General guidelines for the interpretation of samples and controls data

In the next tables, figures, and chapters, we collected guidelines and some general concepts to assist the users of Venor®GeM qEP in the process of data handling and interpretation. At the end of this document, a few basic terms are briefly clarified (glossary).

Table 1. Main interpretation of results obtained in FAM™ (*Mollicutes*) and HEX™ (IC) channels.
Pre-requisite for this analysis is the appropriate setting of the thresholds (see below).

Detection of *Mollicutes* FAM™ Channel	Internal Control (IC) HEX™ Channel	Interpretation
Ct < 40: Positive	Irrelevant	Positive (*Mollicutes* contamination)
Ct ≥ 40: Negative	Negative	IC as PCR control: PCR inhibition
		IC as process control: Extraction failure and/or PCR inhibition
Ct ≥ 40: Negative	Positive*	Negative (no *Mollicutes* contamination)

*If used as a PCR control, the internal control (HEX™) for negative samples (FAM™ ≥ 40) must show comparable Ct values to those obtained for the negative controls (no-template, NTC) (± 2 cycles). If used as a process control, the internal control (HEX™) for negative samples (FAM™ ≥ 40) must show comparable Ct values to those obtained for the NTCs (± 3 cycles).

Table 2. Detailed interpretation of results for EP- & JP-compliant testing.

Sample	Result	Meaning	Actions and next steps
NTC	Negative	Valid PCR	Analyze sample data
	Positive	PCR contamination	Repeat the PCR and check for contamination sources (e.g. master mix, water, cycler etc.)
PC	Negative	Failed PCR	Repeat the PCR and check for reaction failure (e.g. missing or old reagents, pipetting errors, cycler program etc.)
	Positive	Valid PCR	Analyze sample data
NEC	Negative	Valid PCR	Analyze sample data
	Positive	Contamination during extraction or PCR	Repeat the extraction AND the PCR and check for contamination sources (e.g. buffers, master mix, water, cycler etc.). If at the same time, the NTCs are positive, consider PCR contamination and repeat PCR only to assess that possibility.
Sample	Negative (0/2 duplicates)	No contamination	Biological product release
	Positive/Negative (1/2 duplicates)	Possible contamination	Whole process incl. extraction needs to be repeated to assess potential contamination. If at the same time, the NTCs are positive, consider PCR contamination and repeat experiment to assess that possibility. A reiterated ambiguous result halts product release.
	Positive (2/2 duplicates)	Contamination	No biological product release

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Guidelines for data handling and results interpretation

1. Amplification curves, raw fluorescence data and baselines

As for any other qPCRs, the primary output for Venor®GeM qEP are raw fluorescence data (Y axis), which plotted against the PCR cycle number (X axis), results into amplification plots or curves. A positive amplification is often clearly detected as a sigmoidally-shaped curve. Two particularly relevant parameters characterize such a curve: one is the max. fluorescence reached at the plateau, which correlates with the amount of amplicon generated by the reaction; the other is the Ct value: the cycle number where a statistically significant amount of template has been amplified (above a threshold), which correlates with the amount of target originally present in the sample.

At first, data analysis should focus on the obtained amplification curves and their shape. Next, the baseline (background fluorescence normalization/subtraction) as well as the threshold settings (next chapter) should be taken into account for a comprehensive analysis of the data. Evaluating the effect of baseline subtraction by having a look at the raw fluorescence data can help gain additional information on the run. Particularly, raw data can reveal inconsistencies or whether the fluorescence intensity range is appropriate. In Figure 1, for example, the results of a run with samples, all expected to be positive, are shown.

