artus® RG Protocol Sheet

Quantitation of pathogen copy numbers using CE-IVD-marked *artus* RG PCR Kits and Rotor-Gene® instruments

These guidelines are for use with CE-IVD-marked *artus* RG PCR Kits on Rotor-Gene Q MDx, Rotor-Gene Q, Rotor-Gene 3000, or Rotor-Gene 6000 instruments with Rotor-Gene software (version 6.0 or higher) or Rotor-Gene Q software (version 1.7 or higher).

Principle of the procedure

Introduction

Due to its exceptional sensitivity, specificity, and high dynamic range, quantitative, real-time polymerase chain reaction (qPCR) has become an 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 Rotor-Gene Q MDx, Rotor-Gene Q, Rotor-Gene 3000, and Rotor-Gene 6000 — enable kinetic quantitation of DNA during the logarithmic-linear phase of the qPCR.
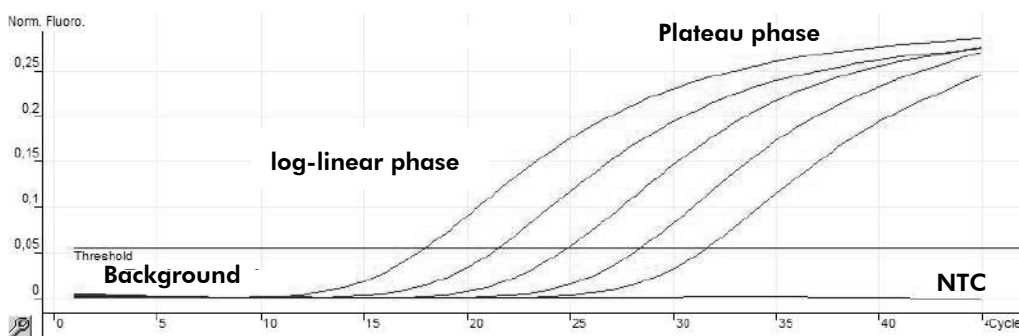

Figure 1. Characteristic PCR amplification curves using 5 quantitation standards of the *artus* HBV RG PCR Kit on the Rotor-Gene 6000. NTC: No template control.

December 2012

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

Three segments can be distinguished in each amplification curve:

- Initial background phase
- Exponential growth 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 phase starts. The log-linear phase commences when sufficient product has accumulated to be detected above background and terminates when the reaction enters the plateau phase and the reaction amplification efficiency decreases.

The intensity of the fluorescence signal is directly dependent on the DNA concentration. However, reactions with a low initial copy number can reach the same plateau as reactions that started with higher template concentrations. In contrast to end-point analysis following conventional gel-based PCR, analysis in the log-linear phase 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 using external standards is referred to as "absolute quantitation" because a defined number of nucleic acid molecules per volume unit is obtained. The relationship between dilutions of the target and the threshold cycle (C_T) values (number of PCR cycles at which the fluorescence signal of a reaction exceeds the threshold level, see "Methods to generate a standard curve, threshold, and C_T values", page 4) 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/μl or IU/μl). However, the final result (i.e., copies or IU per milliliter of original sample material, such as blood, serum, or plasma) still needs to be calculated (see "Quantitation of pathogen copy numbers using *artus* RG PCR Kits", page 6).

