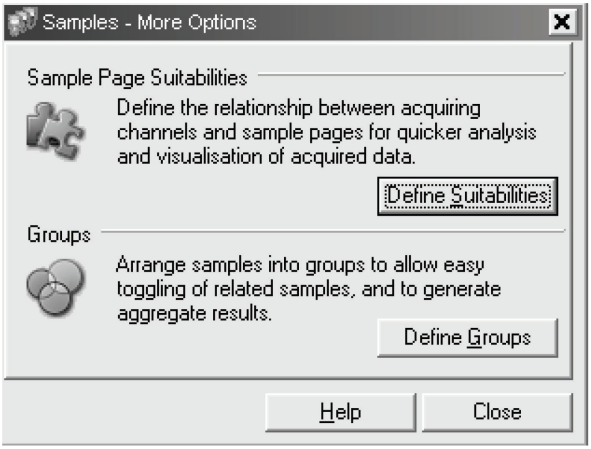

**Create a new sample page.**

9. On Page 2/Male DNA, deselect the “Synchronize pages” check box. In the “Given Conc.” column, enter the DNA concentration of the male standards according to Table 7 and define the unit (ng/µl) from the dropdown menu.

   **Important**: If you do not deselect the “Synchronize pages” check box, the same concentrations will be adopted for both the human and the male standards, leading to a wrong calculation of the unknown concentrations.

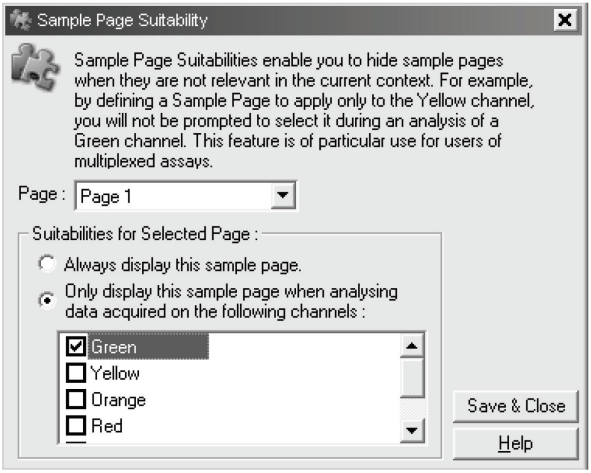
10. On Page 3/IC, leave all the samples as “Unknown” in the Type column.

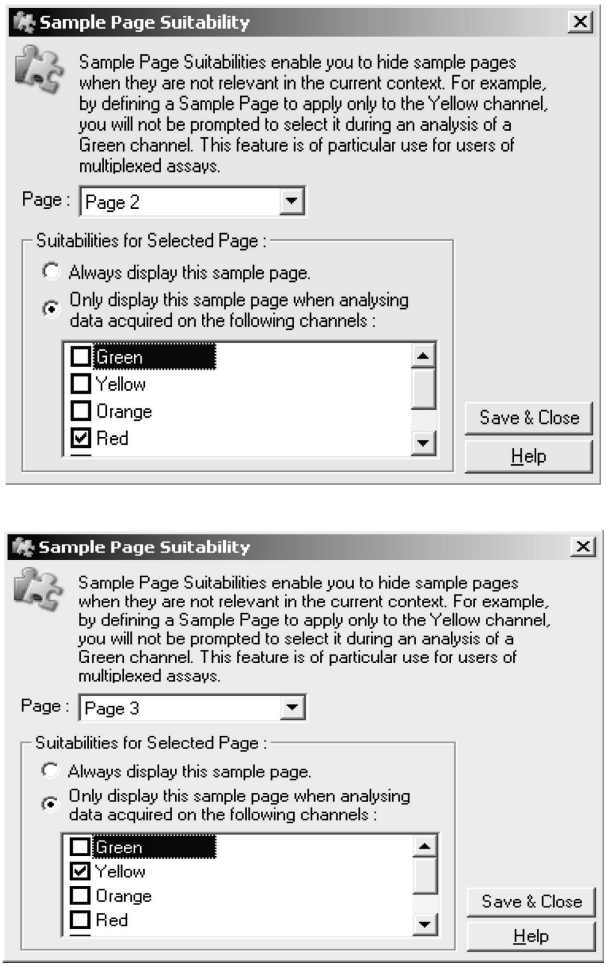
11. Open the "More Options" menu and define the Sample Page Suitabilities. Select the option "Only display this sample page when analyzing data."




**Sample Page Suitabilities.**

12. Select the green channel for page 1, the red channel for page 2, and the yellow channel for page 3. Click "Save and Close." Close the Sample Page Suitabilities by clicking "Close."

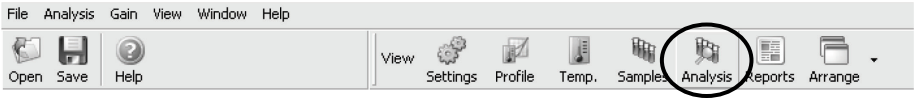





**Selecting color channels.**

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

14. To analyze the samples, open the analysis tool by clicking "Analysis."

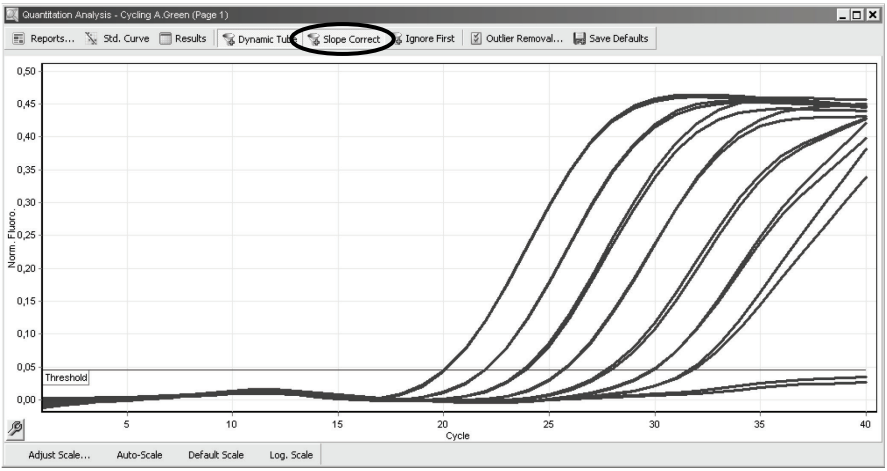



15. On the Quantitation tab, select "Page 1/Human DNA" for the green channel and click "Show." Select "Page 2/Male DNA" for the red channel and "Page 3/IC" for the yellow channel and click "Show." If the Autofind Threshold window opens automatically, click "Cancel."

    The amplification plots for both the green and yellow channels are displayed in the "Quantitation Analysis" window.

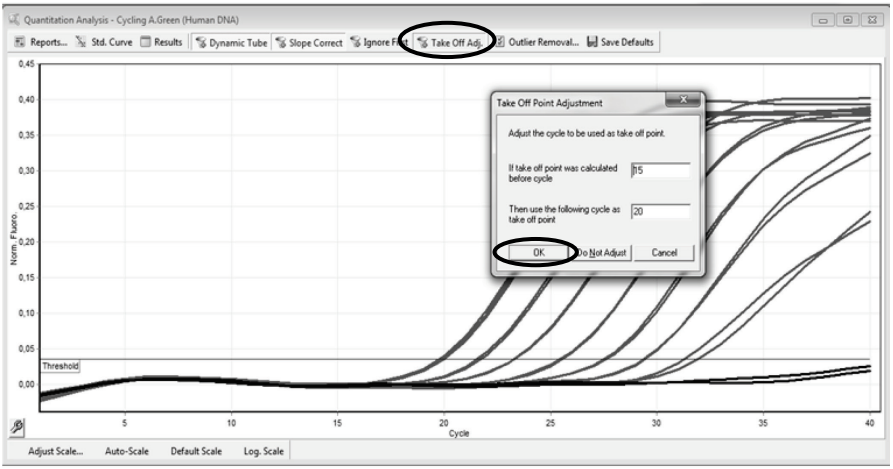

**Quantitation tab in the Analysis tool.**

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




**Slope correction tool.**

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

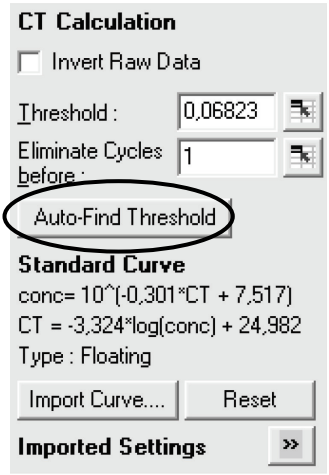



**Take Off Point Adjustment tool.**

18. 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.

