DNA: 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® Duo 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

Chemical hazard

Quantifiler® Duo 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.

Materials and reagents

The following materials and reagents are used in this procedure:

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

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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.

Preparing the DNA quantitation standard

QAS 9.5.1

Applied Biosystems recommends a three-fold dilution series with eight 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).

Standard Dilution Series for Quantifiler Duo:

Standard	Conc. (ng/µl)	Amounts
Std 1	50.00	10 µl (200 ng/µl stock) + 30 µl Quantifiler Duo dilution buffer
Std 2	16.700	10 µl (std 1) + 20 µl Quantifiler Duo dilution buffer
Std 3	5.560	10 µl (std 2) + 20 µl Quantifiler Duo dilution buffer
Std 4	1.850	10 µl (std 3) + 20 µl Quantifiler Duo dilution buffer
Std 5	0.620	10 µl (std 4) + 20 µl Quantifiler Duo dilution buffer
Std 6	0.210	10 µl (std 5) + 20 µl Quantifiler Duo dilution buffer
Std 7	0.068	10 µl (std 6) + 20 µl Quantifiler Duo dilution buffer
Std 8	0.023	10 µl (std 7) + 20 µl Quantifiler Duo dilution buffer

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Preparing DNA standard dilution series

To prepare the DNA standard dilution series:

Step	Action
1	Label eight sterile microcentrifuge tubes with the appropriate standard (1-8 or A-H).
2	Dispense the required amount of dilution buffer from the kit.
	Prepare Standard 1(A).
3	Vortex the DNA Standard 3 to 5 seconds.
4	Using a new pipette tip, add 10µl of the standard to the first tube.
5	Mix the dilution thoroughly.
	Prepare Standards 2 – 8 (B-H).
6	Using a new pipette tip, add 10µl of the prepared standard to the tube for the next standard.
7	Mix thoroughly.
8	Repeat steps until you complete the dilution series.

Preparing reactions

To prepare the reactions:

Step	Action
1	If unopened, thaw the kit completely. (Open stock is stored in the refrigerator.) Vortex primer mix 3 to 5 seconds and centrifuge briefly before opening the tube.
2	Swirl the PCR Reaction Mix gently. Do not vortex.
3	Pipet 10.5 µL of the Primer Mix and 12.5 µL of the PCR Reaction Mix into a sterile microcentrifuge tube per sample. Prepare reaction mix for n+3 samples.
4	Vortex for 3 to 5 seconds and centrifuge briefly.
5	Dispense 23µl 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	Open the instrument and place the plate in the instrument thermal block so that well A1 is in the upper left corner. NOTE: No compression pad is used with this instrument.
2	Close the instrument door.
3	Open the ABI Prism HID Real-Time PCR Analysis Software. When the software is open, select the Quantifiler Duo assay. A new experiment can also be opened from the *File Menu* by clicking *New Experiment*.
4	Under *File* click on *Import* and select the plate setup file from the portable drive, press *Start Import*, then select *YES.*
5	Highlight wells with no sample (if appropriate) and right click to choose *Clear*. This step can also be done after the run is complete.
6	Under *Setup* select the *Run Method* tab. The *sample volume field* should indicate 25µl and the *Thermal Profile* should indicate the thermal cycler parameters listed below. Hold: 50°C, 2 minutes Pre-heat: 95°C, 10 minutes 40 cycles: 95°C, 15 seconds 60°C, 1 minute
7	Save plate as an ".eds" file. The plate name can only contain letters and numbers.
8	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 settings are as shown below, then click OK. Threshold: 0.200000 Baseline Start (cycle): 3 Baseline End (cycle): 15

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

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

Kit	Typical Slope (range)	Average Slope
Quantifiler Duo	-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 differ slightly between kit lots and should be monitored as an indicator of the quality of the standard curve. An analyst should evaluate the Y-intercept value for a shift from the typical value. 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.

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Viewing the report

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

Step	Action
1	Highlight unknown samples and NTC.
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 male and total human DNA 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
- if performing Y-STR analysis, samples may be amplified using the modified Yfiler® PCR amplification procedure

- Normal Range for IPC: The range of C_T values for the IPC can be examined by viewing the NED 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 NED C_T value can range from 20 to 30.
- True Negative Results: With a true negative result, the VIC & FAM dye signal indicates that the human-specific target failed to amplify and the NED dye signal (NED C_T value between 20 and 30) 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 (>10ng/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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Assessing male/female mixture data

For samples containing a mixture of male and female DNA, the male contribution to the mixture can be calculated as a percentage by dividing the reported male DNA concentration by the human DNA concentration. The following are guidelines for choosing an amplification strategy:

- When the male contribution is greater than 10% (1:9) of the total human DNA concentration, the Identifiler® Plus kit is recommended for amplification.
- When the male contribution is less than 10% (1:9) of the total human DNA concentration and there is enough DNA extract to amplify both Identifiler® Plus and Yfiler®, Identifiler® Plus should be used first followed by Yfiler®, if necessary.
- When the male contribution is less than 10% (1:9) of the total human DNA concentration and there is not enough DNA extract to amplify both Identifiler® Plus and Yfiler®, the analyst should use discretion in selecting the amplification kit. The reported male concentration value may provide an expectation of whether a minor male contributor can be detected in an Identifiler® Plus mixture profile.

Preparing for STR amplification

After viewing quantitation results and assessing whether sufficient DNA or inhibited DNA is present in the samples, the analyst may proceed to STR amplification. Amplification using the Identifiler® Plus kit should be based on the Human quantitation values. Amplification using the Yfiler® kit should be based on the Male quantitation values.