Absolute Real-Time PCR: A Comparison of Spectrophotometric and On-Chip Methods for External Standard Curve Construction from Different Nucleic Acid Dosages

Application Note

Agriculture Biotechnology and Nucleic Acid Analysis

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Abstract

Concentration determination of DNA using real-time quantitative PCR technology plays a substantial role for identification and quantitation of microbes in unknown samples in many research areas. Absolute quantitation with qPCR fully depends on the accuracy of the external standards. This Application Note focuses on establishing calibration standards by two different approaches – spectrophotometry and on-chip electrophoresis. Conventional spectrophotometric techniques for the evaluation of DNA concentration may lead to greater variation between standard curve replicates and bring more variability to final results. In contrast, the Agilent 2100 Bioanalyzer System provides accurate and precise quantitation of DNA, enabling the preparation of reliable standard curves for absolute quantitation with qPCR assays.

Introduction

Quantitation of DNA using real-time quantitative PCR (qPCR) represents a significant contribution in plant pathology by allowing identification and calculation of pathogens in the air, soil, water, seeds, plants, and so forth. Two major strategies for qPCR are based on relative or absolute quantitation[1]. Absolute quantitation qPCR assays are increasingly adopted to track and monitor airborne fungi in fruit and vegetable production areas. The accuracy of this method depends on the accuracy of the standard curves. Typical challenges include standard design, production, determination of the exact concentration, and storage stability. A precise quantitation by reliable independent methods is mandatory for successful qPCR.

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This Application Note compares the initial concentration determination of standards by two different approaches, spectrophotometry and on-chip electrophoresis. Based on the quantitative values obtained, the reproducibility of standard curve creation was evaluated. To compare these different approaches, absolute quantitation with qPCR was performed with standard curves generated from the DNA of two species of plant pathogens, *Erysiphe necator* and *Botrytis cinerea.* Those pathogens cause powdery mildew and grey mold on grapes and other plants, leading to potential crop loss and poor wine quality[3,4]. Specific amplicons were generated using standard PCR procedures. The purified amplicons were quantified using two spectrophotometric instruments, NanoDrop and NanoVue, and an Agilent 2100 Bioanalyzer System, an on-chip electrophoresis instrument. Based on the quantitation values given by each system, standard curves were generated by qPCR.

Experimental

Materials

E. necator and *B. cinerea* isolate, Internal Transcribe Spacer (ITS) specific primers for *E. necator,* Intergenic Spacer (IGS) specific primer for *B. cinerea,* specific forward primers, reverse primers, and probes were from Agriculture and Agri-Food Canada, St-Jean-sur-Richelieu, Qc, Canada. Details on specific primers and probes are not yet published. The NanoDrop 2000 was purchased from Thermo Scientific (Wilmington, DE, USA), the NanoVue Plus from Sigma-Aldrich (St. Louis, MO, USA). The NucleoSpin Gel and PCR Clean-up kit from MACHEREY-NAGEL GmbH & Co (Düren, Germany) was used for sample clean-up. SurePRIME DNA Polymerase was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). SureCycler 8800 Thermal Cycler (Cat#G8800A) with 96-well module (Cat#G8810A), Mx3005P qPCR system with MxPro software (Cat#401513), Brilliant II QPCR Low ROX Master Mix (Cat#600806), Agilent 2100 Bioanalyzer system (Cat# G2939AA), and Agilent DNA 1000 kit (Cat#5067-1504) were obtained from Agilent Technologies (Santa Clara, CA, USA).

PCR

For amplicon generation, the SureCycler 8800 Thermal Cycler equipped with a 96-well block was used. A 1 µL amount of DNA extracted from fungus isolates *E. necator* and *B. cinerea* was used for PCR. The concentration of the starting material was not determined. Specific primers were used depending on the species (Table 1).

The PCR reaction was performed following a conventional Taq protocol with SurePRIME polymerase. The 25 µL PCR reaction was started with a pre-incubation step at 95 °C for 10 minutes to activate the polymerase, followed by 45 cycles (94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 45 seconds), and finally 72 °C for 5 minutes.