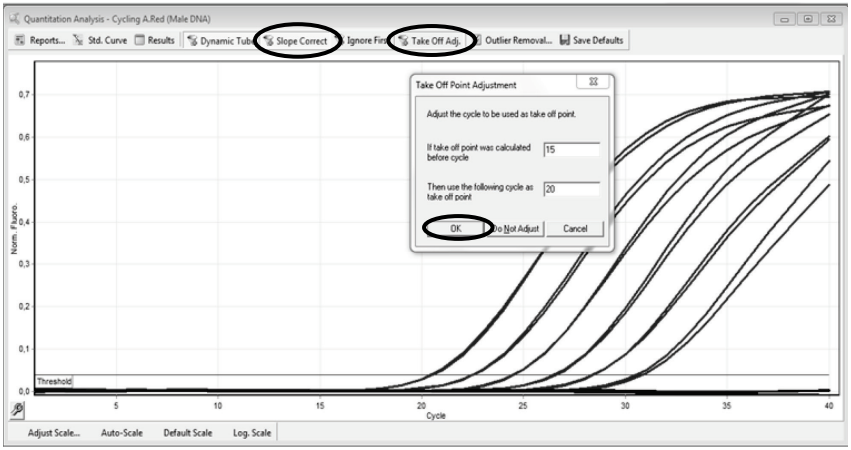



**Setting the $C_T$ value for the Green Channel using the Auto-Find Threshold.**

19. For the red channel, activate the slope correction tool. If available, activate the Take Off Point Adjustment tool for the red channel. Choose the default settings and click "OK." Select "Auto-Find Threshold" on the right side at the bottom of the panel as shown for the green channel above.

    The $C_T$ values are reported in the "Quantitation Results" window.

    We recommend the Auto-Find threshold. If you need to set a fixed threshold, further internal validation at your facility may be required to determine the appropriate threshold value.

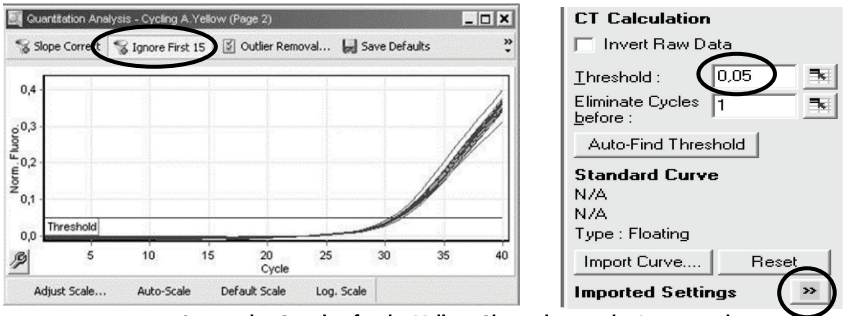



**Slope correction and Take Off Point Adjustment tool.**

20. 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 15 for information on how to download this file. Alternatively, select a $C_T$ threshold value of 0.05 and ignore the first 15 cycles.

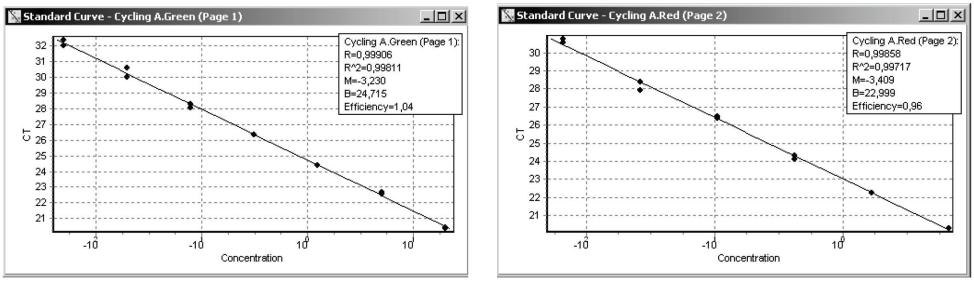
    The $C_T$ values are reported in the "Quantitation Results" window.

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




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

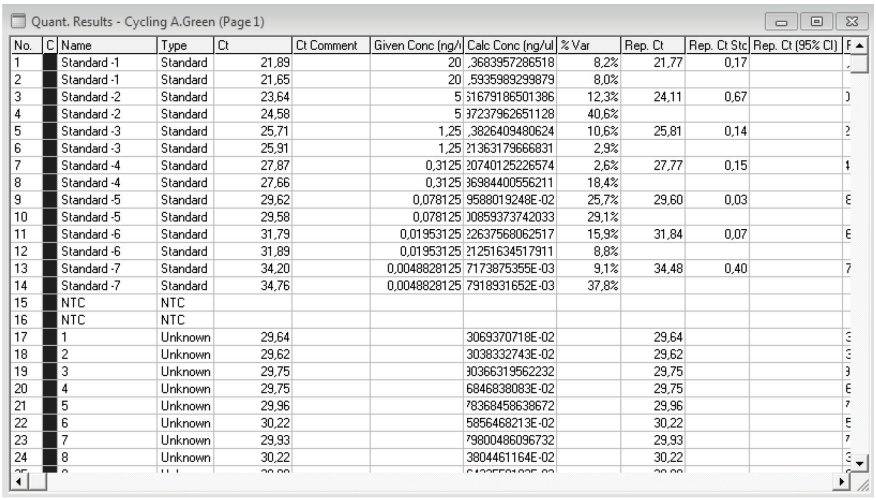
21. The Standard Curves are shown in the "Standard Curve" windows for the green and the red channels. View the calculated regression line, slope (M), y-intercept (B), and $R^2$ values.




**Standard curves.**

22. View the concentration of the unknown samples.

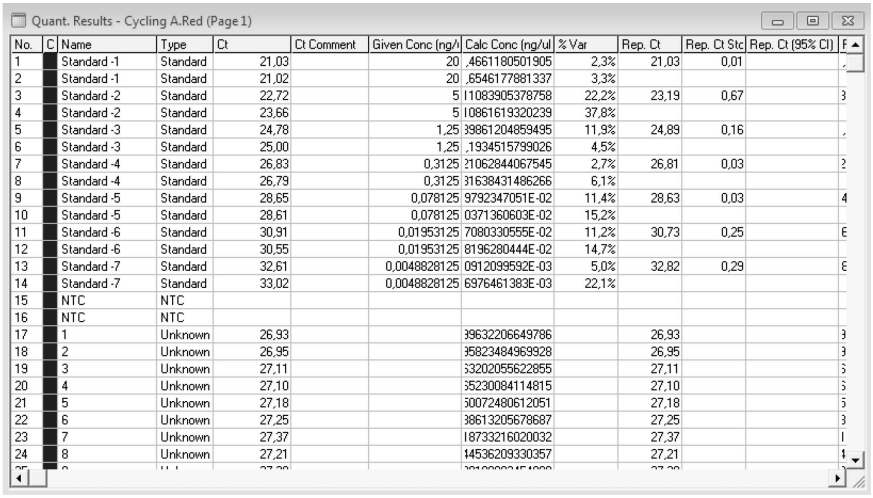
    The "Quantitation Results — Cycling A. Green" and the "Quantitation Results — Cycling A. Red" windows display data for selected wells, and respectively summarize the quantity of the total and male DNA present in the unknown samples. The unit is shown at the top of the column.