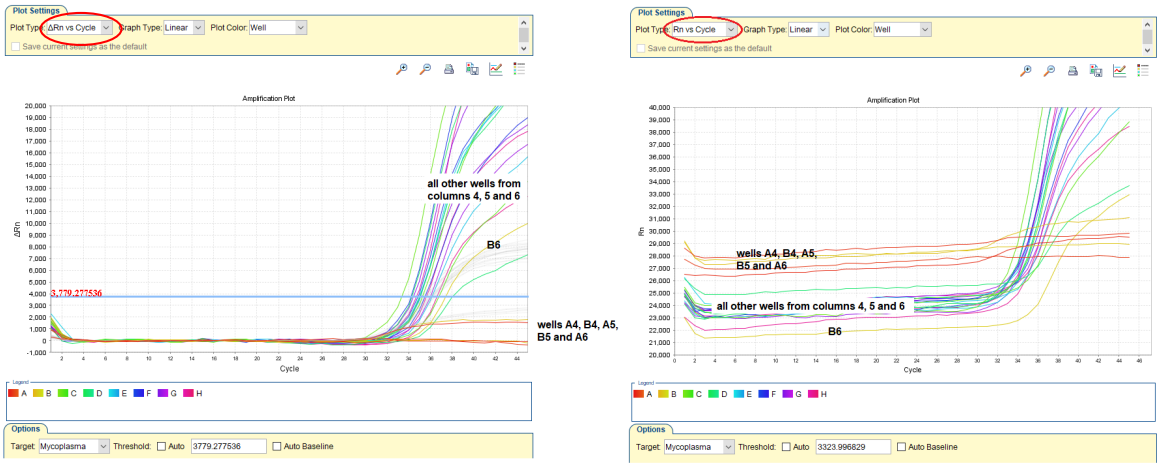


Figure 1. Amplification curves displayed after (left) and before (right) baseline subtraction.

The analysis of baseline-subtracted data (normalized, graph on the left) show significantly reduced fluorescent signals at plateau for wells A4, B4, A5, B5, A6, which are wrongly classified as negative. Interestingly, after raw data are retrieved (before normalization, graph on the right), it appears that these same wells rather display higher background fluorescence levels compared to the other curves. Such higher background fluorescence combined with the lack of clear sigmoidal curves hints at set-up errors or at the issues with the samples/DNA templates.

Notably, the Venor®GeM qEP kit includes a very convenient tool to control and troubleshoot the results of qPCR runs, the internal control (or internal amplification control, IC). Already in the first phases of the analysis, we recommend correlating the amplification plots obtained for target and IC, as generally described in Table 1. For more information about the IC, please see below the chapters about PCR inhibition and competition.

2. Threshold Setting

In general, a number of factors are considered when setting the threshold (manually or automatically), surely among others the sensitivity, the reproducibility, the background noise, and the specific run performance. For example, setting a threshold set too low (e.g. background fluorescence) maximizes sensitivity but increases the probability of obtaining false positive results. On the other hand, setting it too high (e.g. in the upper exponential phase) might dramatically affect sensitivity and lead to data misinterpretation as well (e.g. false negative).

Just as for every other assay that requires it, threshold setting can be a complex process, where the experimental conditions, the cycler, its software as well as the user experience play a role.

Again, similarly to any other qPCR assay, the possible strategies for threshold setting can be schematically described as listed below. Each approach has obviously its pros and cons and must be carefully assessed by the user according to the obtained data and processed samples. Importantly, no matter which approach is selected, this should be systematically, consistently adopted for all samples of the run.

Automatic threshold – Automatic setting of the threshold relies on the cycler-software algorithm and is, therefore, software-dependent. In many cases, it is defined by the baseline plus a certain number of standard deviations, in a variable manner for distinct instrument software. It is a quick, useful approach that often preserves the assay sensitivity and, in some cases, helps avoiding gross errors. However, automatic setting cannot be successfully applied to every run or sample set and can lead to misinterpretation of the results.

Manual threshold (fixed, constant value) – Setting the threshold to an arbitrary value after (automatic or manual) baseline subtraction is also a conceivable strategy. This approach must be carefully established through detailed analysis of data sets, including the definition of acceptance criteria for fluorescence levels.

Manual threshold (using as a reference: a % of the max fluorescence of a standard) – The threshold can also be set to a defined value represented by a X percentage of the max fluorescence of a standard. Ideally, this standard might be a well-established positive control, which is always tested in parallel with the samples for a certain assay. Also this approach requires a certain degree of methodological experience.