Sample purification

The NucleoSpin Gel and PCR Clean-up kit was used according to the manufacturer's recommendation for for PCR clean-up.

DNA quantification with a spectrophotometer

For spectrophotometric DNA quantification, the NanoDrop 2000 and the GE NanoVue Plus were used. As suggested by the manufacturers, a 2 µL droplet of each purified amplicon was deposited on the measurement surface of the spectrophotometer. NucleoSpin kit elution buffer was used as a blank.

DNA analysis with on-chip electrophoresis

DNA electrophoresis was performed on the 2100 Bioanalyzer System in combination with the Agilent DNA 1000 Kit, according to the manufacturer's protocol[2].

Copy number calculation

The DNA copy number was calculated based on the quantitation results using the following equation:

Copy number = (concentration)/(molar mass) × (6.022×10^{23})

Standard preparation

The purified amplicon was used to prepare five standards ranging from 2×10^5 to 2×10^1 copies/µL.

qPCR

The Mx3005P qPCR system with MxPro software was used for real-time qPCR using Brilliant II QPCR Low ROX Master Mix with multiple standards. The 25 µL PCR reaction was performed according to the manufacturer's protocol, containing 1X-Brilliant II PCR buffer, 300 nM specific forward primers and reverse primers, 200 nM probe, and 2 µL DNA from 10x dilution of a standard. Specific primers and probes used are described in Table 1.

Results and Discussion

PCR was performed for two different genes from plant fungus species *E. necator* and *B. cinerea* as summarized in Table 1. Five independent PCR reactions for amplicon generation were performed using the same DNA as starting material. Internal Transcribe Spacer (ITS) specific primers for *E. necator* give an amplicon of 438 bp and Intergenic Spacer (IGS) specific primers

Table 1. Two species of plant fungus were used to test the absolute quantitation approach.

Source	*Erysiphe necator*	*Botrytis cinerea*
PCR	Internal Transcribed Spacer (ITS) specific primers for *E. necator*	Intergenic Spacer (IGS) specific primers for *B. cinerea*
RT-PCR	Internal Transcribed Spacer (ITS) specific qPCR primers and probe for *E. necator*. Size = 95 bp.	Intergenic Spacer (IGS) specific qPCR primers and probe for *B. cinerea*. Size = also 95 bp.

for *B. cinerea* give an amplicon of 576 bp. The five PCR replicates obtained for each species were purified and analyzed using agarose gel electrophoresis (Figure 1).

The agarose gel shows reproducible results for the five replicates for both species. As expected, only one PCR product was detected and nonspecific PCR products were not observed. For *E. necator*, the PCR product migrates between 400 and 500 bp, which is in good agreement with the expected 483 bp. For *B. cinerea*, the PCR product migrates above 500 bp, again in good agreement with the expected size of 576 bp.

The PCR replicates were quantified using two different spectrophotometric instruments, NanoVue and NanoDrop. DNA concentration was also determined on the 2100 Bioanalyzer System with the DNA 1000 kit, which is based on on-chip electrophoresis with fl uorescence detection. The PCR samples were analyzed in duplicate, and the average was calculated (Table 2).

As expected, quantitation with the 2100 Bioanalyzer System results in significantly lower values compared to both of the spectrophotometric methods. The DNA quantitation with the DNA 1000 Kit is limited to the size range from 25 to 1,000 bp and is based on a fluorescent dye specific for double-stranded nucleic acids. In contrast, spectrophotometers detect all molecules absorbing at a specific wavelength, including different types of nucleic acids or contaminants, which may yield inflated results. The 2100 Bioanalyzer System provides not only DNA quantitation, but also DNA sizing and purity analysis in a single step (Figure 2).

As shown in Figure 2, the 2100 Bioanalyzer System provides electropherograms and gel-like images. The separation of the five PCR reactions, ran in duplicate, are shown as gel-like images. None of the samples show the presence of nonspecific PRC products. The obtained results for the PCR product size and purity are in good agreement with the observations made after agarose gel electrophoresis (Figure 1).

