

artus[®] LC Protocol Sheet

Quantitation of pathogen copy numbers using CE-IVD-marked *artus* LC PCR Kits and the LightCycler[®] 2.0 Instrument

These guidelines are for use with CE-IVD-marked *artus* LC PCR Kits on the LightCycler 2.0 Instrument.

Principle of the procedure

Introduction

Due to its exceptional sensitivity, specificity, and high dynamic range, quantitative, real-time polymerase chain reaction (qPCR) is the essential technique for nucleic acid quantitation. Before qPCR was available, the copy number of a specific target had to be determined by conventional methods such as:

- Competitive PCR
- Limiting dilution PCR
- Radioactive assays (e.g., Southern blot analysis)

These conventional techniques only allow end-point analysis to detect PCR products within the plateau phase (Figure 1). However, PCR exhibits its lowest amplification efficiency at this stage. Detection systems based on qPCR instruments — such as the LightCycler 2.0 Instrument — enable quantitation of DNA during the logarithmic-linear phase of the qPCR.

qPCR allows data analysis by comparison of individual reactions, with the same amplification efficiency, at the beginning of the logarithmic-linear phase. As explained below, this quantitation provides accurate results for external, internal, or relative quantitation methods.

Three segments can be distinguished in each amplification curve:

- Initial background phase
- Exponential amplification phase (or log-linear phase)
- Plateau phase

The background phase lasts until the fluorescence signal from the PCR product exceeds the background fluorescence of the probe system. Then the exponential amplification phase starts. The subsequent log-linear phase terminates when the reaction enters the plateau phase and the reaction amplification efficiency decreases.

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Sample & Assay Technologies

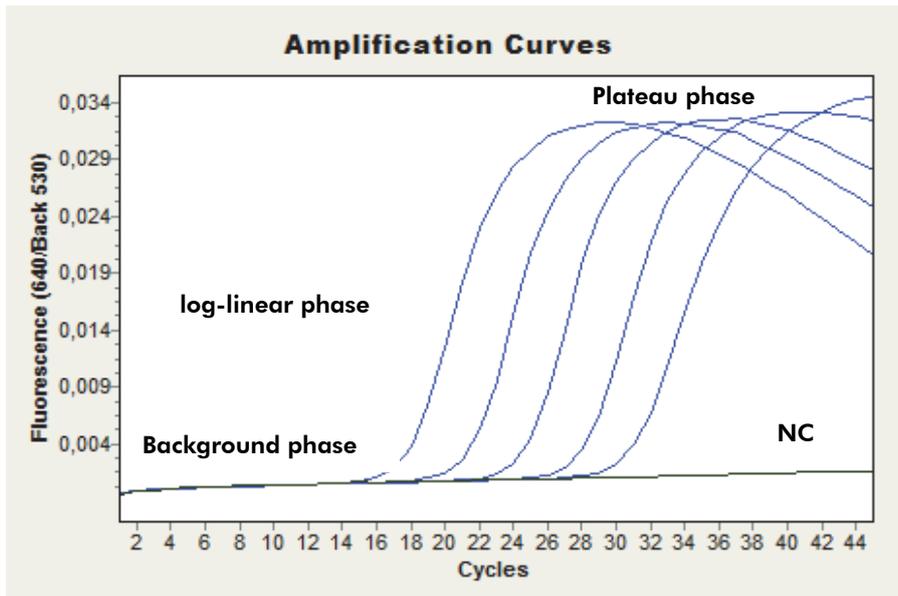


Figure 1. Characteristic PCR amplification curves using the 5 quantitation standards of the artus HBV LC PCR Kit and the LightCycler 2.0 Instrument. NC: Negative control.

The intensity of the fluorescence signal is directly dependent on the DNA concentration. However, reactions with a low initial copy number may reach the same plateau as reactions with higher template concentrations, albeit at a later cycle.

In contrast to end-point analysis following conventional PCR, analysis in the log-linear phase of qPCR produces much more accurate data. Since amplification efficiency is constant throughout the analysis, the amount of starting material can be determined very precisely. An increase of the fluorescence signal during the log-linear phase is directly proportional to an increase in PCR product. It is relatively difficult to identify and measure the few detectable cycles of the log-linear phase by conventional methods such as agarose gel electrophoresis. In contrast, qPCR monitoring (measuring fluorescence intensity in each PCR cycle) offers a convenient way to identify this log-linear phase and to quantify the pathogen load.

