Preparation of Defined Reference Material for Molecular Testing of "Bois Noir" Phytoplasma

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***Abstract*: In the field of plant health, and in particular for unculturable pathogens or pathogens difficult to grow, there is a lack of availability of reference materials, here defined as well-characterized material suitable to be used as reference positive test control. Reference materials differ in their commutability and complexity, as well as the effort required for their preparation and characterization. Here the preparation of two types of reference materials based on synthetic DNA and well characterized DNA of naturally contaminated plant material of grapevine on an example of "stolbur" phytoplasma associated with "bois noir" disease of grapevine is described. The materials are suitable as controls for quantitative PCR with the specific target of the 16SrXII group and for a wide variety of molecular tests. They provide comparability among tests, laboratories and over time, and are a useful reference in validation studies, test performance studies and proficiency tests.**

***Key words*: absolute quantification, "bois noir" phytoplasma, digital PCR, reference material, synthetic DNA.**

I. INTRODUCTION

Molecular methods are an important component of many diagnostic schemes or even the only method allowing sensitive and high throughput detection of certain plant pathogens. One of the main challenges encountered in both diagnostics, development of methods and research is the absence of certified (or other) reference materials. This requires that laboratories themselves prepare in-house controls for both validation and routine testing. While this is relatively straightforward for some pathogens which can be efficiently grown in artificial media, the preparation of reliable controls for obligate parasites is much more demanding, time consuming or not feasible at all. However, characterized reference materials are necessary to allow comparative analyses in different laboratories. Two levels of reference materials were defined: (i) synthetic dsDNA, and (ii) well-characterized DNA extracted from naturally infected plant material.

II. MATERIAL AND METHODS

Design of synthetic DNA control

The sequences of synthetic dsDNA which may serve as reference material for the specific test (in this case Hren *et al.* 2007) were selected based on blast analysis of the primers and probes used.

Plant samples

For the preparation of reference material, three plant extracts (DNA) prepared from grapevine leaf veins positive for 16SrXII group phytoplasmas, a group which includes '*Candidatus* Phytoplasma solani', agent of "bois noir" (BN) by quantitative PCR (qPCR; Hren *et al.* 2007) were used. The phytoplasma was characterized in *tuf* gene by RFLP and sequencing of TufAY nested PCR product as described by Aryan *et al.* (2014) (Vidmar, 2017). DNA was extracted from samples as described in Mehle *et al*. (2013a). The test is based on Hren *et al.* (2007) with the testing protocol published by Mehle *et al*. (2013b). Additional validation data for the diagnostic test used are available in the EPPO database on diagnostic expertise (http://dc.eppo.int/validationlist.php, NIB report Detection of FD and BN by real time PCR).

All of the qPCR reactions for phytoplasma testing were performed in triplicates on an ABI 7900 HT Fast Sequence Detection system (Life Technologies) using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C, and 1 min at 60°C, using standard temperature ramping mode. The reaction volumes of 10 µL contained, as final concentrations: 900 nM primers, 20 nM FAM and BHQ-1 labelled probes (all Integrated DNA Technologies), 1×TaqMan Universal PCR Master Mix (Life Technologies), and 2 µL 10-fold diluted sample DNA. The qPCR data were analysed using the SDS software 2.4 (Life Technologies) with automatic baseline and a manual threshold of 0.065. The qPCR data are given as average Cq values (qPCR quantification cycle).

Digital PCR for quantification of the target copies of phytoplasma

A previously validated test for the detection of BN phytoplasma was directly transferred to droplet digital format and used for quantification of target copy numbers. The DNA was detected and quantified using the droplet digital PCR system QX100™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA, USA). dPCR Supermix for probes (12 µL, Bio-Rad) and sample DNA (8 µL) were used, with the primer

and probe concentrations as for the qPCR. After droplet generation, 40 µL of the generated droplet emulsion was transferred to a new 96-well PCR plate (Eppendorf) and amplified in a T100™ Thermal Cycler (Bio-Rad). The amplification conditions were 10 min DNA polymerase activation at 95°C, followed by 45 cycles of a two-step thermal profile of 30 s at 94°C for denaturation, and 60 s at 60°C for annealing and extension, followed by a final hold of 10 min at 98°C for droplet stabilization and cooling to 4°C. The temperature ramp rate was set to 3°C/s, and the lid was heated to 105 °C, according to the Bio-Rad recommendations. After the thermal cycling, the plates were transferred to a droplet reader (Bio-Rad). The software package provided with the dPCR system (QuantaSoft 1.7.4.0917, Bio-Rad) was used for data acquisition and raw data analysis. Microsoft Excel was used for further data analysis and visualization.

