DNA: Quantifiler Trio Quantitation

Introduction

QAS 9.4

Real-time quantitative PCR is defined as the detection of products generated at each cycle of PCR so that the accumulation of PCR products from an unknown sample can be compared to the corresponding accumulation for a series of standard samples containing known quantities of template DNA. Through this comparison, real-time qPCR is used to estimate the initial quantity of specific template DNA in the unknown sample.

The Quantifiler® Trio DNA Quantification Kit is designed to quantify both human DNA and human male DNA simultaneously, in a single sample. The results from using the kit can aid in determining:

- if sufficient human DNA or human male DNA is present to proceed with short tandem repeat (STR) analysis
- how much sample to use in STR analysis applications
- DNA quality (inhibition/degradation)

NOTE: DNA extracts that have been previously quantitated and require concentration before amplification do not need to be re-analyzed with the Quantifiler® Trio DNA Quantification Kit.

Chemical hazard

Quantifiler® Trio PCR Reaction Mix may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled.

Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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Materials and reagents

The following materials and reagents are used in this procedure:

- Quantifiler® Trio DNA Quantification Kit
- pipettors
- barrier pipette tips
- 96-well reaction plate
- sterile microcentrifuge tubes
- Optical Adhesive Cover
- Quantifiler® Trio THP DNA dilution buffer
- extracted DNA
- centrifuge
- vortex

Plate document set-up

The template for the *DNA Quantitation Load Sheet* is located in DNA Manager on the S drive.

- After filling out the load sheet (run samples in duplicate when appropriate and name the duplicate samples in the same manner), click the *Quant File* tab.
- Select the entire sheet and *Copy*.
- Open Notepad and *Paste*.
- Save as a ".txt" file on a removable drive.
- Turn on the ABI Prism 7500 System and allow to warm up for approximately 15 minutes before beginning the run. While the instrument is warming up, prepare the plate and set up the appropriate plate record. The plate record can be imported using the *Import* tab in the *File* menu.

Alternatively, the plate record information can be manually entered directly onto the 7500 computer.

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Preparing the DNA quantitation standard

QAS 9.5.1

Applied Biosystems recommends a 10-fold dilution series with five concentration points in the standard series for each assay and a minimum input volume of 10 µl DNA for dilutions (to ensure accuracy in pipetting). The Alternate Dilution Series that enables saving of Quantifiler® THP DNA dilution buffer is also acceptable.

Standard Dilution Series for Quantifiler Trio:

Standard	Conc. (ng/µl)	Amounts
Std 1	50.00	10 µl (100 ng/µl stock) + 10 µl Quantifiler® THP DNA dilution buffer
Std 2	5.000	10 µl (Std 1) + 90 µl Quantifiler® THP DNA dilution buffer
Std 3	0.500	10 µl (Std 2) + 90 µl Quantifiler® THP DNA dilution buffer
Std 4	0.050	10 µl (Std 3) + 90 µl Quantifiler® THP DNA dilution buffer
Std 5	0.005	10 µl (Std 4) + 90 µl Quantifiler® THP DNA dilution buffer

Alternate Dilution Series for Quantifiler Trio:

Standard	Conc. (ng/µl)	Amounts
Std 1	50.00	10 µl (100 ng/µl stock) + 10 µl Quantifiler® THP DNA dilution buffer
Std 2	5.00	2 µl (Std 1) + 18 µl Quantifiler® THP DNA dilution buffer
Std 3	0.500	2 µl (Std 2) + 18 µl Quantifiler® THP DNA dilution buffer
Std 4	0.050	2 µl (Std 3) + 18 µl Quantifiler® THP DNA dilution buffer
Std 5	0.005	2 µl (Std 4) + 18 µl Quantifiler® THP DNA dilution buffer

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Preparing DNA standard dilution series

To prepare the DNA standard dilution series:

Step	Action
1	If unopened, thaw the kit completely. (Open stock is stored in the refrigerator.)
2	Label five sterile microcentrifuge tubes with the appropriate standard (1-5 or A-E).
3	Dispense the required amount of dilution buffer from the kit for each tube.
	Prepare Standard 1(A).
4	Vortex the DNA Standard 3 to 5 seconds.
5	Using a new pipette tip, add 10µl of the standard to the first tube.
6	Mix the dilution thoroughly.
	Prepare Standards 2-5 (B-E).
7	Using a new pipette tip, add 10µl (or 2µl for alternate preparation) of the prepared standard to the tube for the next standard.
8	Mix thoroughly.
9	Repeat steps until you complete the dilution series.

