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CROP PROTECTION

Absolute quantification of viruses by TaqMan real-time RT-PCR in grapevines

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ABSTRACT: *The absolute quantification determines the absolute amount of a targeted nucleic acid expressed as a copy number or concentration. The knowledge of virus concentrations in commercial crops possesses high relevance to ensure a reliable diagnosis. The objective of this study was to perform an absolute quantification of five viruses in infected grapevines (Vitis spp.). Different known amounts of the standard sample (cloned viral cDNA or in vitro transcribed viral RNA) were quantified by TaqMan RT-qPCR. Based on these data, standard curves were generated plotting Ct values (threshold cycle) against the log of the standard sample amount. Infected grapevine samples were evaluated to determine virus titers, which were highly variable. This result may contribute to improve virus diagnosis by accurately quantifying virus titre variations in grapevines.*
Key words: *RT-qPCR, GRSPaV, GVA, GVD, GLRaV-3 and -4.*

Quantificação absoluta de vírus por RT-PCR em tempo real (TaqMan) em videiras

RESUMO: *A quantificação absoluta determina a quantidade absoluta de um ácido nucleico alvo expressa como número de cópias ou concentração. O conhecimento das concentrações virais em culturas comerciais tem grande relevância para assegurar um diagnóstico confiável. O objetivo deste trabalho foi realizar uma quantificação absoluta de cinco vírus em videiras infectadas (Vitis spp.). Diferentes quantidades conhecidas da amostra padrão (cDNA viral clonado ou RNA viral transcrito in vitro) foram quantificadas por RT-qPCR TaqMan. A partir destes dados, curvas padrão foram geradas plotando-se os valores de Ct (ciclo limiar) contra o log da quantidade da amostra padrão. Amostras de videiras infectadas foram avaliadas visando-se determinar os títulos virais que foram bastante variáveis. Este resultado contribui para melhorar o diagnóstico viral ao quantificar com precisão variações no título viral em videiras.*
Palavras-chave: *RT-qPCR, GRSPaV, GVA, GVD, GLRaV-3 e -4.*

Among grapevine pathogens, viruses stand out, considering that this crop is susceptible to numerous graft-transmissible agents that cause several diseases, negatively affecting plant vigor, yield, productivity and fruit quality. Grapevine leafroll disease (GLRD) is the most economically damaging and widespread viral disease of grapevine throughout the world. Several viral species designated *Grapevine leafroll-associated virus* (GLRaV) belonging to the family Closteroviridae are related to GLRD, which can occur alone or as a viral complex. GLRaV-3 is the most prevalent and widely spread worldwide similarly to GLRaV-4, both classified in the genus *Ampelovirus*. Grapevine rugose wood disease (GRWD) is a complex disease, occurring in several grapevine growing regions, associated with four distinct syndromes: rupestris stem pitting, kober stem grooving, corky bark and LN33 stem grooving. Among the associated viral agents, there are *Grapevine rupestris stem pitting-associated virus* (GRSPaV, genus *Foveavirus*), *Grapevine virus A* and *D* (GVA and GVD, *Vitivirus*), all belonging to the family Betaflexiviridae (MARTELLI, 2014).

There are two possibilities for performing viral quantification: the absolute quantification, which determines the absolute amount of a DNA or RNA target, expressed as a number of virus copies or concentration and the relative quantification, which determines the rate between the amount of a DNA or RNA target and the amount of a reference nucleic acid. In both cases a standard sample with a known concentration is required (QIAGEN, 2014), such as: viral RNA extracted from purified viral particles, viral RNA transcribed *in vitro* or cloned viral cDNA (a recombinant plasmid inserted into *Escherichia coli*). The difficulty in obtaining a standard sample with known concentration lies

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in two features: the total RNA, extracted from the infected plant, consists of plant RNAs and the viruses infecting it and, viral RNA purified from particles, usually obtained from purification of a viral complex that is infecting the plant. In both situations, it is not possible to quantify the viral RNA of a particular virus. This type of extracts is not suitable as standard sample. To overcome this obstacle it is possible to use a standard sample obtained from a cloned viral cDNA or viral RNA transcribed *in vitro* (RUIZ-RUIZ et al., 2009; FERRIOL et al., 2011). These two possibilities were explored in this work. The aim of this research was to generate standard curves for absolute quantification of five viruses by quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) in infected grapevines (*Vitis* spp.).

