Technical Note

Quantitation for Pathogen Copy Numbers Using *artus*® Kits and *artus 3000*™/Rotor-Gene™ 2000/3000

1 Introduction

Due to its unsurpassed sensitivity, specificity and high dynamic range real-time PCR (polymerase chain reaction) has become an essential technique for nucleic acid quantitation. Before real-time PCR 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)

These conventional techniques only allow an end-point analysis detecting PCR products within the plateau phase (Fig. 1). At this stage PCR exhibits the lowest amplification efficiency. Detection systems based on real-time PCR instruments, like the *artus 3000* and the Rotor-Gene 2000/3000, allow a kinetic quantitation of DNA/RNA **during** the logarithmic-linear phase of the PCR.

Real-time PCR allows data analysis by comparison of individual reactions at the beginning of the logarithmic-linear phase (see below). 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. Here, the exponential growth 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 concentration of the nucleic acid of a reaction. However, reactions with a low initial copy number can reach the same plateau as reactions that started with higher template concentrations.

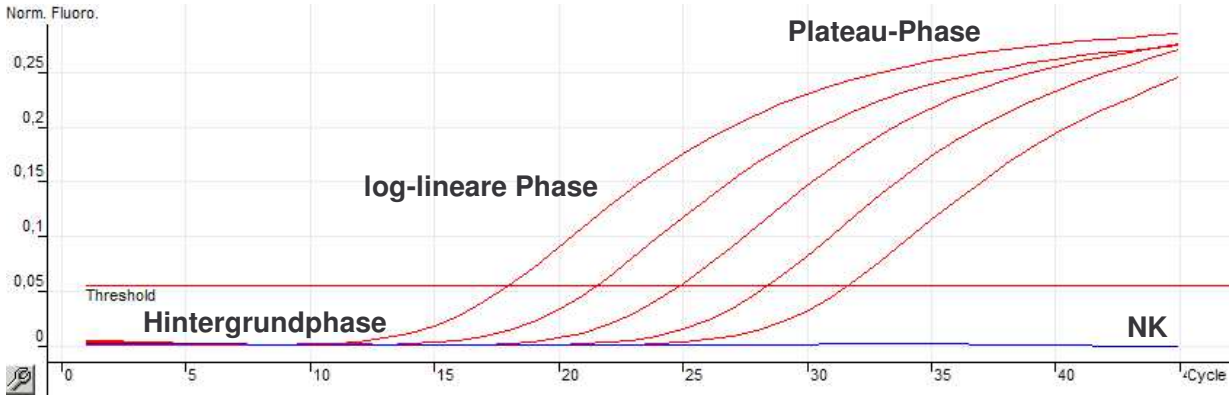

Fig. 1: Characteristic PCR amplification curves using two quantitation standards of the *artus* HSV1/2 RG PCR Kit and the Rotor-Gene 3000. **NTC**: non-template control.

In contrast to end-point analysis following conventional gel-based PCR, analyses in the log-linear phase produce 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 comparatively difficult to identify and measure the few detectable cycles of the log-linear phase by conventional methods such as agarose gel electrophoresis. In contrast, real-time PCR 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 (concentration in copies/µl or international units (IU)/µl) 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 Ct values (Ct: threshold cycle, number of PCR cycles at which the fluorescence signal of a reaction exceeds the threshold level, see chapter 3) of the amplification curves is highly reproducible. As a result of precise absolute quantitation the concentration of the PCR product is stated as copies/µl of IU/µl. However, the final result (i. e. copies or IU per ml sample material such as blood, serum or plasma) still has to be calculated (for equation please refer to chapter 6).

2 The Standard Curve

The following information and details refer to the Rotor-Gene software version 6.0.34. Older software versions may not contain all data analysis options given here.