The standard curve

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 Rotor-Gene 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 reaction tubes but in the same Rotor-Gene 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* RG PCR Kits cover a range of 4 to 5 orders of magnitude (QS1–4/5), where 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 dynamic range of quantitation, which exceeds the linear range of the standard curve by several log values (see the corresponding *artus* Kit handbooks for further information). Unknown samples of a concentration higher than QS1 (e.g., 10,000 copies/µl) are recommended to be diluted down to a concentration within the linear range of the standard curve to allow precise quantitation. A valid determination of copy numbers (or international units) of an unknown sample can be done within the range of the quantitation standards (QS1–4/5) or, if available, within the determined linear range of that assay. Samples with concentrations lower than the lowest standard or the lower end of the linear range should therefore be considered as positive with a concentration ≤QS4 (or ≤QS5) or ≤ lower end of the linear range, respectively.
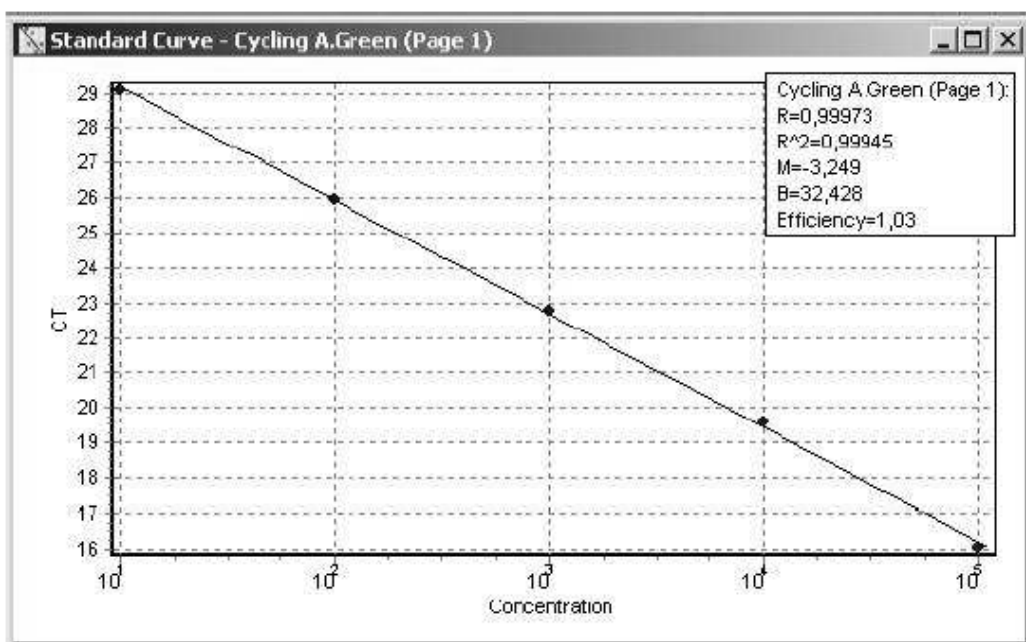

Figure 2. Characteristic standard curve generated with *artus* HBV RG PCR Kit quantitation standard series (QS5–QS1) on the Rotor-Gene 6000 instrument. B: Intercept with the ordinate. **Efficiency**: Reaction efficiency. **M**: Slope of the standard curve. **R** and **R^2**: Correlation coefficients.

In the window of the standard curve several values indicate the quality of the curve (Figure 2). The correlation coefficient "R^2" and its square root "R" indicate the percentage of the data that matches the hypothesis that the given standards form a regression line or a standard curve (1.0 is equivalent to 100%). "Efficiency" relates to the reaction efficiency of the PCR. A value of 1.0 means a duplication of the DNA concentration after each cycle of the log-linear phase. "M" is the slope of the standard curve, and its value gives an indication of the reaction efficiency (Efficiency = $10^{-M} - 1$). The intercept of the curve "B" is a mathematical value. It calculates the next C_T value of a sample concentration one magnitude lower than the last QS used under ideal circumstances (linear PCR). For example, if QS5 has a concentration of 10 IU/µl and a C_T value of 35, then the next sample

with a concentration one magnitude lower (1 IU/μl) would result in a C_T value of approximately 32.4.

For calculation of target concentration, use the following formula.

$$x = \frac{C_T - B}{M}$$

Where:

- x = concentration
- C_T = threshold cycle
- B = intercept of the curve
- M = slope of the curve

Methods to generate a standard curve, threshold, and C_T values

The Rotor-Gene software requires determination of the background fluorescence signal for calculation of the amplification curves of the quantitation standards. Standard normalization calculates the mean of the fluorescence intensity of the first 5 cycles of the PCR. All data points for the samples are then divided by this value to normalize the data. On the basis of the fluorescence signals, the software further fixes a threshold, which is required for the determination of the C_T values (see below). This threshold is defined as a level just above background fluorescence of all samples and is set automatically or manually in the exponential or log-linear phase of amplification. The cycle number of the PCR, at which the fluorescence signal exceeds the threshold, is termed the threshold cycle (C_T). A difference in nucleic acid concentration of 2 samples by one log unit is equivalent to a C_T difference of 3.322. The C_T values will be correlated with the concentrations of the standards, and from these data a standard curve will be generated to determine unknown nucleic acid concentrations (see also "Quantitation of pathogen copy numbers using *artus* RG PCR Kits", page 6). The threshold is automatically determined by optimizing the correlation coefficient (see "The standard curve", page 2) or can be influenced by the user. To optimize the background signal and the C_T values, the following 4 options are available.