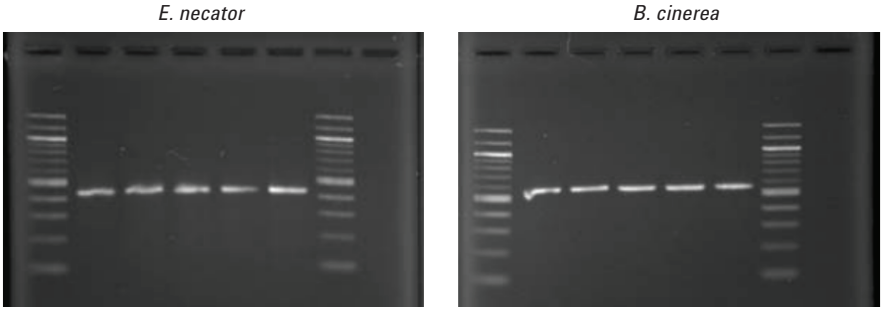

Figure 1. The purified PCR samples obtained for *E. necator* and *B. cinerea* were analyzed using agarose gels stained with ethidium bromide. Five replicates from each system were analyzed on each gel. A 100 bp ladder was used as a size standard (100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,200, and 1,500 bp).

Table 2. Summary of DNA quantitation obtained with different methods, on-chip electrophoresis with the Agilent 2100 Bioanalyzer system, and spectrophotometry with the NanoDrop 2000 and the NanoVue Plus instruments. The average of replicate measurements was determined.

	E. necator		*B. cinerea*	
System	DNA concentration (ng/µL)	Standard deviation	DNA concentration (ng/µL)	Standard deviation
Agilent 2100 Bioanalyzer System	12.96	0.98	10.75	1.14
NanoVue Plus	38.40	9.06	35.10	9.89
NanoDrop 2000	38.75	8.87	35.01	9.84

The copy number of pathogens is typically calculated from standard curves generated by qPCR. Based on the quantitation results obtained with a spectrophotometer and the 2100 Bioanalyzer System, samples of differing concentrations were prepared and subjected to qPCR. The reactions were performed as five replicate measurements to obtain the standard curves shown in Figure 3.

Regardless of amplicons used for the creation of the standard curve, comparable copy numbers would have comparable Ct values. Hence, the qPCR experiment was expected to show similar standard curves. However, Figure 3 shows differences in the standard curves for *E. necator* and *B. cinerea* obtained when using the copy number determination based on the spectrophotometric measurement. This unexpected difference between the standard curves clearly indicates an inconsistency with the quantitation precision of the spectrophotometric approach.

In contrast, when the standard curves are created and calculated based on the quantitation with the 2100 Bioanalyzer System, much less variation between the five standard curves is observed (Figure 3) providing needed confidence in copy number calculations. The 2100 Bioanalyzer System provides reproducible and reliable quantitation of amplicons compared tospectrophotometry.

Conclusion

Absolute quantitation by real-time PCR is a widely accepted technique in numerous research applications allowing for copy number determination of microbes or genes in many sample types. Airborne plant pathogen detection was used as an example of a critical fieldinpla nt biology requiring sensitive and accurate methods for absolute quantitation and copy number calculation. The results presented in this Application Note reveal that two independent spectrophotometric methods used for DNA quantitation measurement lack the consistency