The easiest way to obtain an absolute value for an unknown nucleic acid concentration of a target includes the use of external standards. In the quantitation method described in this note, the Rotor-Gene software compares the amplification of target nucleic acids in an unknown sample with a standard curve (Fig. 2) prepared with known concentrations of the same target (homologous standards). The standard samples are amplified in separate reaction tubes but within the same Rotor-Gene run (external standards). The concentrations chosen for the standard curve should cover the expected concentration range of unknown samples to allow a valid quantitation. Usually, a standard curve is generated using at least four 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) of which QS1 is the standard of the highest 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 logs (please refer to the *artus* user manual, chapter **11. Specifications** for further information). If the data of linear quantitation should not yet be available for a particular *artus* TM PCR Kit, the standard curve has to be referred to as the linear dynamic range. Unknown samples with a calculated concentration greater than QS1 (e. g. 10.000 cop/µl) has to be diluted to fit the log-linear range of the standard curve to enable a precise calculation. A reaction of an unknown sample with a nucleic acid concentration lower than QS4/5 can be considered positive, with a concentration of lower than QS4 or QS5.

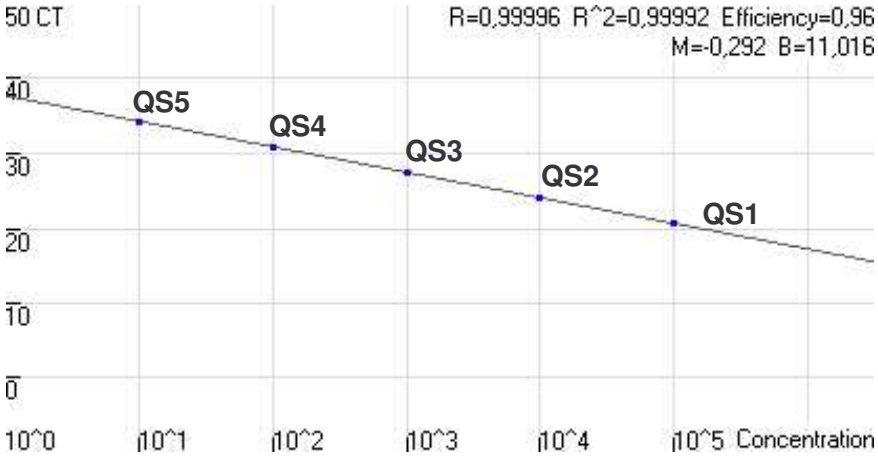

Fig. 2: Characteristic standard curve generated with *artus* C. trachomatis RG PCR Kit quantitation standard series (QS4-QS1) and Rotor-Gene 3000. *B*: Intercept with the ordinate, *Efficiency*: Reaction efficiency, *M*: Slope of the standard curve, *R* and *R^2*: correlation coefficients.

In the window of the standard curve values are indicated which allow to assess the quality of the curve (Fig. 2): The correlation coefficient *R^2* and its square root *R* provide information regarding the correlation of the QS concentrations entered into the sample sheet and those calculated on basis of the 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 (i. e. conc.=10^(M*C_t+B) or y=mx+b), its value gives a clue to the reaction efficiency (=10^{-M}-1). The intercept of the curve termed *B* is a theoretical value of the nucleic acid concentration of a sample after the first cycle of the PCR. The decisive value for the assessment of the standard curve is the *R^2* value which should not be lower than 0.98. Lower values are usually indicative of either incorrect concentration values or of pipetting errors.

3 Rotor-Gene Methods to Generate a Standard Curve, the Threshold and the Ct Values

The Rotor-Gene software requires the determination of the background fluorescence signal or the calculation of the amplification curves of the quantitation standards. Standard normalization calculates the mean of the fluorescence intensity of the first five cycles of the PCR. All data points for the samples are then divided by this value to normalize the data. On basis of the fluorescence signals, the software further fixes a threshold which is required for the determination of the Ct 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 and exceeds the threshold is termed threshold cycle (Ct). A difference in nucleic acid concentration of two samples by one log is equivalent to a Ct difference of 3.322. The threshold is automatically determined by optimising the correlation coefficient (see chapter 2) or user influenced (*Auto-find Threshold*).

To optimise the background signal and the Ct values four options are offered: *Dynamic Tube*, *Slope Correction*, *Ignore First* and *Quant. Settings*:

- *Dynamic Tube* normalization uses the 2nd 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 number for each reaction. This option gives the most precise normalisation 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 normalises to that instead.
- *Ignore First* allows to exclude the first up to ten cycles of the PCR data. This function allows the exclusion of potential signal intensity fluctuations within the initial cycles of PCR which are frequently not representative for normalisation. In addition to that, if the background signal exceeds the threshold prior to the starting point of amplification (Ct), it may be useful to ignore the first cycles for the determination of the Ct values (*Eliminate Cycles before*).
- The *Quant. Settings* option allows excluding samples, which have a slight drift upwards. All samples with a change in fluorescence intensity below a user defined NTC threshold (none-template control) will not be reported (percentage of the largest maximum change found in any tube). Further, this function provides a tool to ignore samples with a reaction efficiency below a user defined value (Rotor-Gene software version 5.0 only).