Quantitation by external standards is referred to as "absolute quantitation" because a defined number of DNA molecules per volume unit is obtained. The relationship between different dilutions of the target DNA and the crossing point (CP) or threshold cycle (C_T) value (number of PCR cycles at which the fluorescence signal of a reaction exceeds the threshold level, see "Methods to generate a standard curve and determine threshold and CP values", page 4) of the amplification curves is highly reproducible. As a result of precise absolute quantitation, the concentration of the PCR product is obtained as copies or international units per microliter (copies/ μ l or IU/ μ l). However, the final result (i.e., copies or IU per milliliter sample material such as blood, serum, or plasma) still needs to be calculated (see "Quantitation of pathogen copy numbers using artus LC PCR Kits", page 8).

The standard curve (using LightCycler software 4.0 or 4.05)

The easiest way to obtain an absolute value for an unknown nucleic acid concentration of a target is to use external standards. The quantitation method described in this protocol sheet describes how

the LightCycler software compares the amplification of target nucleic acids in an unknown sample with a standard curve (Figure 2) prepared with known concentrations of the same target (homologous standards). The standard samples are amplified in separate capillaries but in the same LightCycler run (external standards). The concentrations chosen for the standard curve should cover the expected concentration range of unknown samples, so as to allow a valid quantitation. Usually, a standard curve is generated using at least 4 dilutions of known concentration. The quantitation standards of the *artus* LC PCR Kits cover a range of 4 to 5 orders of magnitude (QS1–4/5), the concentration of which is stated as copies per microliter (copies/ μ l) or international units per microliter (IU/ μ l). QS1 is the standard of the highest concentration and QS4 or QS5 of the lowest concentration. A precise determination of the PCR product amount is possible within the linear range of quantitation, which exceeds the linear dynamic range of the standard curve by several logarithmic orders of magnitude. Samples of a concentration higher than QS1 (e.g., 10,000 copies/ μ l) are recommended to be diluted down to a concentration within the linear range of the standard curve to allow a more precise quantitation. Samples with a concentration lower than QS4 or QS5 should be considered as positive with a nucleic acid concentration of <QS4 or <QS5.

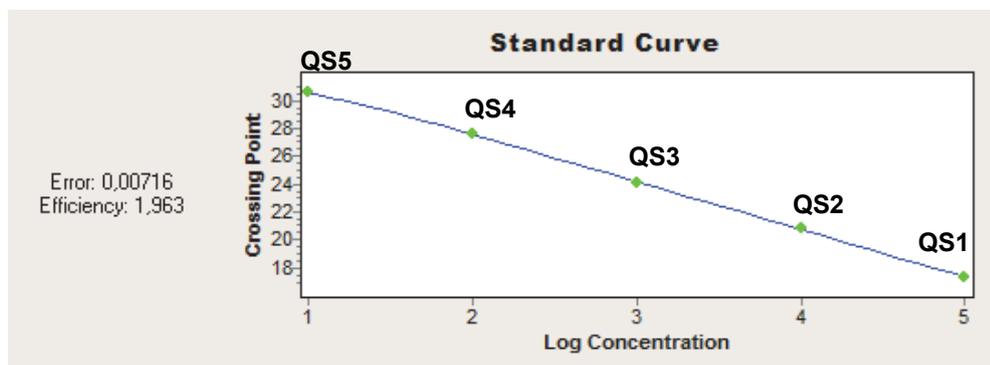


Figure 2. Characteristic standard curve generated with the quantitation standard series (QS5–QS1) of the *artus* HBV LC Kit on the LightCycler 2.0 Instrument.

In the window of the standard curve, 2 values are quoted that allow assessing the validity of the curve (Figure 2). The error value is of particular interest when performing quantitation using external standards (absolute quantitation). The value should be as low as possible and not higher than 0.1. An error value of 0.6 is equivalent to a data deviation of 50%. "Efficiency" refers to the amplification efficiency of the PCR, which is indicated by the slope of the standard curve according to the equation $E = 10^{-1/\text{slope}}$. An amplification efficiency of 2.0 (maximum value) corresponds to a doubling of PCR products (DNA) after each cycle and is only important in the case of relative quantitation, where the amplification efficiencies of 2 quantitative amplification systems are compared to each other.