III. RESULTS

Design of a synthetic DNA control

The target sequence includes flanking regions that improve the efficiency of amplification and mimic more natural conditions were selected (Fig. 1). Synthetic dsDNA (e.g. gBlock, IDT) is cheap and provides high amounts of target sequence suitable for both (i) technical assessment of tests, particularly in the initial stages of evaluation, and (ii) suitable positive control for testing. While a potential drawback is that, the synthesized DNA is sequence i.e. target specific and therefore test specific, the known size and amount allow to prepare material with relatively well defined target concentration without additional testing.

Characterization of naturally contaminated plant samples with digital PCR

Protocols were designed to characterize the DNA samples using digital PCR and to assign reference values i.e. target DNA concentration. As a method enabling absolute quantification without the need for standards, digital PCR (dPCR) is currently used as a better method in metrological and clinical fields. An important advantage of dPCR over quantitative PCR is that it can be used to absolutely quantify target's concentrations without the need for calibration, which simplifies both experiments and data comparability. Based on previous experiences with dPCR for quantification of phytoplasmas (Mehle *et al*. 2013a, 2014), reference material was prepared for the "bois noir" phytoplasma (BN) as a test case.

The direct transfer of the assay was successful, with positive and negative controls giving the expected results (data not shown). In addition, amplification was successful in samples of grapevine leaf vein tissues with DNA extracted and purified as described above (Fig. 2). The number of accepted droplets ranged from 12,493 to 16,312 indicating good quality of reaction preparation. The number of positive droplets in the three positive samples ranged from minimum 66 (in a 100x diluted sample) to maximum 1144 (in a 10x diluted sample).

Using dPCR, it was possible to assign reference values to the samples i.e. the absolute concentration of the target copy numbers in each DNA sample (Table 1).

Original samples all contained high concentrations of phytoplasma. In the plant extracts used, the concentration of the target DNA was above log 6 DNA target copies/mL of undiluted DNA in all cases. Assuming that the target DNA fragment is present in one copy per genome and phytoplasma cell, the concentration of phytoplasma in plant material was above 10^6 cells/mL of plant extract. The high concentration of phytoplasma means that it is possible to prepare reference material (control samples) containing a range of defined concentrations in relatively large quantities by mixing the DNA samples quantified with DNA of healthy plant material.

Based on the described test case it is possible to conclude that the approach and the resulting material is suitable for further use. The protocol itself can be easily transferred to other phytoplasmas or other harmful organisms, and can be used to assign values to naturally infected samples or artificially prepared defined mixtures.

> gBlock control on KT281865.1 '*Candidatus* Phytoplasma solani' strain STOL3 1-acyl-sn-glycerol-3-phosphate acyltransferase gene, partial cds

ACGTAAAACAGCTTTAAGTTTAATAAAGGCAATTCCAAAAGTAA**AAGCAGGTTTAGCGATGGTTGT**T**TTTCCTGAAGGTGGTATTAA**AGATCG**AAATGATGAAGCAACGGTACCA**CTTTTAGAGGGGTCTTTTAAAATTGCTTTTAAAACGCAAGCCGA

Figure 1. DNA sequence of the proposed synthetic DNA control for the BN amplicon targeted by qPCR described by Hren et al. (2007). Bold and underlined text areas correspond to primer and probe annealing sites.