Note: The *Quant. Settings* are recommended with restrictions only, since the samples of very low pathogen concentrations may not be identified.

Finally, the Ct values determined with either of the two methods will be correlated with the concentrations of the standards and from these data a standard curve (refer to chapter 2) will be generated to determine unknown nucleic concentrations (refer to chapter 6).

Remark: If you use only one standard per concentration (no replicates) to generate a standard curve you have the option to optimise your date by using the *Singlet Concentration* function (click on the standard curve and then use the right mouse button). With this function the standard curve shows a line between the given concentration and the calculated concentration for the standards. The threshold is subsequently optimised (please use *Auto-find Threshold*) to minimise the distance between each given and calculated standard concentration.

4 Import of 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 (Fig. 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 channels of the same experiment (*Current Experiment*) or from other experiments (*From Other Experiment*). Please make sure that the inserted curve was generated in the same fluorescence channel as the current experiment to be analysed. The following option *Adjust* allows the adaption of the imported standard curve to the current experiment using the calibrator as a fix point (see above). The nucleic acid concentrations of the samples will then be calculated automatically. With this setting the threshold can not be modified manually (*Standard Curve Type: Fixed*). As a consequence, C_t's may not be assigned to samples with a very low DNA/RNA concentration and, hence, reported negative.

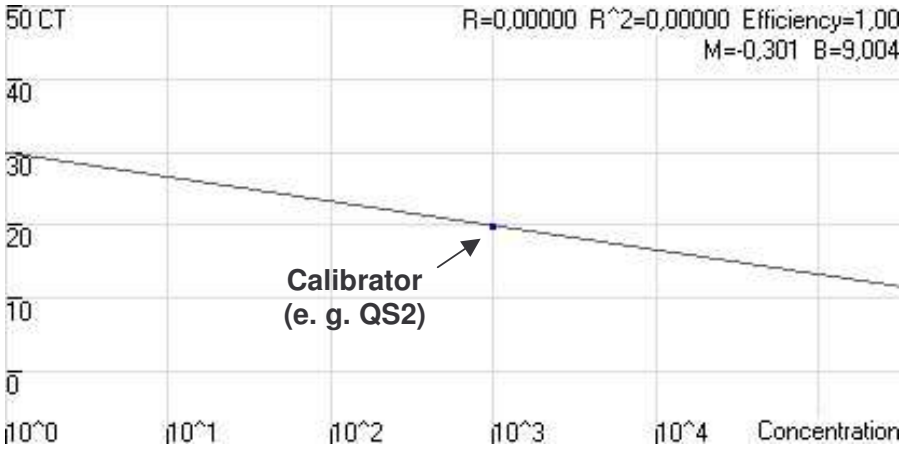

Fig. 3: A previously generated standard curve can be imported provided at least one quantitation standard (QS) of **one** given concentration is used as a calibrator. The slope will remain unchanged whereas the intercept value will be aligned according to the calibrator.

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.

5 Quantitation Report

The *Reports* menu provides a result summary, and the option *Report Settings* allows the selection of various options to document the results:

- Among other data the *Standard Report* provides general information about experimental parameters. Further, a graphical and tabular documentation of the results is presented.
- The *Full Report* comprises additional information about the temperature profile and gives an overview about the *Messages* that display e. g. the gain adjustment and also warnings received during the experiment.
- The *Concise Report* briefly summarises the essential results (like the quantitation of the samples) in a graphical and tabular report.

All report formats can be saved as a Microsoft Word® document or forwarded by e-mail.

6 Quantitation of Pathogen Copy Numbers using *artus* RG PCR Kits

Where possible *artus* PCR Kits' 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 the control plasmids.