Crosstalk Color Compensation

The optical filters of the LightCycler 2.0 have a band width of 20 nm to 40 nm around the specified filter area. Due to the relatively large emission spectra of the fluorescence dyes used, irradiation effects may occur from one detection channel to another. A strong fluorescence signal in channel 530 (e.g., FAM™) may thus also be detected in channel 705, resulting in difficulties in data

interpretation. This irradiation effect is called crosstalk. It appears if more than 2 different fluorescence dyes are used in one PCR (e.g., multiplex PCR). *artus* LC PCR Kits generally contain at least 2 different PCR systems (duplex PCR) that react in one LightCycler capillary: one PCR for the detection of the pathogen and another for the detection of the internal control (IC). Since both systems contain different fluorescence dyes, a LightCycler Multicolor Demo Set (Roche) has to be used for compensation of the irradiation effect (Crosstalk Color Compensation). For installation and use of this set, follow the instructions of the manufacturer.

Methods to generate a standard curve and determine threshold and CP values

The LightCycler 2.0 software calculates a threshold value from the fluorescence signals of the individual reaction, which allows determination of the CP value (see below). The threshold is set at a level at which the amplification curves of the standards show comparable fluorescence intensities. The intercept at which the amplification signal exceeds the background signal (see below) and crosses the threshold is known as the threshold cycle (C_T) or crossing point (CP). A difference in the nucleic acid concentration of 2 samples by one log order of magnitude is equivalent to a CP difference of 3.322. The calculated CP values of the quantitation standards will be correlated with their concentrations, and from these data a standard curve will be generated. This allows the quantitation of the nucleic acid concentration of an unknown sample (see "Quantitation of pathogen copy numbers using *artus* LC PCR Kits", page 8).

The LightCycler 2.0 software provides the following 2 analysis algorithms for generating a standard curve for absolute quantitation.

- The "Automated (F" max)" method (LightCycler software 4.0 and 4.05) does not differ from the "Second Derivative Maximum" method of the LightCycler software version 3.5. This method is based on the maximum of the second derivative curve, by which a crossing point is determined. It offers automated data calculation without any user influence (except for the selection/deselection of standards) and provides the advantage of high reproducibility. It is easy to handle and is especially suited for a quick analysis of samples that have to be detected frequently (high sample throughput).
- The calculation of the "Fit Points" method (LightCycler software 4.0 and 4.05) has been improved compared to previous software versions (improved calculation algorithm). It also allows manual optimization of data analysis. Refer also to the indications in the *LightCycler 2.0 Instrument Operator's Manual*, Version 4.0. For data analysis carry out the following procedure.

1. Choose the "Fit Points" option under the analysis mode "Absolute Quantification" in the "Method" menu (Figure 3).

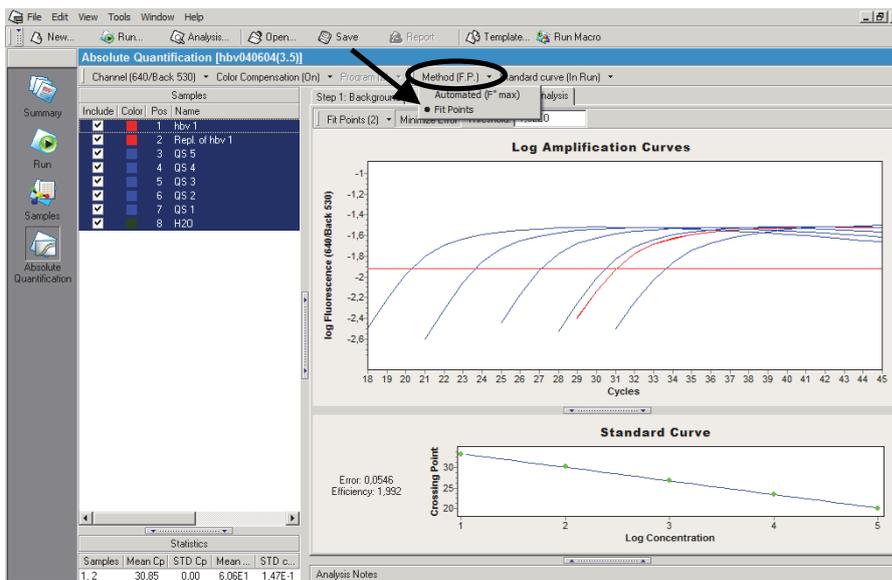


Figure 3. Setting "Fit Points" as the analysis option in the analysis mode "Absolute Quantification".