Quant. Results - Cycling A.Green (Page 1)

| No. | C | Name | Type | Ct | Ct Comment | Given Conc (ng/ | Calc Conc (ng/ul | % Var | Rep. Ct | Rep. Ct Stc | Rep. Ct (95% CI) | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | Standard -1 | Standard | 21,89 | | 20 | ,3683957286518 | 8,2% | 21,77 | 0,17 | | |
| 2 | | Standard -1 | Standard | 21,65 | | 20 | ,5935989299879 | 8,0% | | | | |
| 3 | | Standard -2 | Standard | 23,64 | | 5 | 51679186501386 | 12,3% | 24,11 | 0,67 | | ) |
| 4 | | Standard -2 | Standard | 24,58 | | 5 | 97237962651128 | 40,6% | | | | |
| 5 | | Standard -3 | Standard | 25,71 | | 1,25 | ,3826409480624 | 10,6% | 25,81 | 0,14 | | 2 |
| 6 | | Standard -3 | Standard | 25,91 | | 1,25 | 21363179666831 | 2,9% | | | | |
| 7 | | Standard -4 | Standard | 27,87 | | 0,3125 | 20740125226574 | 2,6% | 27,77 | 0,15 | | 1 |
| 8 | | Standard -4 | Standard | 27,66 | | 0,3125 | 36984400556211 | 18,4% | | | | |
| 9 | | Standard -5 | Standard | 29,62 | | 0,078125 | 9588019248E-02 | 25,7% | 29,60 | 0,03 | | 8 |
| 10 | | Standard -5 | Standard | 29,58 | | 0,078125 | 0859373742033 | 29,1% | | | | |
| 11 | | Standard -6 | Standard | 31,79 | | 0,01953125 | 22637568062517 | 15,9% | 31,84 | 0,07 | | 6 |
| 12 | | Standard -6 | Standard | 31,89 | | 0,01953125 | 21251634517911 | 8,8% | | | | |
| 13 | | Standard -7 | Standard | 34,20 | | 0,0048828125 | 7173875355E-03 | 9,1% | 34,48 | 0,40 | | 7 |
| 14 | | Standard -7 | Standard | 34,76 | | 0,0048828125 | 7918931652E-03 | 37,8% | | | | |
| 15 | | NTC | NTC | | | | | | | | | |
| 16 | | NTC | NTC | | | | | | | | | |
| 17 | | 1 | Unknown | 29,64 | | | 3069370718E-02 | | 29,64 | | | 3 |
| 18 | | 2 | Unknown | 29,62 | | | 3038332743E-02 | | 29,62 | | | 3 |
| 19 | | 3 | Unknown | 29,75 | | | 30366319562232 | | 29,75 | | | 3 |
| 20 | | 4 | Unknown | 29,75 | | | 6846838083E-02 | | 29,75 | | | 6 |
| 21 | | 5 | Unknown | 29,96 | | | 78368458638672 | | 29,96 | | | 7 |
| 22 | | 6 | Unknown | 30,22 | | | 5856468213E-02 | | 30,22 | | | 5 |
| 23 | | 7 | Unknown | 29,93 | | | 79800486096732 | | 29,93 | | | 7 |
| 24 | | 8 | Unknown | 30,22 | | | 3804461164E-02 | | 30,22 | | | 3 |

**The "Quantitation Results — Cycling A. Green" window.**

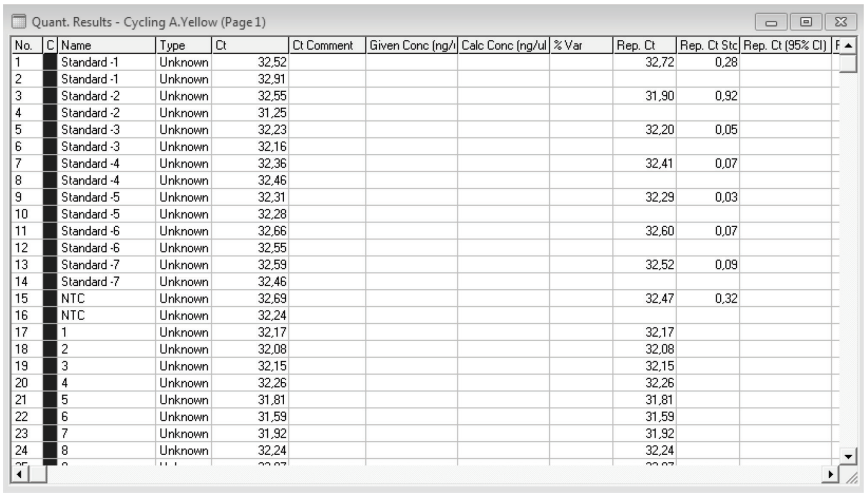


Quant. Results - Cycling A.Red (Page 1)

| No. | C | Name | Type | Ct | Ct Comment | Given Conc (ng/ | Calc Conc (ng/ul | % Var | Rep. Ct | Rep. Ct Stc | Rep. Ct (95% CI) | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | Standard -1 | Standard | 21,03 | | 20 | ,4661180501905 | 2,3% | 21,03 | 0,01 | | |
| 2 | | Standard -1 | Standard | 21,02 | | 20 | ,6546177881337 | 3,3% | | | | |
| 3 | | Standard -2 | Standard | 22,72 | | 5 | 11083905378758 | 22,2% | 23,19 | 0,67 | | 3 |
| 4 | | Standard -2 | Standard | 23,66 | | 5 | 10861619320239 | 37,8% | | | | |
| 5 | | Standard -3 | Standard | 24,78 | | 1,25 | 39861204859495 | 11,9% | 24,89 | 0,16 | | |
| 6 | | Standard -3 | Standard | 25,00 | | 1,25 | ,1934515799026 | 4,5% | | | | |
| 7 | | Standard -4 | Standard | 26,83 | | 0,3125 | 21062844067545 | 2,7% | 26,81 | 0,03 | | 2 |
| 8 | | Standard -4 | Standard | 26,79 | | 0,3125 | 31638431486266 | 6,1% | | | | |
| 9 | | Standard -5 | Standard | 28,65 | | 0,078125 | 9792347051E-02 | 11,4% | 28,63 | 0,03 | | 4 |
| 10 | | Standard -5 | Standard | 28,61 | | 0,078125 | 0371360603E-02 | 15,2% | | | | |
| 11 | | Standard -6 | Standard | 30,91 | | 0,01953125 | 7080330555E-02 | 11,2% | 30,73 | 0,25 | | E |
| 12 | | Standard -6 | Standard | 30,55 | | 0,01953125 | 8196280444E-02 | 14,7% | | | | |
| 13 | | Standard -7 | Standard | 32,61 | | 0,0048828125 | 0912099592E-03 | 5,0% | 32,82 | 0,29 | | E |
| 14 | | Standard -7 | Standard | 33,02 | | 0,0048828125 | 6976461383E-03 | 22,1% | | | | |
| 15 | | NTC | NTC | | | | | | | | | |
| 16 | | NTC | NTC | | | | | | | | | |
| 17 | | 1 | Unknown | 26,93 | | | 39632206649786 | | 26,93 | | | 3 |
| 18 | | 2 | Unknown | 26,95 | | | 35823484969928 | | 26,95 | | | 3 |
| 19 | | 3 | Unknown | 27,11 | | | 33202055622855 | | 27,11 | | | 3 |
| 20 | | 4 | Unknown | 27,10 | | | 35230084114815 | | 27,10 | | | 3 |
| 21 | | 5 | Unknown | 27,18 | | | 30072480612051 | | 27,18 | | | 3 |
| 22 | | 6 | Unknown | 27,25 | | | 38613205678687 | | 27,25 | | | 3 |
| 23 | | 7 | Unknown | 27,37 | | | 18733216020032 | | 27,37 | | | I |
| 24 | | 8 | Unknown | 27,21 | | | 14536209330357 | | 27,21 | | | 1 |

**The "Quantitation Results – Cycling A. Red" window.**

The "Quantitation Results — Cycling A. Yellow" window displays data for selected wells and summarizes the $C_T$ values for the Internal Control.

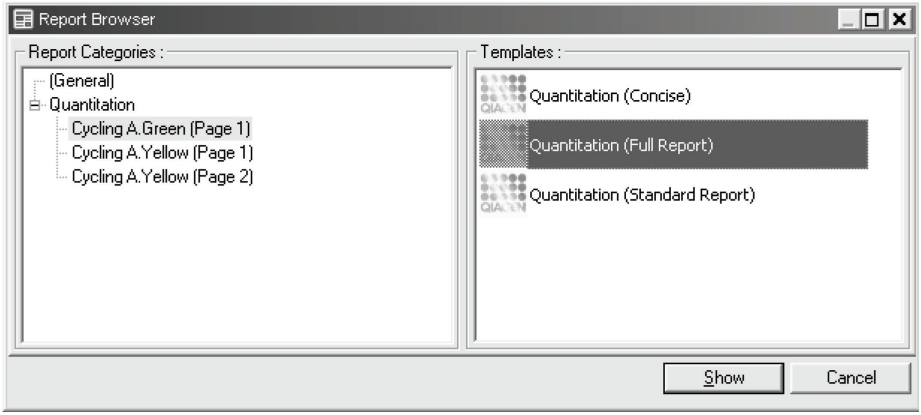


Quant. Results - Cycling A.Yellow (Page 1)