Manual threshold (using as a reference the lower limit of the inflection point of a standard) – This approach is similar to the previous one but relies on the ability of the experimenter to visually identify the point of inflection of the curve Due to the intrinsic subjective nature of this method, its disadvantage is the potential lack of accuracy.

For all variants the following applies: if a Ct < 40 is obtained, the sample is to be evaluated positively; if no Ct or Ct ≥ 40 is obtained, the sample is to be evaluated as negative or more precisely as „below the detection limit“.

Figure 2A and B illustrate how choosing one approach over another affects the outcome. In A, automatic thresholding with the cycler software (Applied Biosystems® 7500) results into detection of 2 positive samples (yellow and red curves) with fluorescence levels which way outside the fluorescence range of the positive control (pink curve). Switching to a manual thresholding approach and setting this value at about the 5 % of the positive control max fluorescence alters this result and turns one sample into a negative sample.

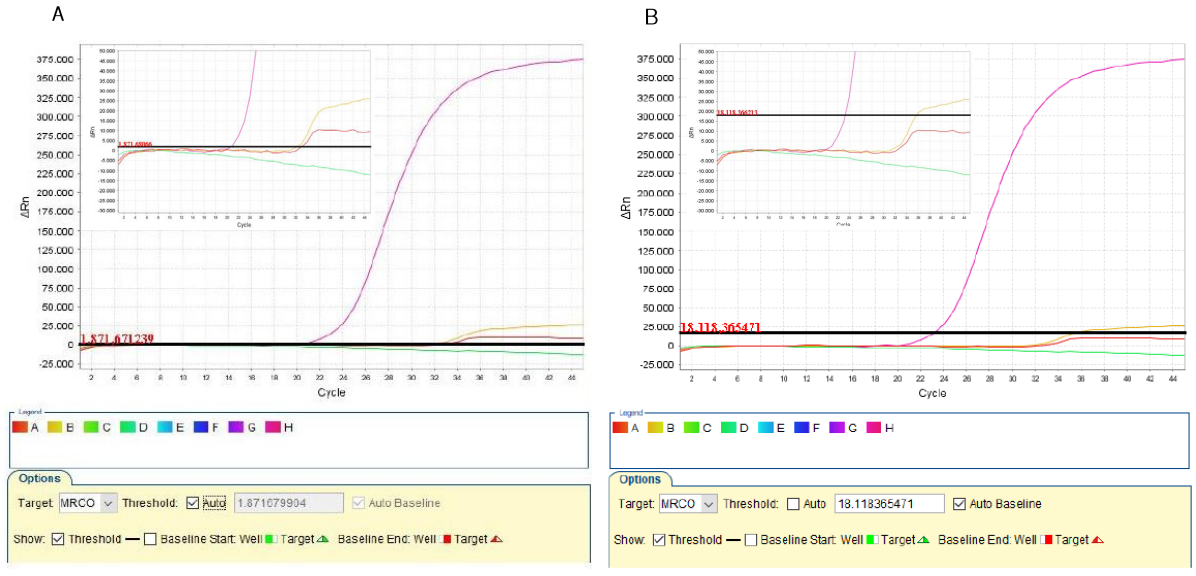


Figure 2. Amplification curves displayed after automatic (A) and manual (about 5 % of max fluorescence for positive control) (B) threshold setting. In the insets, a zoomed view of the intersection point between threshold line and amplification plots.

3. PCR Inhibition

As for any other PCR-based assay, also for mycoplasma detection in biologicals and cell cultures, components of the sample matrix can cause PCR inhibition. This increases the probability of obtaining false negative results and is due to interference with the enzyme/enzyme activity, with the template DNA, or other essential PCR reagents. Immunoglobulins (e.g. blood components) but also some salts or detergents can act as PCR inhibitors. In order to minimize this phenomenon and control its occurrence, we recommend mainly performing the following:

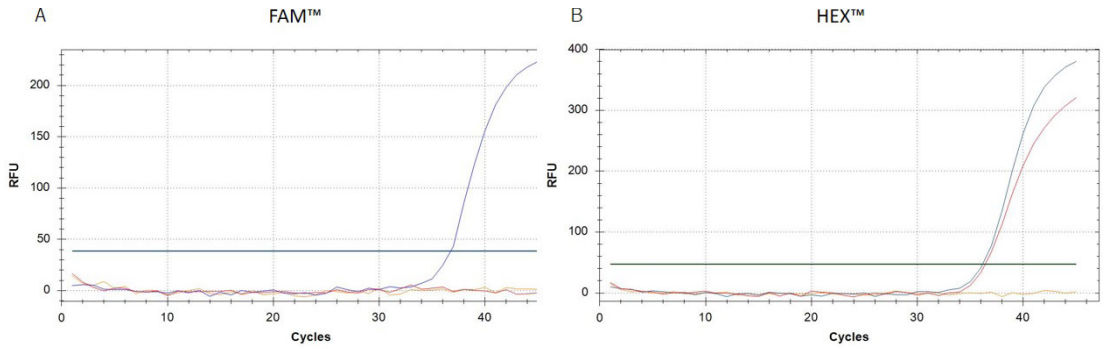
(1) Efficient DNA purification (in order to remove sample-related inhibitors)

(2) Careful analysis of the results obtained with the IC

In the Venor®GeM qEP kit, we suggest two possible methods to use the IC. One where the IC is added to the samples before the extraction and co-extracted with the mycoplasma DNA. This will allow the user to check on the performance of the extraction procedure as well as of the qPCR. Alternatively, the user simply adds the IC after the extraction, directly to the reaction tubes (both samples and controls). This allows the user to check exclusively on the qPCR reaction performance.

In case of fully or partly impaired qPCR reaction for the IC, the user should carefully consider the possibility that also target amplification might have fully or partly failed for that sample.

In Figure 3, a typical example of full qPCR inhibition is illustrated. In this case, a lack of FAM™ signal is accompanied by a lack of signal in the HEX™ channel (flat curves/lines below threshold). As a consequence, „No Ct“ values are obtained (Figure 3, table), which makes the interpretation of the results for these samples impossible.



	A: Ct Target	B: Ct IC
Weakly contaminated sample (blue)	36.75	36.18
Fully inhibited sample (orange)	No Ct	No Ct
Negative control (red)	No Ct	36.42

Figure 3. Amplification plots and corresponding Ct values obtained in a qPCR experiment, where full inhibition occurred. In this run, one sample showed weak but successful target amplification (A, blue curve) and one where qPCR was full inhibited (A and B, orange curve), resulting in flat, below-threshold curves. Internal control amplification for the negative control (B, red curve) is shown as an indicator of assay validity. In the table beneath the graphs the corresponding Ct values are reported.