2. "Step 1: Background". Make sure that in step 1 of the analysis the background fluorescence will be calculated arithmetically ("Arithmetic", Figure 4).

When choosing the option "Arithmetic" the software calculates the mean value of the fluorescence signals between cycles 2 and 6 of the PCR and subtracts this value from each data point of the amplification curve (recommended!). This option may be deactivated by choosing the option "None" (not recommended!).

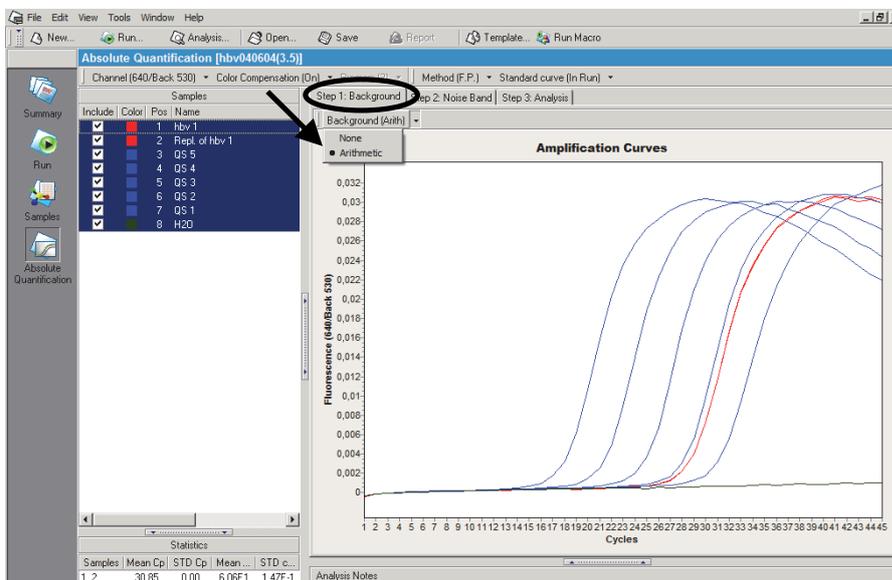


Figure 4. "Step 1: Background". Setting the calculation mode to "Arithmetic" to calculate the background fluorescence (Background).

3. **“Step 2: Noise Band”**. If necessary, position the noise band in step 2 of the analysis above the background fluorescence so that the noise band crosses all positive amplification curves in the lower third of the log-linear phase of the PCR (Figure 5).

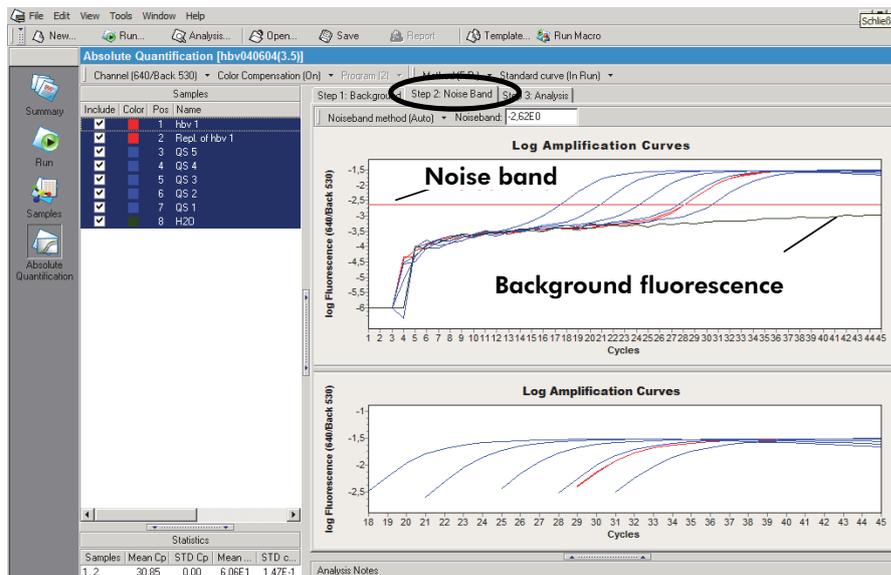


Figure 5. “Step 2: Noise Band”. Positioning the noise band for determination of the background fluorescence.