- "Dynamic Tube" normalization uses the second derivative of each sample trace to determine a starting point for each sample. The background level is then averaged from cycle one up to this starting cycle for each reaction. This option gives the most precise normalization of data and is recommended to use by default.
- The "Slope Correction" option improves the data when raw data backgrounds are observed to slope upward or downward before the amplification inflection point. This option uses a line-of-best-fit to determine the background instead of an average and normalizes to that instead.
- "Ignore First" allows exclusion of up to the first 10 cycles of the PCR data. This function allows the exclusion of potential signal intensity fluctuations within the initial cycles of PCR that are frequently not representative for normalization. In addition, if the background signal exceeds the threshold prior to the starting point of amplification (C_T), it may be useful to ignore the first cycles for the determination of the C_T values ("Eliminate Cycles before").

- The "Outlier Removal…" option allows exclusion of samples that have a slight drift upwards. All samples with a change in fluorescence intensity below a user-defined NTC (no template control) threshold will not be reported (percentage of the largest maximum change found in any tube). Furthermore, this function provides a tool to ignore samples with a reaction efficiency below a user defined value.

Note: The "Outlier Removal…" option is only recommended with reservation since samples with very low pathogen concentrations may not be identified.

Importing an external standard curve

The Rotor-Gene software allows the import of an external standard curve when no adequate standard curve is available in the current experiment. A strict requirement for this method is to include at least one quantitation standard of one given concentration (within the range covered by the standard curve) in each run as a calibrator (Figure 3). It is recommended to use replicates of this calibrator to improve the precision of the calculation. The Rotor-Gene software allows the import of a standard curve from other experiments ("From Other Experiment"). Make sure that the inserted curve was generated in the same fluorescence channel as the current experiment to be analyzed. The following option "Adjust" allows the adaption of the imported standard curve to the current experiment using the calibrator as a fixed point (see above). The nucleic acid concentrations of the samples will then be calculated automatically. With this setting the threshold cannot be modified manually ("Standard Curve Type: Fixed"). Consequently, C_T values may not be assigned to samples with a very low DNA/RNA concentration and, hence, reported negative. By enabling the option "Reset" the threshold can be altered by the user to improve the analysis ("Standard Curve Type: Floating"), but no calculated concentrations are displayed at the "Quant. Results" window.
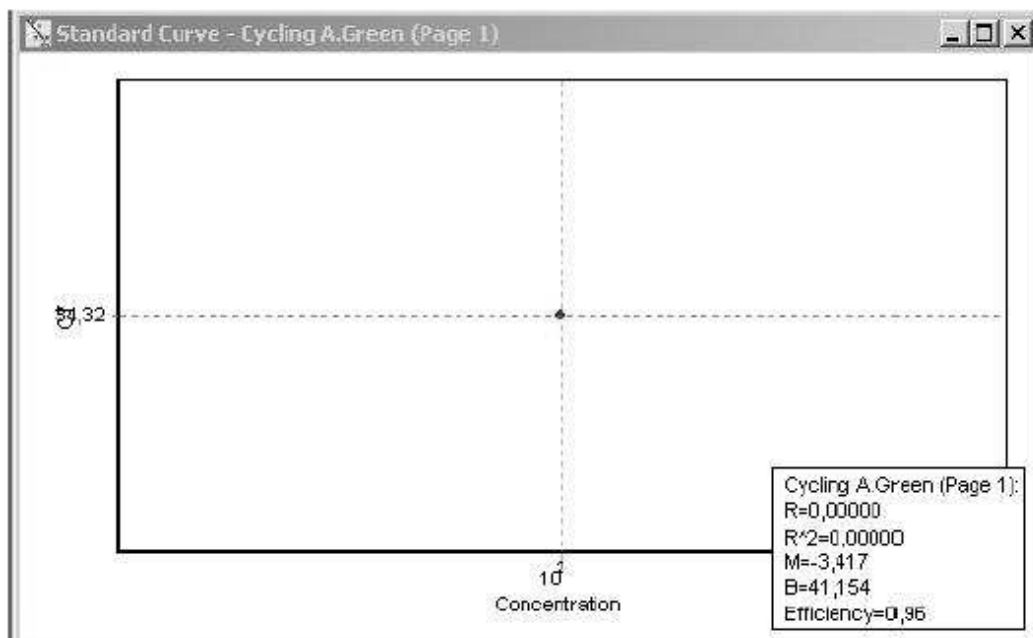

Figure 3. Importing a previously generated standard curve. A previously generated standard curve can be imported provided that at least one quantitation standard (QS) of one given concentration is used as a calibrator. The slope will remain unchanged.