| No. | C | Name | Type | Ct | Ct Comment | Given Conc (ng/ | Calc Conc (ng/ul | % Var | Rep. Ct | Rep. Ct Stc | Rep. Ct (95% CI) | F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | Standard -1 | Unknown | 32,52 | | | | | 32,72 | 0,28 | | |
| 2 | | Standard -1 | Unknown | 32,91 | | | | | | | | |
| 3 | | Standard -2 | Unknown | 32,55 | | | | | 31,90 | 0,92 | | |
| 4 | | Standard -2 | Unknown | 31,25 | | | | | | | | |
| 5 | | Standard -3 | Unknown | 32,23 | | | | | 32,20 | 0,05 | | |
| 6 | | Standard -3 | Unknown | 32,16 | | | | | | | | |
| 7 | | Standard -4 | Unknown | 32,36 | | | | | 32,41 | 0,07 | | |
| 8 | | Standard -4 | Unknown | 32,46 | | | | | | | | |
| 9 | | Standard -5 | Unknown | 32,31 | | | | | 32,29 | 0,03 | | |
| 10 | | Standard -5 | Unknown | 32,28 | | | | | | | | |
| 11 | | Standard -6 | Unknown | 32,66 | | | | | 32,60 | 0,07 | | |
| 12 | | Standard -6 | Unknown | 32,55 | | | | | | | | |
| 13 | | Standard -7 | Unknown | 32,59 | | | | | 32,52 | 0,09 | | |
| 14 | | Standard -7 | Unknown | 32,46 | | | | | | | | |
| 15 | | NTC | Unknown | 32,69 | | | | | 32,47 | 0,32 | | |
| 16 | | NTC | Unknown | 32,24 | | | | | | | | |
| 17 | | 1 | Unknown | 32,17 | | | | | 32,17 | | | |
| 18 | | 2 | Unknown | 32,08 | | | | | 32,08 | | | |
| 19 | | 3 | Unknown | 32,15 | | | | | 32,15 | | | |
| 20 | | 4 | Unknown | 32,26 | | | | | 32,26 | | | |
| 21 | | 5 | Unknown | 31,81 | | | | | 31,81 | | | |
| 22 | | 6 | Unknown | 31,59 | | | | | 31,59 | | | |
| 23 | | 7 | Unknown | 31,92 | | | | | 31,92 | | | |
| 24 | | 8 | Unknown | 32,24 | | | | | 32,24 | | | |

**The "Quantitation Results — Cycling A. Yellow" window.**

23. To export the results to Excel, go to File, followed by “Save As” and then “Excel Analysis Sheet.” The results will be saved in *.csv format. To export a full report, go to “File,” followed by “Reports” and then “Quantitation.”




24. To interpret the results, see “Interpretation of Results” on page 73.

# 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 HYres 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 sharing

- 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.
- With Investigator Quantiplex HYres, the new 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, FAM, and Cy5 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 20 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 8. 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.

   Please see Table 8 for complete concentration information for the standard curve dilutions.

**Table 8. 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 81.

3. Thaw the template nucleic acids.

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

4. Prepare a master mix according to Table 9.
   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 9. Master mix for DNA qualification**

| Component | Volume per 20 µl reaction | Final concentration |
|---|---|---|
| Reaction Mix YQ | 9 µl | 1x |
| Primer Mix IC YQ | 9 µl | 1x |
| **Total volume of master mix** | **18 µl** | **–** |

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

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

   Mix carefully in order to avoid localized concentrations of salt.

   Table 10 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.

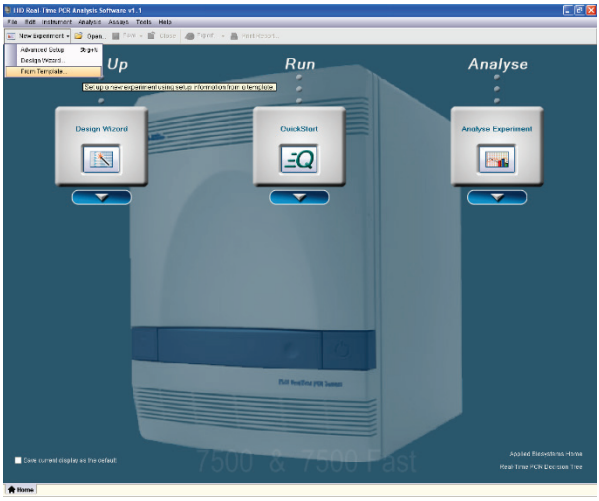
**Table 10. Possible plate setup of reactions on the Applied Biosystems 7500 Real-Time PCR System for Human Identification**

| 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.

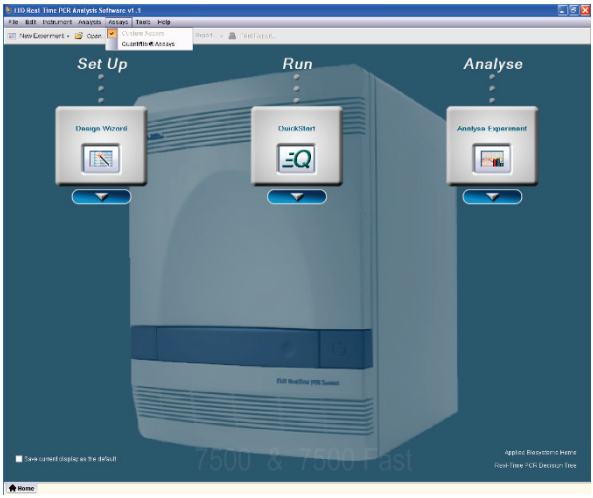
8. Open the HID Real-Time PCR Analysis Software v1.1 or v1.2 in the Custom Assays mode.
9. If you are using a template file, then select "New Experiment From Template" and proceed to step 13 to assign the Targets to the Plate Layout. Then proceed to step 17 to save and start the run.

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

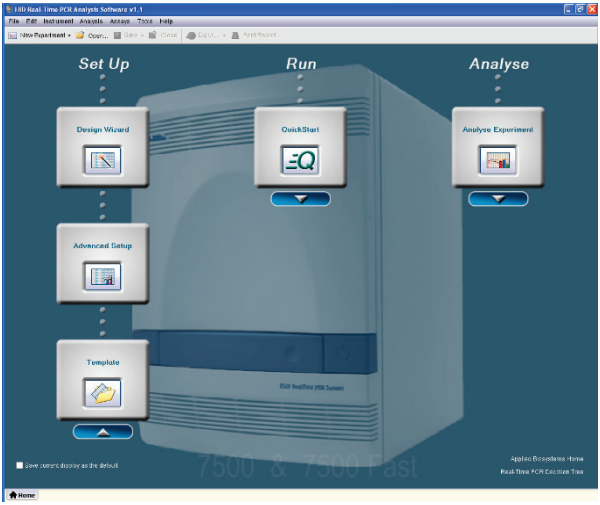



**Starting a new experiment from a template.**

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




**Starting a new experiment.**




**Starting a new experiment using the advanced setup.**

11. Once the window opens, enter a new Experiment Name in the appropriate field.

    Select the following settings:

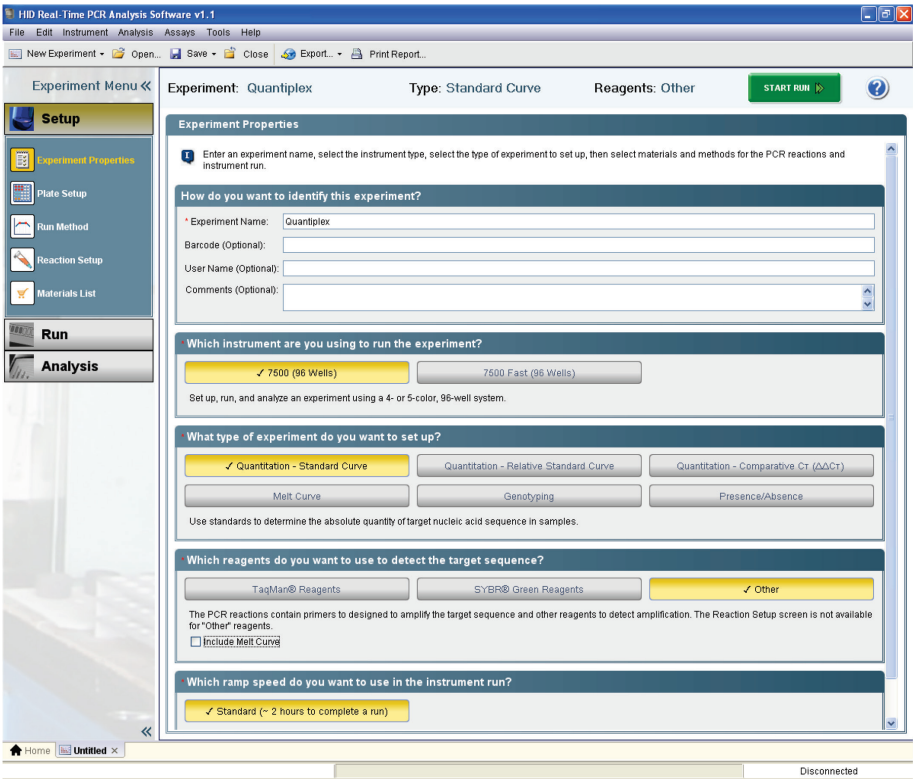
    **Instrument**: 7500 (96 Wells)

    **Experiment Type**: Quantitation — Standard curve

    **Reagents**: Other

    **Ramp Speed**: Standard

    Unselect the "Include Melt Curve" option.