4. **“Step 3: Analysis”**. The threshold will automatically be positioned in step 3 of the analysis.

The threshold should cross all positive amplification curves in the log-linear range of the PCR. If this is not the case, it may be placed in a suitable position using the cursor (Figure 6). When choosing the option “Minimize Error”, the error value (see “The standard curve (using LightCycler software 4.0 or 4.05)”, page 2) will automatically be minimized. This manual adjusting of the parameters may be advantageous if the data show a severely fluctuating fluorescence background. However, this is only possible if the analysis contains at least 2 defined standards.

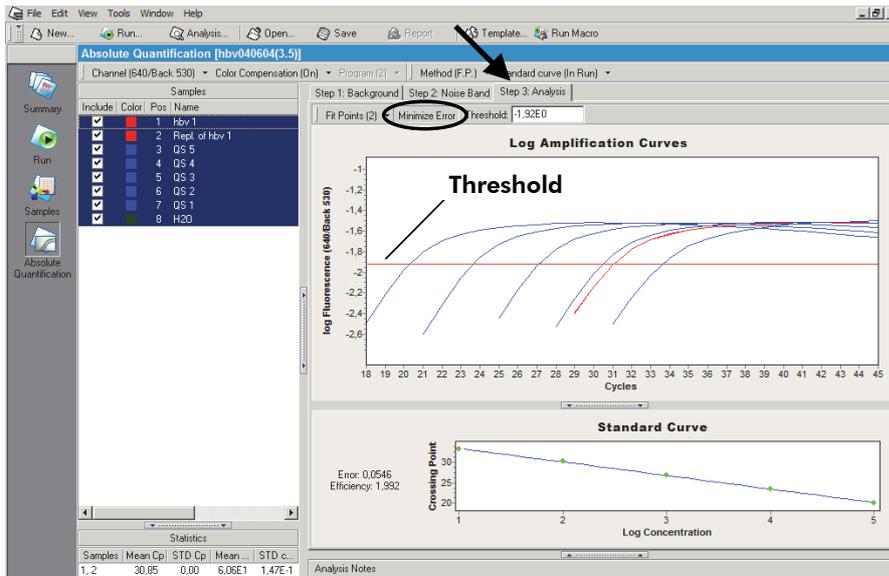


Figure 6. Positioning the threshold for determination of the crossing points (CP).

In both procedures (Automated (F" max) and Fit Points) all further calculation steps necessary to generate a standard curve are performed by the software. Subsequently, the concentration of an unknown sample is determined according to the standard curve, and the corresponding concentrations are indicated in the sample table (Samples → Concentration). For further information, refer to the *LightCycler 2.0 Instrument Operator's Manual*.

Note: In the analysis mode "Abs Quant (Absolute Quantification)" of software versions 4.0 and 4.05, the channel selection "530" corresponds to channel F1 (e.g., FAM) of older software versions (e.g., version 3.5). The channel "640" corresponds to channel F2 (e.g., LC-Red 640), and "705" corresponds to channel F3 (e.g., LC-Red 705). The analysis channel "F2/Back-F1" of older LightCycler software versions corresponds to channel "640/Back 530" of LightCycler software versions 4.0 and 4.05.

Note: Use of the function "Enable Controls in the analysis mode "Abs Quant" is not recommended. Due to competition between the pathogen PCR and the internal control (IC), failure of the amplification curve of an IC may result in an "Invalid Combined" result. In addition, the analysis mode "Fit Points" is not available when this option is used.

Importing an external standard curve

LightCycler 2.0 software versions 4.0 and 4.05 allow the use of external standard curves that have been generated in a previous PCR run and stored under Standard Curve → Save as external ... When such a file is loaded (Standard curve → Use external), the quantitative analysis of PCR runs without a standard curve is possible.