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

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.

Quantitation report

The following "Reports" are provided for documentation of the analyzed data.

- Besides other data, the "Standard Report" provides general information about experimental parameters. A graphical and tabular documentation of the results is also presented in the report.
- The "Full Report" comprises additional information about the temperature profile and gives an overview about the "Messages" that display, such as the gain adjustment and any warnings received during the experiment.
- The "Concise Report" briefly summarizes the essential results (such as the quantitation of the samples) in a graphical and tabular report. All reports can be saved as Microsoft® Word documents or can be forwarded by email.

Interpretation of results

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

Whenever possible, *artus* RG PCR Kit quantitation standards (QS1–4/5) are calibrated against the WHO international standards available from the National Institute for Biological Standards and Control (NIBSC, UK). All other standard concentrations are determined by UV spectrophotometry and by quantitation of a marker gene of control plasmids or in vitro transcripts. Following the instructions in the kit handbooks, each quantitation standard is added directly to the reaction tube and designated as a standard in the "Edit Samples" menu. After the run the fluorescence data are analyzed as described above, and a standard curve is generated (see "The standard curve", page 2, and "Methods to generate a standard curve, threshold, and C_T values", page 4). For unknown samples (patient samples) showing a positive amplification signal, a C_T value is determined and the relative amount of pathogen RNA/DNA per microliter is calculated according to the standard regression plot (Figure 4). 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.

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

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 4 shows an example of quantitation of an unknown sample on the basis of comparing C_T values of *artus* quantitation standards and an unknown sample.
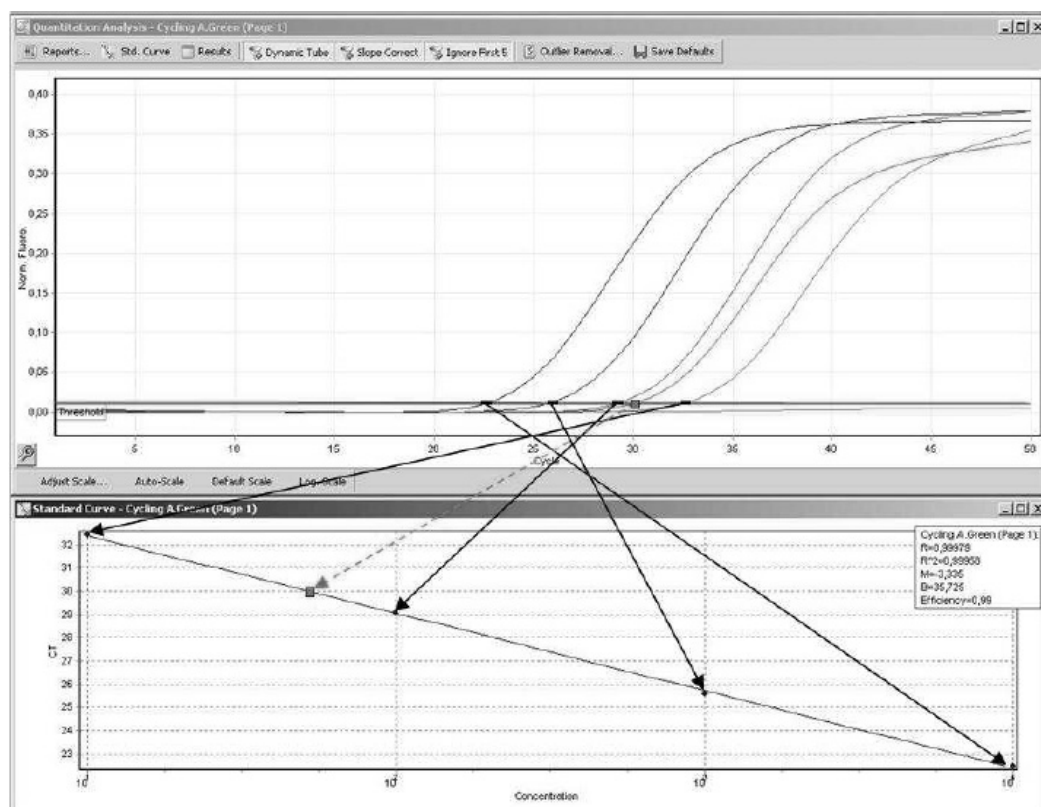

Figure 4. Example of determination of the absolute copy number in an unknown sample using an *artus* assay standard curve and a Rotor-Gene instrument.

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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