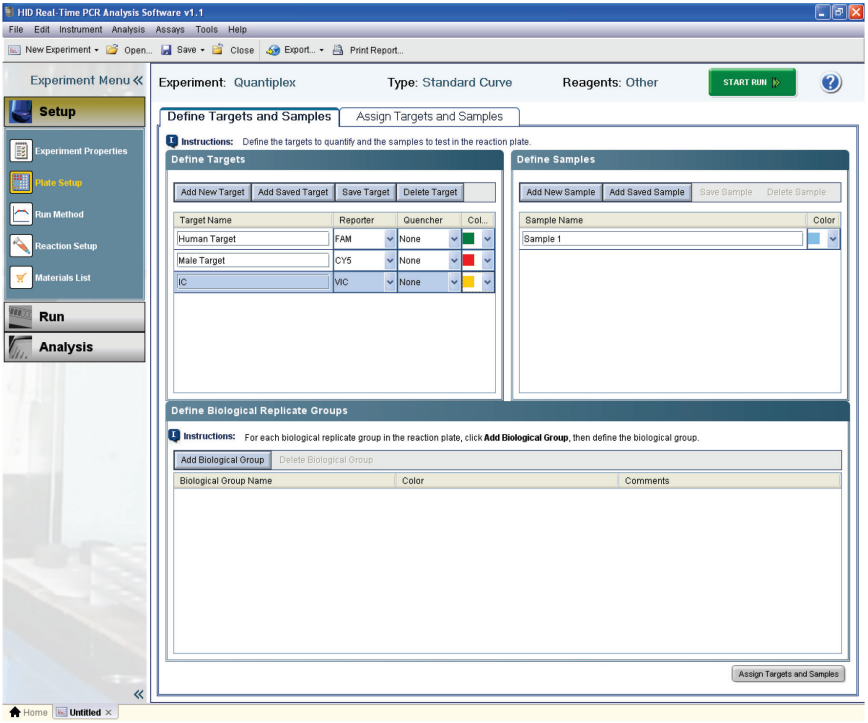

12. Click on Plate Setup and define three Targets by clicking twice on "Add New Target."

Select the following settings:

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

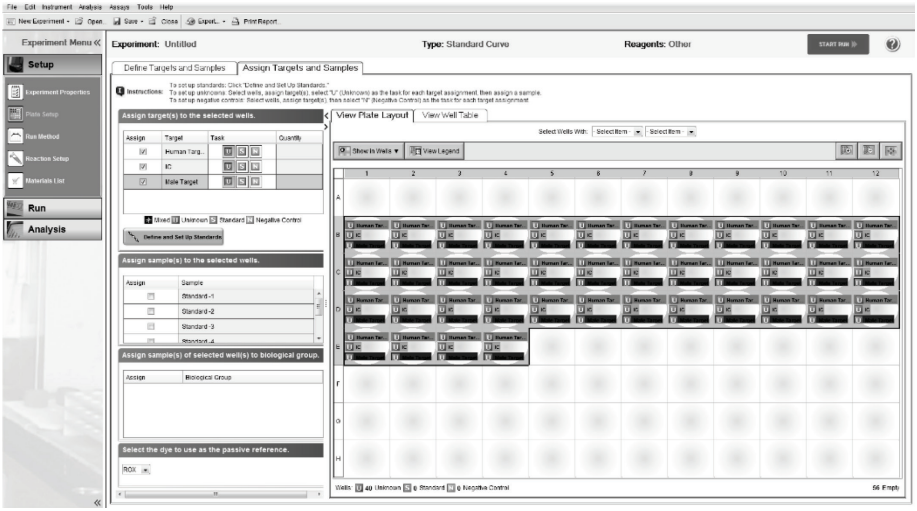
**Male Target**: Reporter Cy5, **Quencher**: None

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




13. Define the Sample names using the "Define Samples" tool on the right panel.
14. Switch to the tab "Assign Targets and Samples." 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.




**Assigning the targets.**

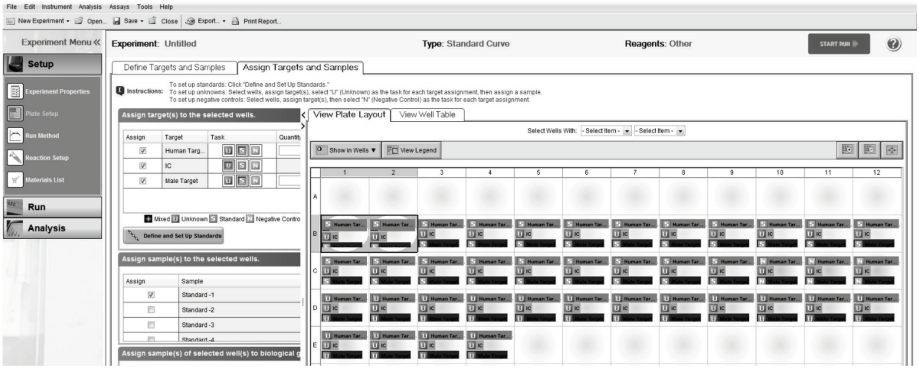
15. Select the wells for the No-Template Controls and flag them as "Negative Control" using the red "N" button.

    **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.




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

**Table 11. Possible plate setup of reactions on the Applied Biosystems 7500 Real-Time PCR System for Human Identification**

| Standard | Human Target (FAM) | Male Target (Cy5) |
|---|---|---|
| Standard -1 | 20 | 20 |
| Standard -2 | 5 | 5 |
| Standard -3 | 1.25 | 1.25 |
| Standard -4 | 0.3125 | 0.3125 |
| Standard -5 | 0.078125 | 0.078125 |
| Standard -6 | 0.01953125 | 0.01953125 |
| Standard -7 | 0.0048828125 | 0.0048828125 |

**Note**: Alternative standard curves are listed in "Appendix: Alternative Standard Curves," page 81.

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 12.

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

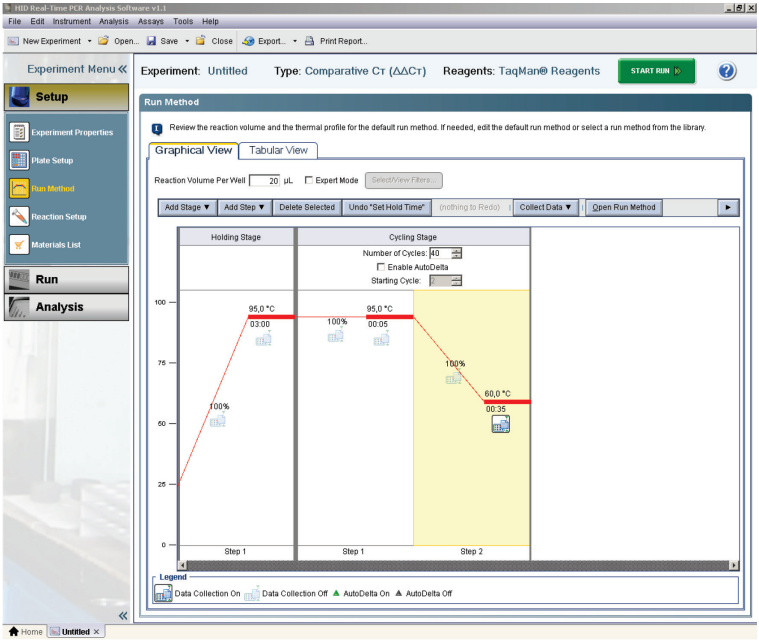
    Two-step cycling PCR requires 40 cycles. Each cycle comprises 2 steps: 95°C for 5 s (denaturation step) and 60°C for 35 s (annealing/extension step).

**Table 12. Cycling conditions for the Applied Biosystems 7500 Real Time PCR System for Human Identification**

| Step | Temperature | Time | Number of cycles | Comment |
|---|---|---|---|---|
| **Initial 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 12. Change the sample volume to 20 µl.

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




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

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 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.