According to the user manual, each quantitation standard is added directly to the reaction tube and designated as standard in the *Edit Samples* menu. After the run the fluorescence data are analysed as described above and a standard curve is generated (see chapter 2 and 3).

For unknown samples (patient samples) showing a positive amplification signal a C_t is determined and the relative amount of pathogen RNA/DNA per µl is calculated according to the standard regression line (Fig. 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 IU per millilitre sample volume (cop/ml or IU/ml), the following equation has to be applied:

$$\text{cop/ml or IU/ml} = \frac{\text{Result (cop/ml or IU/ml) x Elution Volume (µl)}}{\text{Sample Volume (ml)}}$$

Example: If e.g. the QIAamp® DNA Mini Kit is used for the extraction of pathogen DNA from whole blood the extracted volume is generally 0.2 ml. The elution volume for viral DNA extractions is usually 200 µl as given in Qiagen protocol. If we assume a result of the above described amplification analysis of 54 cop/µl, then the above equation can be applied as follows:

$$\frac{\text{54 cop/µl x 200 µl}}{\text{0.2 ml}} = \mathbf{5.4 \times 10^4\ cop/ml}$$

Note: If the sample material has been concentrated (e. g. by centrifugation), please use the initial sample volume for the equation above.

Figure 4 shows the exemplary quantitation of an unknown sample on the basis of comparing C_t values of quantitation standards, and an unknown sample.

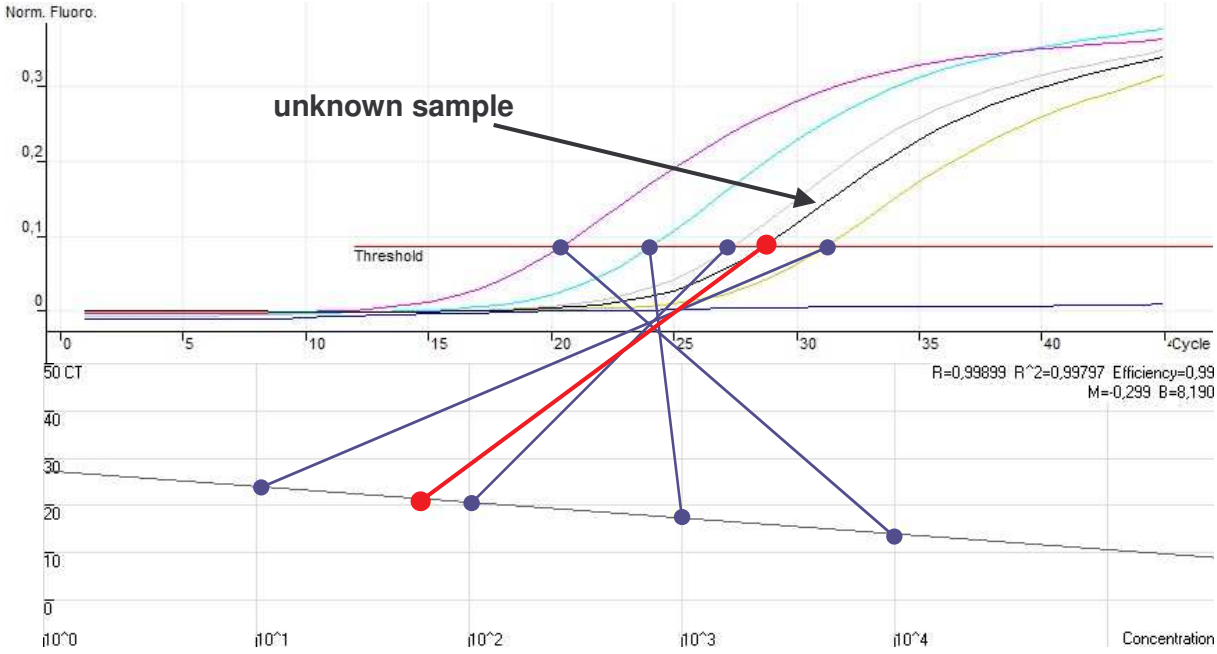

Fig. 4: Determination of the absolute copy number in an unknown sample using a standard curve and the Rotor-Gene 3000 instrument.

If you have further questions regarding real-time PCR quantitation or other technical specifications of the *artus* systems, please contact our technical service.

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