The total RNA extractions from 1g of scrapings of mature stems were performed using the adsorption of nucleic acids on silica particles, grinding plant tissues in liquid nitrogen (DUBIELA et al., 2013). In all performed analyses, healthy grapevines and positive controls from mother stock plants and viral collections at Embrapa Grape and Wine, respectively, were included. RT-qPCR reactions carried out in 96-well plates, consisted of: 3µL of the 4x TaqMan Fast Virus 1-Step Master Mix (Life Technologies), 0.6µL of the mixture of primers and probe (415nM primer and 85nM probe), 3µL of total RNA (ca. 300ng) and 5.4µL of water. Reactions were performed in a thermocycler StepOnePlus Real-time PCR System (Applied Biosystems) as follows: 45ºC for 35min (for reverse transcription), 95ºC for 10min, followed by 40 cycles at 95ºC for 15s and 60ºC for 1min. The reaction data were analyzed using the StepOne Software v.2.3 (Applied Biosystems) by determining the Ct (threshold cycle). For qPCR, 20µL reactions were performed using Platinum *Taq* DNA polymerase (Invitrogen) following manufacturer recommendations, ROX (reference fluorophore) and the same amplification cycle without reverse transcription step. The primers and probes used for viruses detections by RT-qPCR and their specificity and efficiency have been previously mentioned by OSMAN et al. (2007) and DUBIELA et al. (2013). All probes were labeled with 6-FAM or VIC and TAMRA in the 5' and 3' ends, respectively.

Recombinant plasmids containing cloned viral cDNA were purified from transformed *E. coli* cultures using the Wizard Plus SV Minipreps DNA Purification System kit (Promega), and they were quantified by spectrophotometry (1OD to A_{260nm}=50µg dsDNA mL^{-1}). The cDNA clones used to generate equations of standard curves for absolute quantification of Brazilian isolates of viruses infecting grapevines by RT-qPCR were previously obtained and their nucleotide sequences are available at GenBank <http://www.ncbi.nlm.nih.gov>: GVA (accession code AF494187), GVD (JQ031715), GRSPaV (EF636804) and GLRaV-3 (KC519443). The partial virus sequences of these recombinant plasmids were used as templates for amplifications by RT-qPCR using oligonucleotides and probes mentioned by DUBIELA et al. (2013). The number of viral cDNA molecules in the standard sample was calculated using the formula: Y molecules μL^{-1}=(X g μL^{-1} DNA/[base pairs of recombinant plasmid x 660]) x $6.022x10^{23}$ (QIAGEN, 2014).

For GLRaV-4, a standard sample of viral RNA was obtained by *in vitro* transcription from a multiple viral fragments cloned in tandem into a transcriptional recombinant vector (provided by J. A. Sánchez Navarro, IBMCP-CSIC, Spain), aiming to generate the standard curve. After *in vitro* transcription using the T7 RNA polymerase enzyme and dNTP mix (Roche), the template plasmid DNA was removed by *DNase* I digestion (Ambion) and the concentration of the transcribed RNA was determined by spectrophotometry (1OD to A_{260nm}=40µg ssRNA mL^{-1}) and the number of copies of RNA was calculated using the formula: Y molecules μL^{-1}=(X g μL^{-1} RNA/[transcript length in nucleotides x 340]) x $6.022x10^{23}$ (QIAGEN, 2014).

To generate external standard curves, the standard sample was adjusted to known concentrations [5-6 points in triplicates, tenfold serial dilutions at different concentrations of cloned plasmid viral cDNA (from 0.1 to $1x10^{-6}$ng μL^{-1}) and transcribed viral RNA (from 0.3108 to $3.108x10^{-6}$ng μL^{-1}) extracts with these range concentrations also expressed as corresponding viral cDNA or viral RNA molecules] in two independent assays per virus and then detected by RT-qPCR.