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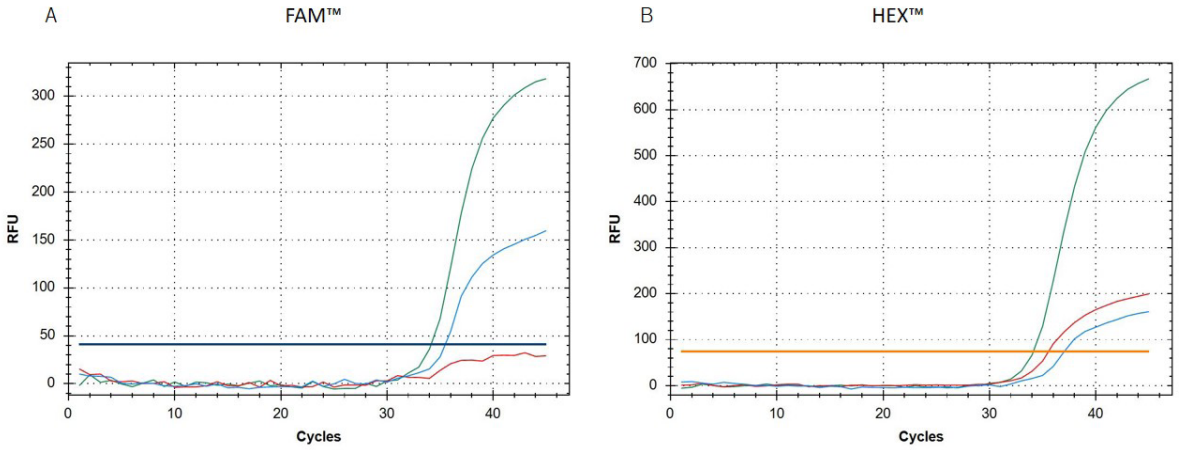
In contrast to this case, unfortunately, recognizing partial inhibition events can often be rather complex. Figure 4 shows three equally contaminated samples with different matrix characteristics. One sample (green) was clearly detected as positive, as demonstrated by the sigmoidally-shaped amplification curves and the Ct value below 40 (Fig. 4A and B). In contrast, the other two samples (red and blue curves) showed strongly reduced max fluorescence levels and flatter curves, in both channels. In one case (Fig. 4A, blue curves), the FAM™ Ct value was also below 40 and the sample was still detected as positive. In the other case (Fig. 4A, red curve), the FAM™ signal was much lower, remained below threshold and the sample was detected as negative. The amplification of the internal control was still detectable for both samples, hinting at the validity of the qPCR. However, the greatly reduced HEX™ max fluorescence levels rather indicate that the amplification was impaired for these two samples (Fig. 4B), regardless of the Ct values and fluorescence signals in the FAM™ channel. More generally, the greater the IC signal and Ct deviate for individual samples, the greater the probability of detecting invalid results due to qPCR perturbations.
This example illustrates how the sample matrix can affect the qPCR performance leading to the potential detection of false negatives (see red curve in A) and how the analysis of the Ct values without the assessing the amplification plots can be misleading when partial inhibition occurs.



	A: Ct Target	B: Ct IC
Contaminated sample (green curve)	34.16	34.13
Partly inhibited sample (blue curve)	35.50	37.03
Partly inhibited sample (red curve)	No Ct	35.55

Figure 4. Amplification plots and corresponding Ct values obtained in a qPCR experiment, where partial inhibition occurred. Despite being all equally contaminated, only one sample showed successful target amplification (A, green curve). The other two presented lower fluorescence levels and flatter curves in both channels (A and B, blue and red curves) as well as distinct Ct shifts in the HEX™ channel (table beneath the graphs, compared to the positive sample, green). According to the applied threshold, a sample (A, blue) was detected as positive/contaminated whereas the other one (A, red) as negative/non-contaminated. In the table beneath the graphs the corresponding Ct values are reported.

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Generally, in the absence of significant inhibitory events, we observe that truly negative samples (FAM™ ≥ 40) show Ct values in the HEX™ channel similar to those obtained for the corresponding negative controls (not more than + 2 or +3 cycles the Ct of NTCs, if used as a PCR control or process control, respectively). Inhibitory events due to the sample matrix can but do not have to correlate with significant rightward shifts of the amplification curves in the HEX™ channel. Also large fluctuations of the IC Ct values in samples with different matrices can indicate the presence of interfering components in the matrices.
However, often, a partly impaired qPCR reaction does not result in dramatic Ct shifts (see Figure 4, Ct value for the red curve). Namely, a partial PCR inhibition might rather be revealed by reduced max. fluorescence levels or flatter curves in the HEX™ channel. Whether or to which extent the corresponding Ct values shift depends ultimately on the threshold settings, as illustrated for example for FAM™ channel in Figure 2.

4. Competition

The IC used in the Venor®GeM qEP kit is an exogenous, homologous internal control: an artificial template included in the kit, containing the same primer binding sites as the main mycoplasma target (but different sequences overall). A logical consequence of this type of approach is the occurrence of a certain degree of competition between target and IC amplification, which depends on (1) how much internal control template is introduced in the reaction and (2) how much main target is available in the sample.
Given a constant, carefully calibrated amount of IC in the qPCR (see manual), the target amplification will depend on the amount of mycoplasma DNA in the samples, within the sensitivity limits of the kit. When the target DNA is in strong excess (strongly contaminated samples or positive controls), the amplification of the IC will be reduced or suppressed (Table 4). In contrast to the case of negative samples/controls, a lack of IC amplification in positive samples/controls represents a valid result. In fact, when a positive result is obtained in the target (FAM™) channel, the amplification of the IC (HEX™ channel) loses its significance and can be ignored in the analysis of that sample.
When correctly interpreted, rather than impairing the validity of the results, the missing amplification of the IC in strongly positive samples can provide additional information on the sample characteristics (e.g. contaminant load).