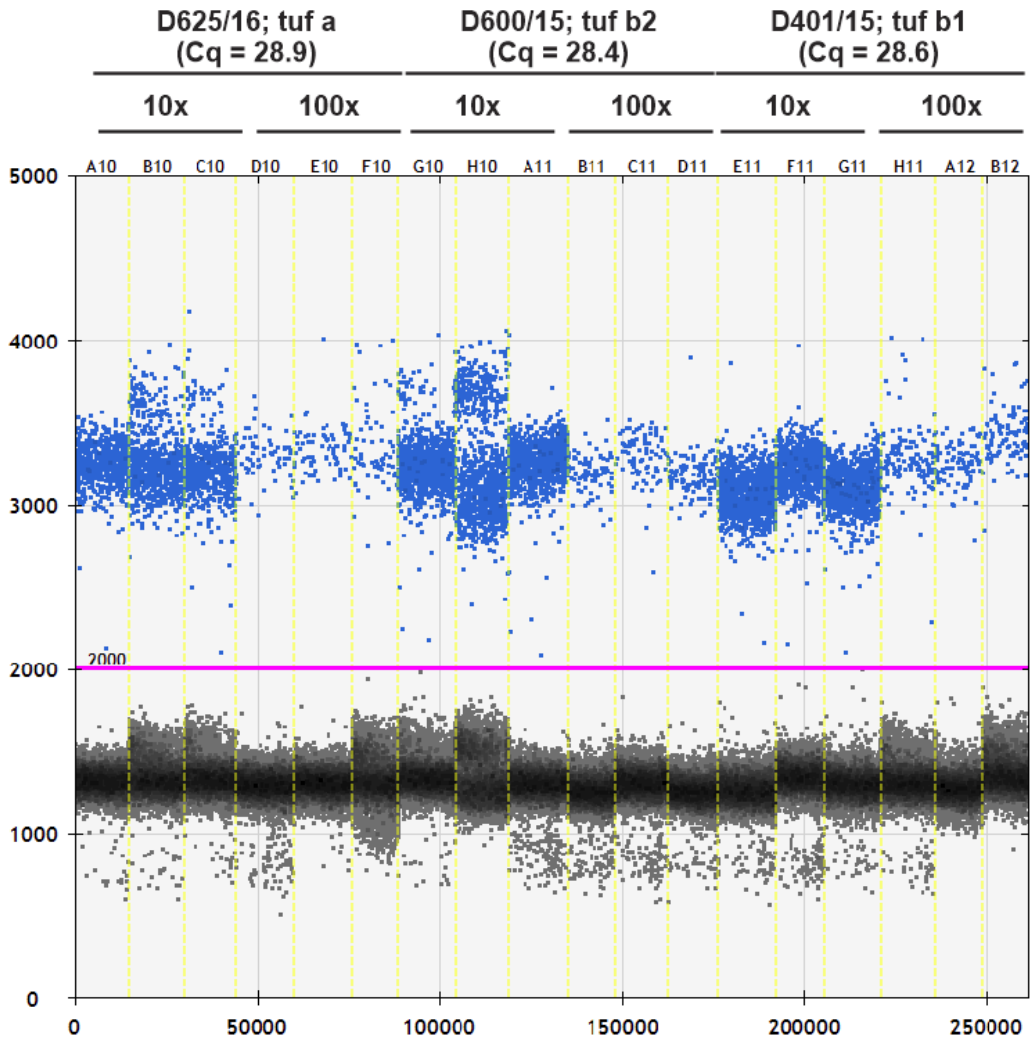

Figure 2. Digital PCR analysis of target copy numbers in three samples of grapevine, known to be infected by "bois noir" phytoplasma. Each sample was analysed in decimal dilutions (10x and 100x diluted in molecular grade water), each in three replicates. For each sample droplets are depicted according to the event (droplet number as read, x-axis) and their fluorescence in FAM channel (y-axis). The threshold discriminating between negative and positive droplets (pink line) was set manually at 2,000 relative fluorescence unit. Type of phytoplasma (tuf-type) and the average Cq value as determined in qPCR using Hren et al. (2007) system on 10-fold diluted DNA sample.

Table . Values assigned to the DNA samples expressed as copies (cps) or logarithm of copies (log cps) per stated volume of the original samples (undiluted DNA extracts, dilution factor taken into account). Min = minimum value, Max = maximum value, CV = coefficient of variation. Average Cq values as determined in qPCR using Hren et al. (2007) on 10-fold diluted DNA sample are shown.