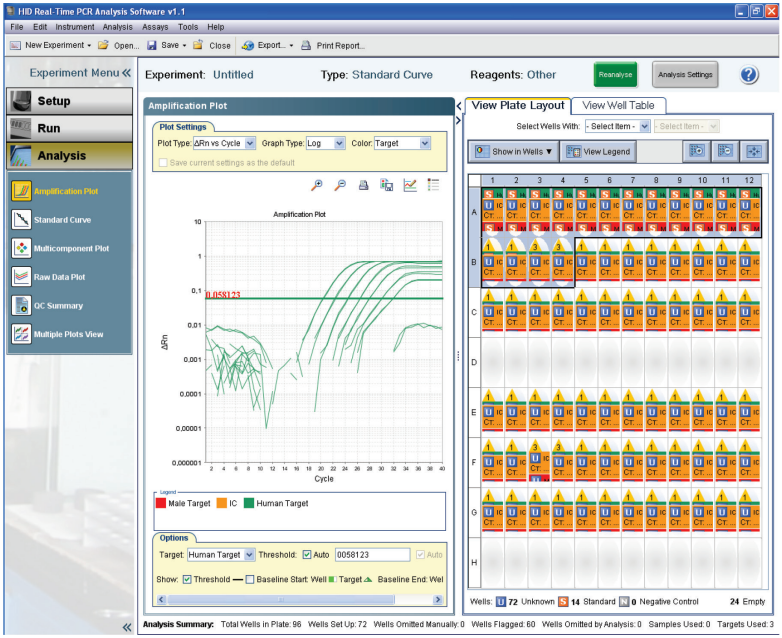
### Procedure

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.

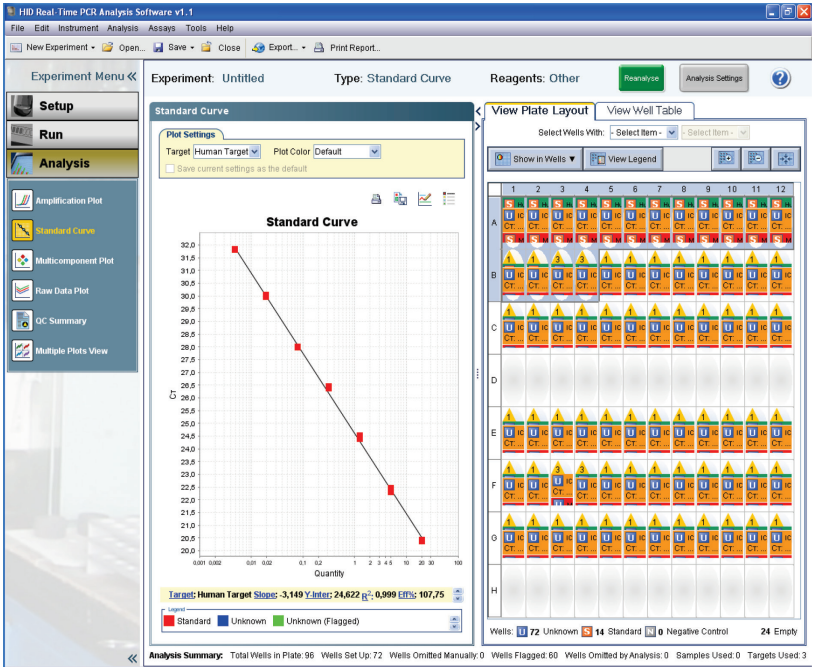
   **Note**: Leave the IC (VIC) Task for standard reactions set to "Unknown." Enter the sample name (e.g., Standard -1).
4. In the "Amplification Plot" tab (found in the "Analysis" tab), select the appropriate samples in the table below the amplification plot. Choose "Auto $C_T$" for both channels and click "Analyze."

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




**Sample analysis for the FAM, Cy5, 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.

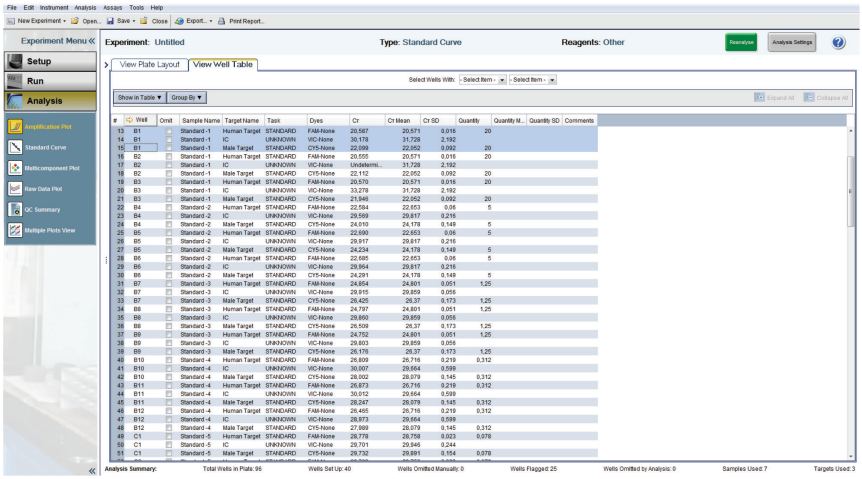



**Standard curve.**

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 Male Target shows the quantity of male DNA present, with the same units as used for the standards.

   The IC Target shows the $C_T$ value for the Internal Control.




**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 73.

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

This protocol is optimized for use of the Investigator Quantiplex HYres 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.
- With Investigator Quantiplex HYres, the new 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, FAM, and Cy5 before starting. Please see the instrument's user manual for the correct setup.
- Use the cycling conditions specified in the protocol. The cycling is optimized for this assay.
- Use the template volume specified in the protocol. The reaction is optimized for use with 2 µl template DNA. Do not use more or less than 2 µl per 20 µl reaction.
- Dilutions of DNA quantification standards in QuantiTect Nucleic Acid Dilution Buffer can be stored at between +2 and +8°C for at least 1 week.
- Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

## Procedure

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 13. 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 cross-contaminate.

   Please see Table 13 for complete concentration information for the standard curve dilutions.

**Table 13. 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 81.

3. Thaw the template nucleic acids.

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

4. Prepare a master mix according to Table 14.
   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 14. Master mix for DNA quantification**

| Component | Volume per 20 µl reaction | Final concentration |
|---|---|---|
| Reaction Mix YQ | 9 µl | 1x |
| Primer Mix IC YQ | 9 µl | 1x |
| **Total volume of master mix** | **18 µl** | **–** |

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

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

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

   Table 15 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 15. Possible plate setup of reactions on the Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems**

| Well contents | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 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 HYres product page, then select the following in the "New Document Wizard" window.

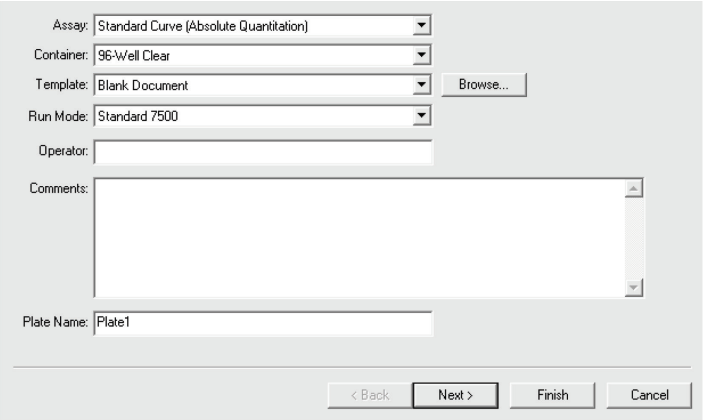
   **Assay**: Standard Curve (Absolute Quantification)

   **Container**: 96-Well Clear

   **Template**: Blank Document

   **Run Mode for the 7500 Real-Time PCR System**: Standard 7500

   **Run Mode for the 7500 Fast Real-Time PCR System**: Standard 7500




**Creating a new document.**

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

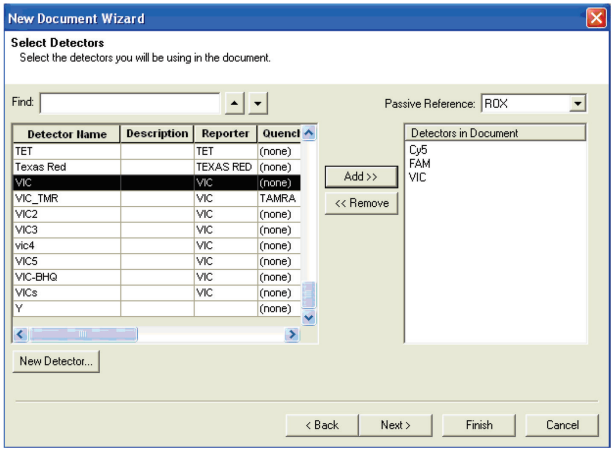
**Assay**: Standard Curve (Absolute Quantification)Container: 96-Well Clear

**Template**: Investigator_Quantiplex HYres_Template_SDS1.4.SDT

**Run Mode for the 7500 Real-Time PCR System**: Standard 7500

**Run Mode for the 7500 Fast Real-Time PCR System**: Standard 7500

9. Add the detectors FAM, VIC, and Cy5 to the document. Click "Next" to continue.




**Selecting the detectors.**

10. Select the wells in use and check the box for all three 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 and the Cy5 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 according to Table 16.