Table 4. Ct values obtained in a routine run with the addition of hypothetically processed samples showing competition effects between target and IC amplification. Here, a leftward shift in the target Ct values is accompanied by a rightward shift of the IC Ct values and correlates with the progressively higher target DNA amounts (*Mycoplasma fermentans* genome copies, GC per µl).

Sample	Mean Ct (Target)	Mean Ct (IC)
No-template control (NTC)	No Ct	32.57
Cell culture supernatant spiked with 40 GC/µl of *M. fermentans*	27.59	31.79
Cell culture supernatant spiked with 400 GC/µl of *M. fermentans*	24.42	33.96
Cell culture supernatant spiked with 4000 GC/µl of *M. fermentans*	20.73	No Ct

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Glossary

NTC: no-template control – In this experimental setting, it is represented by reaction tubes, where a supposedly DNA -negative sample (e.g. PCR grade water) is added to the PCR master mix. The significance of this control is crucial in case of positive samples results, in order to exclude the presence of contaminants in the reagents (PCR master mix and PCR grade water). As a rule, PCR grade water is a sensible choice as negative control. However, in some cases it might be reasonable to control also for the sample matrix. Particularly, if DNA extraction is omitted, a sample of culture medium (as an additional NTC) allows to check simultaneously for mycoplasma contaminations of the medium and for potential PCR interference of its components. After DNA extraction, a more significant sample to use as NTC is the elution buffer used for the extraction.

NEC: negative extraction control – The negative extraction control is a very informative control consisting of a truly negative sample matrix, which is processed in parallel with the test samples. A negative result for the NEC helps ensuring that contamination is not due to the sample matrix or arising during the extraction process.

PC: positive control – another important control to validate your PCR. A well-established template DNA will ensure further reliable verification of PCR performance. It is strongly recommended to add the positive control DNA to the reaction mix in a dedicated area to avoid cross-contamination with other samples. Furthermore, another essential practice regarding the PC is to aliquot it into low-binding plastic tubes, right after rehydration and to freeze immediately it (avoid freeze thawing).

IC: internal control – This control is a DNA template run and simultaneously amplified with the sample target or the positive control, in absence of experimental issues. It is an outstanding validation tool showing that the PCR is amplifying products, as expected and can help rule out false negatives. The IC can be added before extraction (directly to the samples) or after extraction, prior to PCR (to the master mix). In the first case, the IC will function as a process control, pointing at technical problems occurred during the extraction and/or during the PCR. In the latter case, the IC acts as PCR control and helps excluding PCR inhibition.
A successful IC amplification is associated by a fluorescence increase in the HEX™ channel, which validates mycoplasma negative results, lacking amplification in the FAM™ channel. In the case of the Venor®GeM kits, the internal control is designed to compete weakly with the mycoplasma-specific target of the amplification. Consequently, when large amounts of mycoplasma DNA are initially present (e.g. strong sample contamination), the amplification of the IC DNA might not be detected. This is simply due to competitive effects within the reaction mix and not to experimental problems or PCR inhibition. However, if a reaction shows neither target nor internal control amplification, one has to conclude that either the extraction or the PCR reaction did not work properly. In this case, the user should review the experimental conditions to verify the occurrence of false negatives.

Threshold – The threshold is the point where a curve starts rising from the baseline, to a statistically significant degree. This value is assigned to each PCR run separately, and is essential for the definition of the threshold cycle (Ct) and the identification of positive results.

Ct (Threshold cycle) – This is the cycle number at which the fluorescence generated within a reaction crosses the threshold. The Ct value reflects the point expressed in cycles during the PCR run, where a statistically significant amount of template has been amplified. This value is widely used to perform comparisons between samples and correlates to the concentration of the target in the respective sample.

Appendix

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