Plant extract ID	Cq (BN)	cps/µL DNA		log(cps/mL DNA)	
		Min - Max	Average ± CV	Min - Max	Average ± CV
D625/16	28.9	1250 - 1525	1351.7 ± 0.071	6.10 - 6.18	6.13 ± 0.0046
D600/15	28.4	1675 - 2300	1972.2 ± 0.126	6.22 - 6.36	6.29 ± 0.0091
D401/15	28.6	2075 - 2600	2245.8 ± 0.091	6.32 - 6.41	6.35 ± 0.0055

IV. Discussion

The preparation of reference materials for molecular detection of “bois noir” “stolbur” phytoplasma using two approaches: (i) synthetic DNA, and (ii) well-characterized DNA of naturally contaminated plant samples was reported.

DNA sequences of the target region of qPCR originally described by Hren *et al*. (2007), together with nucleotide data available in NCBI, were used for designing synthetic DNA. Synthetic dsDNA (e.g. gBlocks, IDT) is affordable and provides high amounts of target sequence DNA suitable for both (i) technical assessment of tests, particularly in the initial stages of evaluation, and (ii) suitable positive control for testing. While the drawback is that the synthesized DNA is sequence specific and therefore test specific, the known size and amount allows to prepare material with relatively well defined target concentration without additional testing. Based on the selected target sequence the dsDNA reference material can be ordered commercially.

As a more commutable alternative (i.e. reference material) more similar to the diagnostic samples, several archived DNA samples originating from naturally contaminated grapevine plant material (leaf veins) were selected. Protocols were designed to characterize the reference material using digital PCR and to assign a reference value to the concentration of target sequences. Quantitative PCR was successfully transferred to digital PCR format and used to determine absolute concentration of the target DNA copies in DNA samples. As a method enabling absolute quantification without the need for standards, digital PCR is the method currently used as a higher order method in several metrological projects based on nucleic acid detection in the clinical field. An important advantage of dPCR over quantitative PCR is that it can be used to quantify target’s concentrations absolutely without the need for calibration. This simplifies both experimentation and data comparability. While controls prepared and characterized in this way are limited in their amounts by the availability of the material and require more effort for DNA extraction, they are more similar to actual samples tested than synthetic DNA and can be used in a variety of molecular tests.

Based on the needs either of the two types of reference material described here can be fit for purpose. The use of reference material for which the exact target concentration is known has the advantage of providing comparability over tests, test performance studies and over time, even for the unculturable harmful organisms. In addition, the approach described here is easily transferrable to all other phytoplasma and DNA targets.

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References

Aryan, A., Brader, G., Mörtel, J., Pastar, M., Riedle-Bauer, M. (2014). An abundant ‘*Candidatus* Phytoplasma solani’ tuf b strain is associated with grapevine, stinging nettle and Hyalesthes obsoletus. *European Journal of Plant Pathology*, 140, 213-227.

Hren, M., Boben, J., Rotter, A., Kralj, P., Gruden, K., Ravnikar, M. (2007). Real-time PCR detection systems for “flavescence dorée” and “bois noir” phytoplasma in grapevine: a comparison with the conventional PCR detection system and their application in diagnostics. *Plant Pathology*, 56, 785-796.

Mehle, N., Nikolić, P., Rupar, M., Boben, J., Ravnikar, M., Dermastia, M. (2013a). Automated DNA extraction for large numbers of plant samples. In: Dickinson, M., Hodgetts, J. (eds.). Phytoplasma: methods and protocols, (Methods in Molecular Biology, vol. 938), (Springer Protocols). New York: Humana Press, 253-268.

Mehle, N., Dreo, T., Ravnikar, M. (2014). Quantitative analysis of “flavescence doreé” phytoplasma with droplet digital PCR. *Phytopathogenic mollicutes* 4 (1): 9-15.

Mehle, N., Prezelj, N., Hren, M., Boben, J., Gruden, K., Ravnikar, M., Dermastia, M. (2013b). A real time PCR detection systems for the “bois noir” and “flavescence dorée” phytoplasmas and quantification of the target DNA. In: Dickinson, M., Hodgetts, J. (eds.). Phytoplasma: methods and protocols, (Methods in Molecular Biology, vol. 938), (Springer Protocols). New York: Humana Press, 253-268.

Vidmar, S. (2017). Molecular diversity of populations of phytoplasma '*Candidatus* Phytoplasma solani' in Slovene grapevine regions. M.Sc. Thesis. Ljubljana, University of Ljubljana, Biotechnical Faculty, Academic Study in Microbiology, 2017.