Standard curves for each virus were constructed by plotting Ct values versus the logarithm of the RNA copy number (Ct vs the log of the standard sample amount) using the StepOne Software (Applied Biosystems). Regression analyses were adjusted to the standard curves using the Excel software (Microsoft). After this, 76 infected grapevines included in the assays were evaluated by RT-qPCR with the obtained Ct values being inserted in the linear equation aiming at determining the viral titers.

Thus, a study was carried out for the absolute quantification (number of copies) of five viruses aiming to support the implementation of improvements in diagnostic tests. The use of a DNA template, in the generation of the standard curve, does not consider the variable efficiency of reverse transcription (RT) reaction in RT-qPCR; however, for GLRaV-4, the use of transcribed RNA standard takes into account the variable efficiency of the RT reaction.

In relation to the absolute quantification, the following equations of the standard curve (plot of Ct value against the log of the standard sample amount) were determined for the five assayed viruses: $y = -1.528 x + 39.43$ for GVA with coefficient of determination (R^2=0.9988), $y = -1.373 x + 36.374$ for GVD (R^2=0.9968), $y = -1.73 x + 46.015$ for GLRaV-3 (R^2=0.9983), $y = -1.348 x + 37.208$ for GRSPaV (R^2=0.9988) and $y = -1.413 x + 39.701$ for GLRaV-4 (R^2=0.9985), where y = Ct value and x = Log copy number (DNA or RNA molecules μL^{-1}) (for example: GLRaV-4; Figure 1).

Regression analysis of the three independent standard curves demonstrates that the assay is highly repeatable (for example: GLRaV-4; Figure 1B), indicating that assay can be used with a standard curve to accurately estimate viral titers. All obtained indices to measure the absolute quantification i.e. amplification efficiency (*E*) value (calculation of efficiency of the PCR amplification; *E* was calculated using the slope of the regression line in the standard curve); slope (regression coefficient calculated from the regression line in the standard curve. The slope indicates the PCR amplification efficiency for the assay) and R^2 value (regression coefficient calculated from the regression line in the standard curve and indicates the closeness of fit between the standard curve regression line and the individual Ct data points from the standard reactions) were compatible with optimum efficiency indicating high adjustment of the obtained curves and equations.

The Ct values of the viral target, determined by RT-qPCR for each infected grapevine assayed in the experiment, were inserted into the standard curve (used as a reference in all subsequent reactions), allowing to determine the viral titers in the analyzed samples (Figure 1). Thus, the absolute amounts of nucleic acids of GRSPaV, GVA, GVD, GLRaV-3 and GLRaV-4 in the analyzed samples were highly variable with values concentrated in the range from ca. 500 to 150,000 copies of virus per µL of total RNA. The viral titer in the host plants and also in insect vectors (aphids, mealybugs) can vary widely, depending on the viral species or strain, plant species, tissue of the plant analyzed, phase of the vegetative cycle, time of the year and environmental conditions (mainly temperature), plant nutrition, presence of multiple viral infections, time since initial infection and, viral replication rate, among other factors (BERTOLINI et al., 2008).

A RT-qPCR procedure using TaqMan probes has been developed for sensitive and specific detection and quantitation of many plant viruses infecting different hosts and insect vectors. In these studies, standard curves using RNA transcripts identical to TaqMan probes enabled absolute quantitation, with a wide dynamic range and high sensitivity. *Broad bean wilt virus* 1 was detected by absolute quantification in the range 10^3-10^{10} (average number of copies of viral RNA per ng of total RNA from infected plants) (FERRIOL et al., 2011). The estimated number of *Citrus tristeza virus* (CTV) RNA-targets detected in different tissues of a CTV-infected tree ranged from $4.5x10^5$ to $6.5x10^8$ copies when purified RNA was used as template (BERTOLINI et al., 2008). RUIZ-RUIZ et al. (2009) detected until 100 copies of *Citrus leaf blotch virus* (CLBV) RNA in various tissues and citrus varieties infected with CLBV isolates. Less than 1fg was consistently detected when RNA transcripts of CTV were diluted in extracts of healthy plants while RNA copies carried by single aphids were estimated to be between 12,000 and 13,000,000 (SAPONARI et al., 2008). A standard curve using RNA transcripts enabled absolute quantitation, with a dynamic range from 10^4 to 10^{10} *Tomato torrado virus* RNA copies per ng of total RNA (HERRERA-VÁSQUEZ et al., 2015). The measurable concentration by RT-qPCR fell to as low as 10^0 copies of *Impatiens necrotic spot virus* per µL, while RT-PCR could detect only 10^2 copies per µL (CHEN et al., 2013).