Preparing reactions

To prepare the reactions:

Step	Action
1	Remove the kit from the refrigerator.
2	Gently vortex the PCR Reaction Mix and Primer Mix.
3	Pipet 10.0 µL of the PCR Reaction Mix and 8.0 µL of the Primer Mix into a sterile microcentrifuge tube per sample. Prepare reaction mix for number of samples + 15%.
4	Vortex for 3 to 5 seconds and centrifuge briefly.
5	Dispense 18 µl of the mix into each reaction well of the plate.
6	Add 2 µl of standard, sample, or NTC control (the DNA dilution buffer) to the appropriate wells.
7	Seal the reaction plate with the Optical Adhesive Cover (minimize contact with the cover).
8	Centrifuge the plate for at least 20 seconds.

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DNA quantitation

This is the procedure for the quantitation of DNA using the ABI 7500 Real-Time PCR Quantification System

Step	Action
1	Turn on ABI 7500 Real-Time PCR Quantification System instrument. The instrument may be turned on 15 minutes prior to the run to allow it to warm up.
2	Open the instrument tray and place the plate in the instrument thermal block so that well A1 is in the upper left corner.
3	Close the instrument tray.
4	Open the ABI Prism HID Real-Time PCR Analysis Software. Login in as "Admin." When the software is open, select the Quantifiler® Trio assay. A new experiment can also be opened from the **File Menu** by clicking **New Experiment**.
5	Under **File**, click on **Import** and select the plate setup file from the portable drive. Press **Start Import** and then select **YES**.
6	Under **Setup**, select the **Experiment Properties** tab. Enter run name into **Experiment Name** field.
7	Under **Setup**, select the **Plate Setup** tab and select the **Assign Targets and Samples** tab. Highlight empty wells (if appropriate) and right click to choose **Clear**. This step can also be done after the run is complete.
8	Under **Setup**, select the **Run Method** tab. The "Reaction Volume per Well" should indicate 20 µl and the Thermal Profile should indicate the thermal cycler parameters listed below. Hold: 95°C, 2 minutes 40 cycles: 95°C, 9 seconds 60°C, 30 seconds
9	Save plate as an ".eds" file. The plate name can only contain letters and numbers.
10	Click **Start** to begin the run.

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Analyzing plate document

This is the procedure to analyze a plate document:

Step	Action
1	Open the plate document to analyze.
2	Verify the analysis settings: • Select Analysis>Analysis Settings>C_T Settings to open the Analysis Settings dialog box. • Verify the C_T settings for the three targets: Large Autosomal/ Small Autosomal/Y are as follows: – Threshold: 0.2 – Baseline Start (cycle): 3 – Baseline End (cycle): 15 • Verify the C_T settings for T. IPC are as follows: – Threshold: 0.1 – Baseline Start (cycle): 3 – Baseline End (cycle): 15 Click **Cancel** or close the window.

Analyzing results

Analyzing the results of data analysis can involve one or more of the following:

- viewing the Standard Curve
- viewing the Amplification Plot
- viewing the Report
- evaluating the IPC (Internal PCR control) for signs of sample inhibition
- evaluating the Quality Index (degradation index and IPCC_T flag)

Viewing the standard curve

To view the standard curve:

Step	Action
1	In the **Analysis** tab, select the **Standard Curve** tab.
2	Under the **Target** tab you can choose to view each curve individually or in the same graph.
3	View the C_T values for the quantification standard reactions and the calculated regression line, slope, y-intercept, and R^2 values.

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About the standard curve results

The standard curve is a graph of the C_T of quantification standard reactions plotted against the starting quantity of the standards. The C_T is the intersection between the amplification curve and a threshold line which provides a relative measure of the concentration of target in the PCR reaction. The *HID Real-Time PCR Analysis Software* calculates the regression by calculating the best fit line with the quantification standard data points.

- R^2 Value: A value of **≥ 0.99** indicates a close fit between the regression line and the data points. If the value is **<0.98,** check the following:
  – values entered for quantification standards during plate document setup
  – preparation of quantification standard dilution series
  – loading of reactions for quantification standards
  – failure of reactions containing quantification standards

- Slope: The slope indicates the PCR amplification efficiency for the assay. The range and average of standard curve slope values are depicted in the table below. If the slope varies beyond the 'typical range,' check the following:
  – assay setup
  – software setup
  – reagents
  – instrument

Quantifiler® Trio Targets	Typical Slope (range)	Average Slope
Small Autosomal (SA)	-3.0 to -3.6	-3.3
Large Autosomal (LA)	-3.1 to -3.7	-3.4
Y Target (Y)	-3.0 to -3.6	-3.3

- Y-intercept: The Y-intercept indicates the expected C_T value for a sample with a quantity of 1.0 ng/µL. The C_T value can and should be monitored as an indicator of the quality of the standard curve.

  The Y-intercept can be affected by:
  – Minor lot-to-lot variations
  – Target-to-target variation (Y-intercept for LA < SA or Y Target)
  – Instrument-to-instrument variation

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About the standard curve results (continued)

- IPC C_T: In the presence of higher concentrations of human genomic DNA (≥ 5 ng/µL), competition between human and male-specific IPC PCR reactions may suppress IPC amplification resulting in an increased value for the IPC C_T.