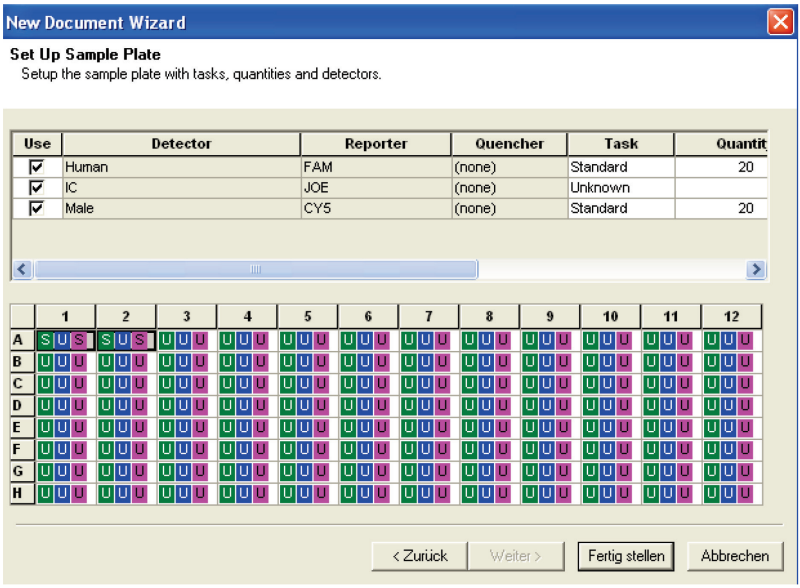
    **Important**: Although units are not entered for Quantity, a common unit must be used for all standard quantities (e.g., ng/µl). The units used for standard quantities define the quantification units for the analysis of results.

    **Note**: Leave the VIC detector Task for standard reactions set to "Unknown." Enter the sample name (e.g., Standard -1).

**Table 16. DNA concentrations of the human and male standards**

| Standard | Human Target (FAM) | Male Target (Cy5) |
|---|---|---|
| Standard -1 | 20 | 20 |
| Standard -2 | 5 | 5 |
| Standard -3 | 1.25 | 1.25 |
| Standard -4 | 0.3125 | 0.3125 |
| Standard -5 | 0.078125 | 0.078125 |
| Standard -6 | 0.01953125 | 0.01953125 |
| Standard -7 | 0.0048828125 | 0.0048828125 |

**Note**: Alternative standard curves are listed in "Appendix: Alternative Standard Curves," page 81.




**Setting up the sample plate.**

12. Click “Finish.” To program the cycler according to Table 17, click on the “Instrument” tab.

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

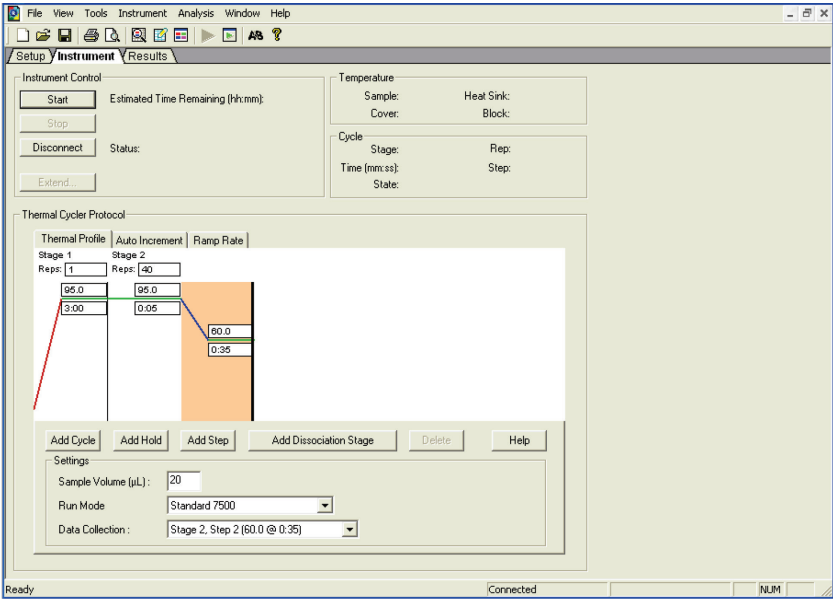
    Two-step cycling PCR requires 40 cycles. Each cycle consists of 2 steps: 95°C for 5 s (denaturation step) and 60°C for 35 s (annealing/extension step).

**Table 17. Cycling conditions for Applied Biosystems 7500 Real-Time PCR System**

| Step | Temperature | Time | Number of cycles | Comment |
|---|---|---|---|---|
| **Initial 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. |

13. 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 17. Be sure to change the Sample volume to 20 µl and select the correct 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.”




**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

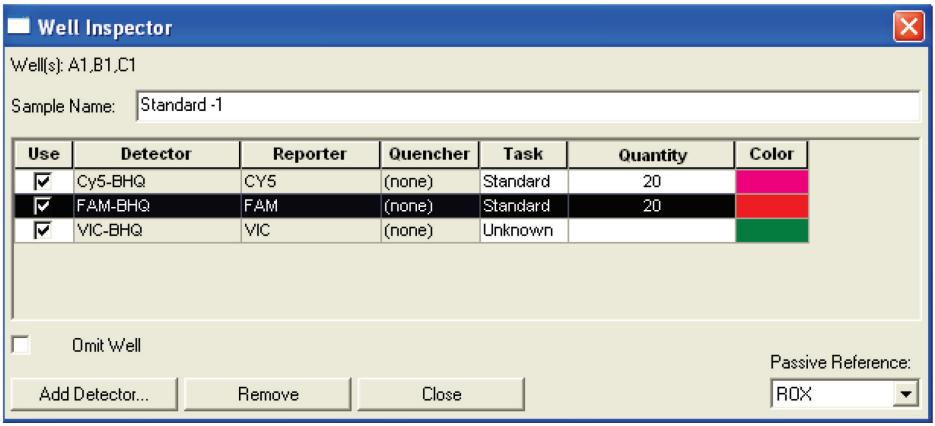
Optimal analysis settings are a prerequisite for accurate quantification data. Readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

## 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 and the Cy5 channel as "Standard" and enter the quantity of DNA in the well according to Table 16 (page 65).

   **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.

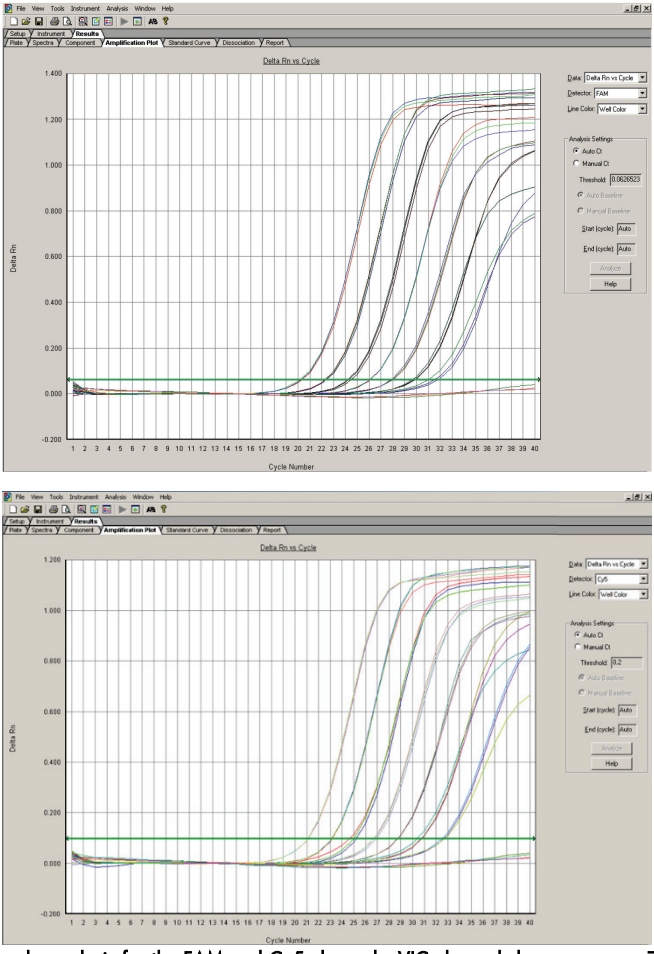
   **Note**: Leave the VIC detector Task for standard reactions set to "Unknown." Quantity values are not needed for the internal control. Enter the sample name (e.g., Standard -1).