Reliable quantitation of five virus targets present in infected grapevine materials was achieved with nucleic acid extraction procedures combined with RT-qPCR. This result contributed to the improvement of the viral diagnosis, since it allows to quantify viral titer variations in infected vines. Use of diagnostic methods such as RT-qPCR, which allows not only the identification and detection of the viral pathogen, but also the quantification of viral titer, is particularly important in certain activities, such as to quantify the genetic resistance of a particular host genotype or assays of the elimination of grapevine viruses using thermotherapy, tissue culture or antiviral drugs.

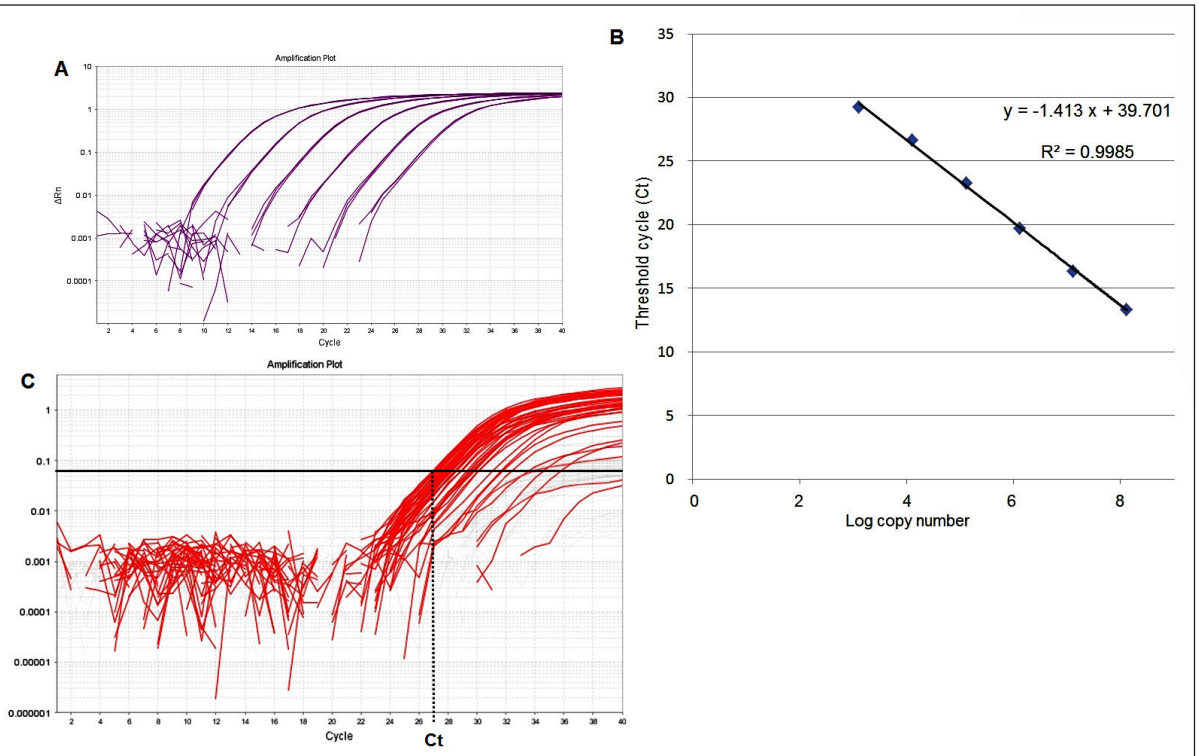

Figure 1 - Quantification of *Grapevine leafroll-associated virus 4* (GLRaV-4) by TaqMan RT-qPCR. A. Amplification plot obtained at different concentrations of transcribed viral RNA (standard sample) in triplicate showing in the Y-axis (ΔRn = normalized fluorescence intensity) and in the X-axis (number of cycles), B. Standard curve prepared with 10-fold serial dilutions of *in vitro* RNA transcripts from GLRaV-4 and obtained by regression analysis plotting the threshold cycle values (Ct) vs. the logarithm of the actual starting RNA copy number. The Ct values plotted for each dilution are the mean of three replicates. Coefficient of determination (R^2), calculated amplification efficiency (E=102.414%) and slope (-3.265) are indicated, C. Amplification results of TaqMan RT-qPCR to evaluate the viral titer in 76 infected grapevine samples highlighting the Ct value position.

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REFERENCES

BERTOLINI, E. et al. Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR. **European Journal of Plant Pathology**, v.120, p.177-188, 2008. Available from: <http://link.springer.com/article/10.1007/s10658-007-9206-9>. Accessed: Mar. 24, 2017.

CHEN, X. et al. Development of a real-time fluorescent quantitative PCR assay for detection of *Impatiens necrotic spot virus*. **Journal of Virological Methods**, v.189, p.299-304, 2013. Available from: <http://www.sciencedirect.com/science/article/pii/S016609341300061X>. Accessed: Mar. 24, 2017.