- If the R^2 and slope are as expected but the reported C_T value has shifted, the analyst can adjust the amplification target concentration. When the C_T value is lower than expected, the target amplification concentration can be decreased; when the C_T value is elevated, the target amplification concentration can be increased.

Viewing the report

The report summarizes the quantity and quality of DNA present in the samples. To view the report:

Step	Action
1	Highlight unknown samples and both NTCs.
2	Select the **Export** tab for results in an Excel document or the **Print Report** tab for a PDF file.
3	View the **Quantity** column to determine the quantity of Y, small autosomal, and large autosomal in each sample.
4	Evaluate the C_T value of the IPC for each sample.
5	Use the quantitation results to proceed to the appropriate STR amplification procedure.

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Evaluating the Internal PCR control system (IPC)

Various compounds may co-extract with DNA in forensic samples and cause PCR inhibition. These PCR inhibitors can interfere with amplification and cause varying levels of reduced PCR efficiency, including complete inhibition of PCR.

The IPC system can distinguish between true negative sample results and reactions affected by the presence of PCR inhibitors. If partial or complete inhibition is noted, the following steps may be taken (if possible):

– samples can be diluted and re-quantitated
– the extraction may be repeated
– additional clean-up of the sample may be performed

- Normal Range for IPC: The range of C_T values for the IPC can be examined by viewing the JUN dye signal in the amplification plots for the quantification standards. Because the IPC system template DNA is added to the reaction at a fixed concentration, the JUN C_T value can range from 20 to 30.
- True Negative Results: With a true negative result, the VIC, ABY, and FAM dye signals indicates that the human-specific target failed to amplify and the JUN dye signal (JUN C_T value between 20 and 40) indicates that the IPC target was amplified. No PCR inhibition.
- Invalid IPC Results: If the target DNA and the IPC target failed to amplify, it is not possible to distinguish between the absence of DNA and PCR inhibition.
- Partial PCR Inhibition: Weak amplification (high C_T value and low ΔR_n value) of the human target and no amplification of the IPC may indicate partial PCR inhibition in the sample.
- Disregarding IPC Results: With extremely high concentrations of human genomic DNA (>5 ng/µl), competition between the human-specific and IPC PCR reactions appears to suppress IPC amplification for that sample, and it is unlikely that PCR inhibitors are present. In these cases, appearance of suppression or failure of IPC amplification can be disregarded.

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Quality Index

The overall quality of a sample can be calculated by evaluating results for the Degradation Index and IPCC$_T$ flag.

- Degradation index: Can indicate whether large DNA fragments may perform more poorly relative to small DNA fragments in a sample. The Degradation Index is automatically calculated by the software and can be displayed in the Well Table view in any of the analysis screens. The degradation index is calculated as follows:

$$\frac{\text{Small autosomal target DNA concentration (ng/uL)}}{\text{Large autosomal target DNA concentration (ng/uL)}}$$

- IPCC$_T$ flag: This quality flag will trigger when the IPC C$_T$ is undetermined or greater than the average IPC C$_T$ for the standards plus the Variance threshold in the Analysis software's HID settings. The flag is displayed in Analysis QC Summary screen and the Plate View or Well Table view in any of the analysis screens.

In general, the Quality Index can be assessed by evaluating the Degradation Index and IPC C$_T$ flag results as follows:

IPCC$_T$ flag triggered?	Degradation Index	Interpretation
No	<1	DNA is not degraded/inhibited.
	1 – 10	DNA may be slightly to moderately degraded. PCR inhibition may be possible but not enough to significantly suppress IPC amplification.
	>10 or blank	DNA may be significantly degraded. PCR inhibition may be possible but not enough to significantly suppress IPC amplification.
Yes	<1	This result is unlikely because PCR inhibition concentration is high enough to trigger the IPCC$_T$ flag, the LA target would also be affected.
	>1 or blank	DNA may be affected by degradation and/or PCR inhibition.

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Assessing male/female mixture data

For samples containing a mixture of male and female DNA, the male to female ratio (M:F) can be calculated as follows:

$$1: \frac{\text{(small autosomal target quantity – Y target quantity)}}{\text{quantity of Y target}}$$

The M:F ratio is automatically calculated by the software. The analyst should use discretion in selecting an amplification kit. Sample analysis can end at quantitation if:

- a male Fusion 6C profile is being sought and the male concentration is <0.0004 ng/µl
- a male Fusion 6C profile is being sought and the M:F ratio >1:50
- a Y23 profile is being sought and the male concentration is <0.001 ng/µl

Preparing for STR amplification

After viewing quantitation results and assessing whether sufficient DNA or degraded/inhibited DNA is present in the samples, the analyst may proceed to STR amplification. Amplification using the Fusion 6C kit should be based on the Small Autosomal Target quantitation values. Amplification using the Y23 kit should be based on the Y Target quantitation values.