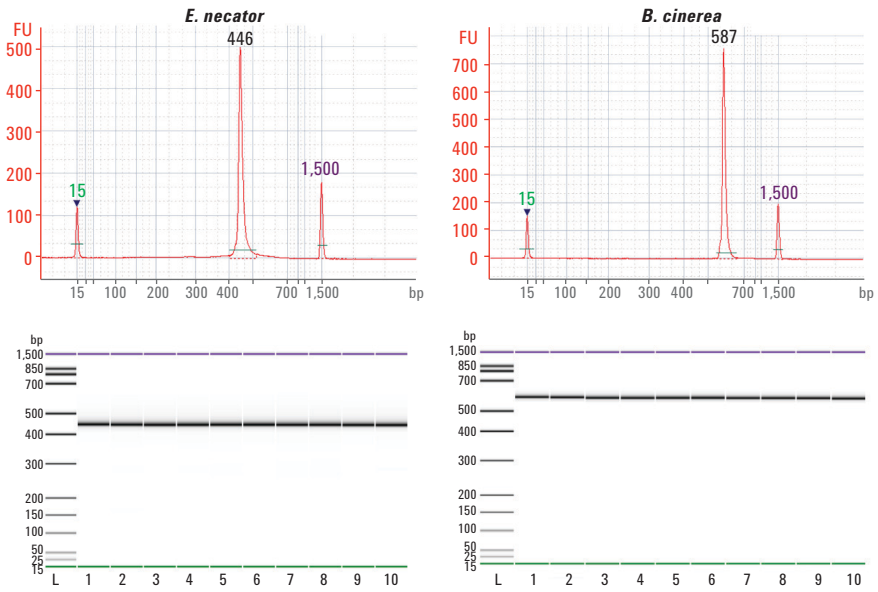

Figure 2. Analysis of PCR samples obtained for *E. necator* and *B. cinerea* using the Agilent 2100 Bioanalyzer System and the Agilent DNA 1000 Kit. Typical electropherograms are shown in the upper panel; the gel-like images from the five replicates, analyzed in duplicates, are shown in the lower panel.

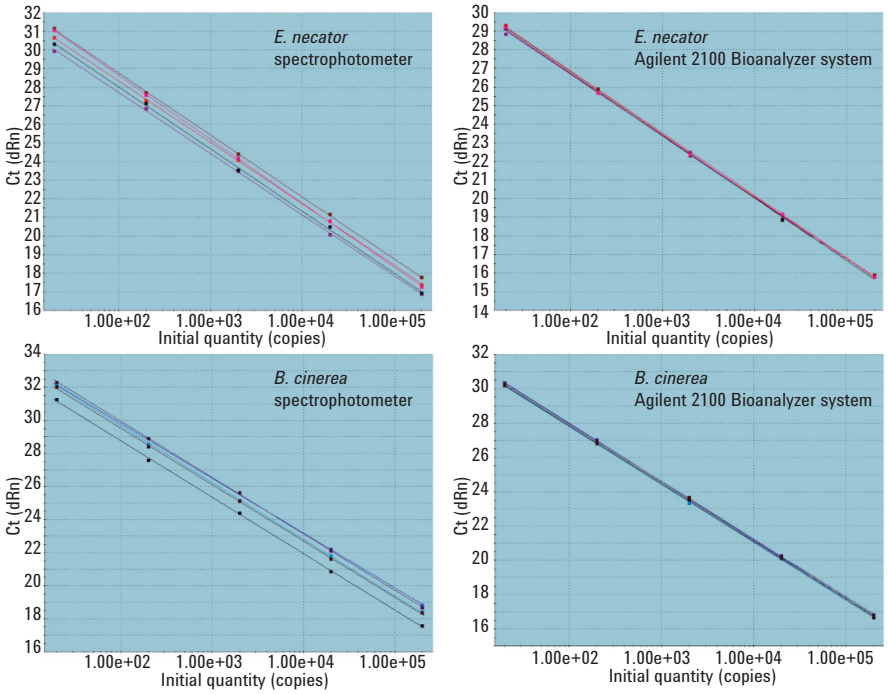

Figure 3. qPCR standard curves for *E. necator* and *B. cinerea* obtained using copy number calculation based on the DNA concentration values obtained by a spectrophotometer and the Agilent 2100 Bioanalyzer System. The initial quantity (copy number) is plotted against the determined Ct (dRn) values. The data for five independent measurements are shown on each graph.

required for meaningful copy number evaluation. In contrast, the Agilent 2100 Bioanalyzer system provides reliable concentration values for qPCR, thereby creating reproducible standard curves and allowing for accurate copy number calculation.

Disclaimer note

Agriculture & Agri-Food Canada is not providing an endorsement for the use of the Agilent 2100 Bioanalyzer system and is not affiliated in any way to Agilent Technologies, Inc.

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