DUBIELA, C.R. et al. Simultaneous detection of Brazilian isolates of grapevine viruses by TaqMan real-time RT-PCR. **Tropical Plant Pathology**, v.38, p.158-165, 2013. Available from: <http://dx.doi.org/10.1590/S1982-56762013000200011>. Accessed: Mar. 24, 2017.

FERRIOL, I. et al. Detection and absolute quantitation of *Broad bean wilt virus 1* (BBWV-1) and BBWV-2 by real time RT-PCR. **Journal of Virological Methods**, v.177, p.202-205, 2011. Available from: <http://www.sciencedirect.com/science/article/pii/S0166093411003211>. Accessed: Mar. 24, 2017.

HERRERA-VÁSQUEZ, J.A. et al. Detection and absolute quantitation of *Tomato torrado virus* (ToTV) by real time RT-PCR. **Journal of Virological Methods**, v.221, p.90-94, 2015. Available from: <http://www.sciencedirect.com/science/article/pii/S0166093415001731>. Accessed: Mar. 24, 2017.

MARTELLI, G.P. Directory of virus and virus-like diseases of the grapevine and their agents. **Journal of Plant Pathology**, v.96, suppl, p.S1-S136, 2014. Available from: <http://dx.doi.org/10.4454/JPP.V96I1SUP>. Accessed: Mar. 24, 2017.

OSMAN, F. et al. Real-time RT-PCR (TaqMan) assays for the detection of *Grapevine leafroll associated viruses* 1-5 and

9. **Journal of Virological Methods**, v.141, p.22-29, 2007. Available from: <http://www.sciencedirect.com/science/article/pii/S0166093406004083>. Accessed: Mar. 24, 2017.

QIAGEN. **QuantiFast SYBR Green PCR Handbook. For fast, quantitative, real-time PCR and two-step RT-PCR using SYBR Green**. Qiagen, 2014. 44p. Available from: <https://www.qiagen.com/us/resources/resourcedetail?id=43137f31-afd9-4916-b53c-c18b0fe505b8&lang=en>. Accessed: Mar. 24, 2017.

RUIZ-RUIZ, S. et al. Detection and quantitation of *Citrus leaf blotch virus* by TaqMan real-time RT-PCR. **Journal of Virological Methods**, v.160, p.57-62, 2009. Available from: <http://www.sciencedirect.com/science/article/pii/S0166093409001931>. Accessed: Mar. 24, 2017.

SAPONARI, M. et al. Quantitative detection of *Citrus tristeza virus* in citrus and aphids by real-time reverse transcription-PCR (TaqMan). **Journal of Virological Methods**, v.147, p.43-53, 2008. Available from: <http://www.sciencedirect.com/science/article/pii/S016609340700300X>. Accessed: Mar. 24, 2017.