Note: Importing standard curves is not validated for CE-IVD-marked *artus* LC PCR Kits and will lead to loss of the CE certification of the kit.

A strict requirement for this method is to include at least one quantitation standard of one known concentration as a calibrator (e.g., QS3) for the imported standard curve (Figure 7). In addition, the

selected signal channel and the analysis mode (Automated (F¹ max) or Fit Points) of the imported standard curve and the actual run must be the same. This option is especially suitable for applications in which the same parameter is repeatedly analyzed in several PCR runs.

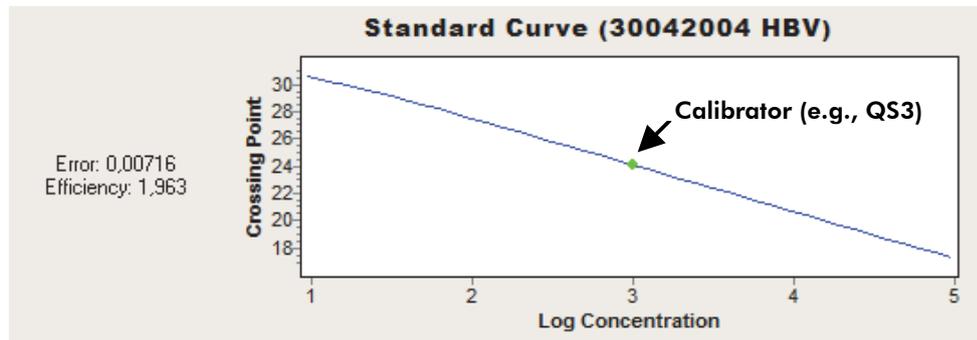


Figure 7. Using an external standard curve. A previously generated standard curve can be imported provided at least one quantitation standard (e.g., QS3) of one concentration is used in the actual run as a calibrator. The slope of the standard curve will remain unchanged while the intercept of the y-axis will be adjusted according to the calibrator.

Note: Importing standard curves is only possible while using the same kit lot number. With different kit lot numbers, the standard curve must be regenerated.

Documentation of quantitation

“Report” (in the menu) compiles the results of the absolute quantitation and, if necessary, the melting curve analysis. It can be directly printed as a data sheet and contains the following information.

- Device adjustments
- PCR profile
- Sample table
- Quantitation results
- Statistics
- Graphs of the amplification curves
- Graphs of the melting curve analysis
- Graphs of the standard curve

Note: The “Navigator” option “Preferences → Sample Preferences” allows changing the default settings of the line style and line color of the graphs. Modification of the sample indications is also partially possible.

Interpretation of results

Quantitation of pathogen copy numbers using *artus* LC PCR Kits

Whenever possible, *artus* LC PCR Kit quantitation standards (QS1–4/5) are calibrated against the WHO international standards available from the National Institute for Biological Standards and

Control (www.nibsc.ac.uk). All other standard concentrations are determined by UV spectrophotometry and by quantitation of a marker gene of control plasmids.

Following the instructions in the kit handbooks, each quantitation standard is added directly to the capillary and designated as a standard in the sample table (Samples → Abs Quant). After the qPCR run, the fluorescence data are analyzed (Absolute Quantification), and a standard curve is generated (see “The standard curve (using LightCycler software 4.0 or 4.05)”, page 2, and “Crosstalk Color Compensation”, page 3).

Note: The use of the function “Enable Controls” in the analysis mode “Abs Quant” is not recommended. Due to competition between the pathogen PCR and the internal control (IC), failure of the amplification curve of an IC may result in an “Invalid Combined” result. In addition, the analysis mode “Fit Points” is not available when this option is used.