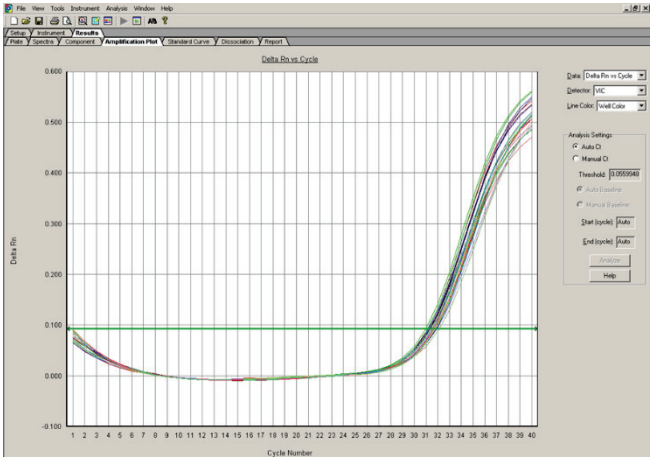

**Well inspector.**

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 all three channels and press "Analyze."

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

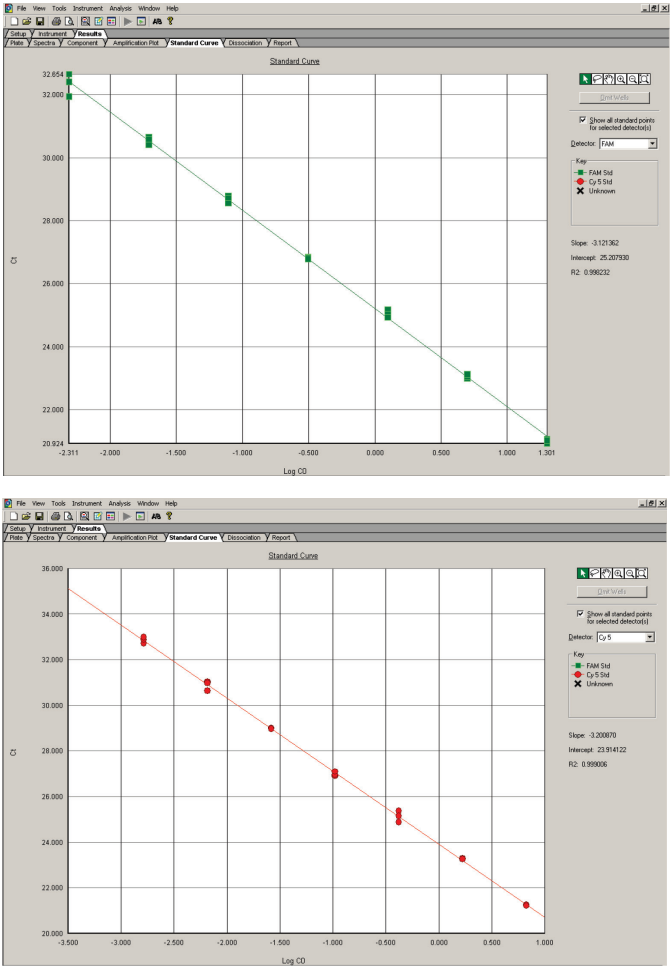



**Sample analysis for the FAM and Cy5 channels. VIC channel shown on page 70.**




Sample analysis for the VIC channel. FAM and Cy5 channels shown on page 69.

5. To view the standard curves, select the "Standard Curve" tab (found in the "Results" tab). View the $C_T$ values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and $R^2$ values for both the FAM and the Cy5 channels.

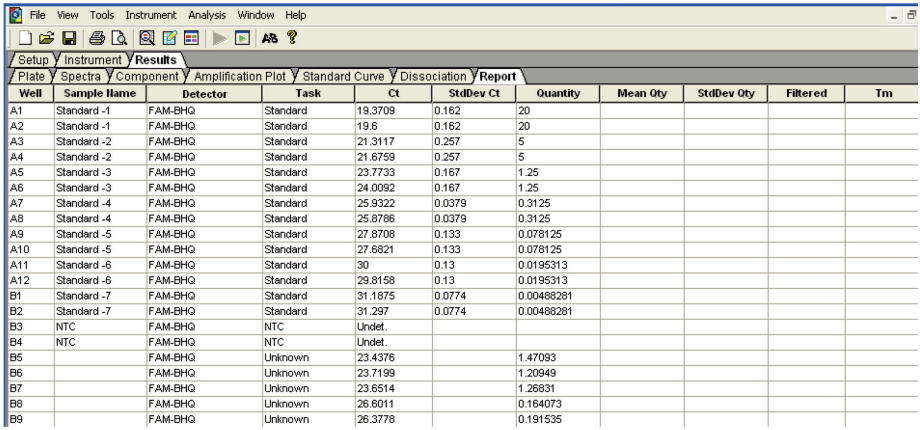



**Standard curve.**

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 total human DNA present, while the Cy5 detector shows the quantity of male DNA present. The quantity is shown with the same unit as used for the standards (i.e., if ng/µl was used for the definition of the standards, then the quantities for the unknowns will be reported in ng/µl).

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


File View Tools Instrument Analysis Window Help

Setup | Instrument | Results

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

| 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 73.

# 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 the Investigator Quantiplex HYres Kit, and may need to be adjusted.

For DNA quantification using the Investigator Quantiplex HYres 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. If the values fall outside of this range, see the Troubleshooting Guide, page 76, 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 ($R^2$ ≤0.98) may occur for many different reasons. In the case of low $R^2$ values, see the Troubleshooting Guide, page 76, 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 (this value is cycler specific and has to be validated in your laboratory). 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, mostly in the higher concentration samples.

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 18 may arise.

**Table 18. 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 |

* This value is indicative and has to be validated within your laboratory.

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

## Quantification of unknowns

The Investigator Quantiplex HYres 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 protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

| Comments and suggestions | |
|---|---|
| **No signal, or one or more signals detected late in PCR (Stylesheet)** | |
| 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.<br>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. |
| 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 cycling conditions | Always start with the optimized cycling conditions specified in the protocols. Ensure that the cycling conditions include the initial step for activation of the DNA Polymerase, and the specified times for denaturation and annealing/extension. |
| Analysis settings (e.g., threshold and baseline settings) not optimal | Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye. |

**No linearity in ratio of $C_T$ value/crossing point to log of the template amount**

| | |
|---|---|
| Amount of template in unknown sample too high | Linearity is guaranteed within the range of the standard curve. 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).<br>Repeat the assay using new components. |
| Minimal probe degradation, leading to sliding increase in fluorescence | Check the amplification plots, and adjust the threshold settings. |
| 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.<br>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. |
| Problem with dilution of standards | Ensure that the DNA standard is completely thawed and mixed thoroughly before use.<br>Ensure dilutions of the DNA standard are mixed thoroughly before removing each aliquot for the serial dilution.<br>Do not use a sample volume other than 2 µl.<br>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

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 19. 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 20. 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 HYres Kit (200) | Reaction Mix YQ, Primer Mix IC YQ, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer | 387116 |
| **Related Products** | | |
| Investigator Quantiplex Kit (200) | Reaction Mix YQ, Primer Mix IC FQ, Male Control DNA M1, QuantiTect Nucleic Acid Dilution Buffer | 387016 |
| Investigator Quantiplex Pro Kit (200) | Quantiplex Pro Reaction Mix, Quantiplex Pro Primer Mix, Male Control DNA M1, QT 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 | 383213 |

* Larger kit sizes available; please inquire.

| | | |
|---|---|---|
| | ladder, 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 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<br>06/2018 | Replacement of Control DNA1 Z1 with Male Control DNA M1 and updated tables for standard DNA dilution series and DNA concentrations. PCR cycling parameters for Rotor-Gene Q updated. Alternative Standard curves added on page 81. |
| R5<br>12/2015 | Male DNA values changed for Control DNA and standard curves. Updated figures and tables. Minor text modifications. |
| R4<br>11/2014 | Replacement of Reaction Mix FQ with Reaction Mix YQ. Content updates for quantification protocols and data/results interpretation. Updated figures and tables. Minor text modifications. |

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