For unknown samples (patient samples) showing a positive amplification signal, a CP value is determined and the relative amount of pathogen RNA/DNA per microliter is calculated according to the standard regression plot (standard curve). However, this result does not represent the number of RNA/DNA copies initially present in the sample material. To obtain the final result in copies or international units per milliliter of original sample volume (copies/ml or IU/ml), the following equation must be applied.

$$\text{Result (copies/ml or IU/ml)} = \frac{\text{Result (copies/}\mu\text{l or IU/}\mu\text{l)} \times \text{Initial Elution Volume (}\mu\text{l)}}{\text{Sample Volume (ml)}}$$

As an example, if the QIAamp® DNA Mini Kit is used for extraction of pathogen DNA from whole blood, the extracted volume (sample volume) is generally 0.2 ml. The elution volume for viral DNA extractions is usually 200 μl , as given in the kit protocol. If the result of the amplification analysis, as described above, is 54 copies/ μl , then the equation above can be applied as follows.

$$\frac{54 \text{ copies/}\mu\text{l} \times 200 \mu\text{l}}{0.2 \text{ ml}} = 5.4 \times 10^4 \text{ copies/ml}$$

Note: If the sample material has been concentrated (e.g., by centrifugation), make sure to use the initial sample volume in the equation above.

Figure 8 shows an example of quantitation of an unknown sample on the basis of comparing CP values of *artus* quantitation standards and an unknown sample.

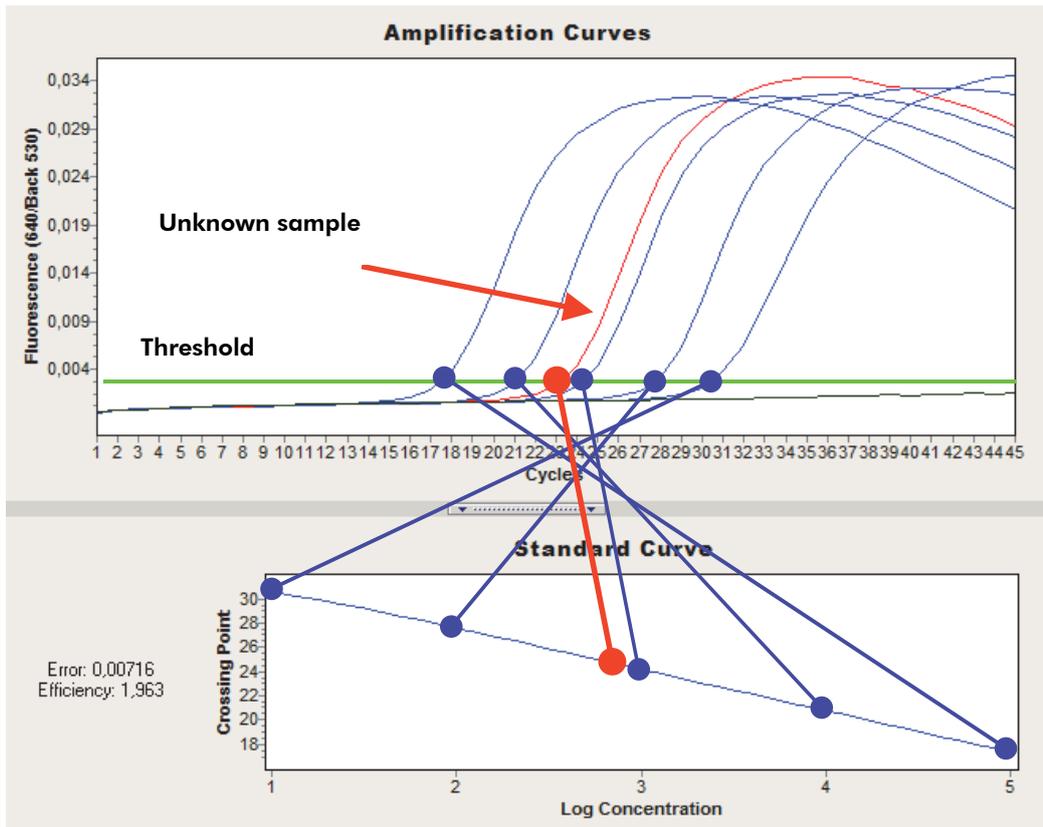


Figure 8. Example of determination of the absolute copy number in an unknown sample using the standard curve.

Contact information

If you have further questions regarding qPCR quantitation or other technical specifications of *artus* assays, please see our Technical Support Center at www.qiagen.com/Support or contact one of the QIAGEN Technical Service Departments or local distributors (see the back cover of QIAGEN® kit handbooks or visit www.qiagen.com).

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