Food Control 20 (2009) 391–401

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Comparison of fluorometric and spectrophotometric DNA quantification for real-time quantitative PCR of degraded DNA

Luke A. Shokere [a], Marcia J. Holden [b], G. Ronald Jenkins [a,*]

[a] United States Department of Agriculture, Grain Inspection, Packers and Stockyards Administration, 10383 N. Ambassador Dr., Kansas City, MO 64153, United States
[b] National Institute of Standards and Technology, Biochemical Science Division, 100 Bureau Drive, MS-8311, Gaithersburg, MD 20899, United States

ARTICLE INFO

Article history:
Received 8 May 2008
Received in revised form 20 June 2008
Accepted 8 July 2008

Keywords:
DNA quantification
A_{260}
Picogreen fluorescence
Hoescht dye
Degraded DNA quantitative real-time PCR
NK603 maize

ABSTRACT

Isogenic NK603 DNA was degraded by sonication or heat and quantified using A_{260} and two fluorescent dye methods. Quantitative PCR (qPCR) experiments were conducted by amplifying an SSIIb-3 endogenous control and an NK603 transgene in untreated, sonicated, and heat treated samples. qPCR reactions on sonicated DNA samples, based on A_{260} quantification, provided 0.125%, 1.14% and 2.15% NK603; while heat treated samples, provided results of 0.128%, 1.42%, and 2.73% NK603. qPCR reactions on sonicated DNA samples, based on the fluorescent dye method, provided results of 0.18%, 0.861% and 1.74% NK603; while heat treated DNA samples, provided results of 0.18%, 1.02%, and 2.16% NK603. The data suggested that fluorescent dye-based quantifications yielded more accurate determinations of the percent genetically engineered (GM) content at higher concentrations, most likely because fluorescent dye quantifications resulted in additional copies of template added into the qPCR. The data in this study suggested that neither fluorescent dye nor spectrophotometric methods of quantification on highly degraded DNA translated into concordant measurements of qPCR amplifiable DNA and accurate C_t values.

Published by Elsevier Ltd.

1. Introduction

Since the advent of the first genetically engineered (GM) crop, commercialized in 1996, modern biotechnology techniques have accelerated the production of crop plants containing protection traits that are absent from the genome of their wild-type cultivars. These crop protection traits are the result of the stable insertion and constituitive expression of genes transferred from bacteria that encode for insecticidal toxins and/or metabolic pathways that deactivate absorbed herbicides (Chassy, 2002; Chassy, Parrott, & Roush, 2005; Nap, Metz, Escaler, & Conner, 2003). Proponents of this technology suggest that GM crops have increased yields and require less crop management resources (ISAAA, 2004). In spite of these beneficial outcomes, some consumer advocacy groups have expressed concerns about the efficacy of introducing biotechnology-derived grains into the marketplace (Conner, Glare, & Nap, 2003; Goodman et al., 2008; Jonas et al., 2001; Union of Concerned Scientists, 2008). Collectively, these issues have generated considerable global regulatory oversight of GM crops and their food products. In the US, the responsibility for evaluating nutritional quality, environmental impact, and conducting risk assessments is shared by the Animal and Plant Health Inspection Service (USDA–APHIS), Environmental Protection Agency (EPA), and Food and Drug Administration (FDA). The established policy in the United States is that grains are considered to be "substantially equivalent" when GM varieties are shown to be similar in nutritional value, toxicity level, and allergenicity characteristics compared to their non-GM counterparts (Ahmed, 2004; Nap et al., 2003; WHO, 2000). Some governmental agencies abroad, however, adhere to regulatory mandates that strictly enforce traceability and labeling systems for GM food products, prior to introducing these commodities into their marketplaces (Directive 79/112/EC; Regulation 1829/2003). Under these regulations, foods containing GM traits are required to be labeled as such if the content of the commodity is above a specified threshold, i.e., 0.9% in the European Union (ACREN, 2000; Directive 79/112/EC; Regulation 1829/2003). The results of quantitative analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes (European Commission 787/2004, 2004). It becomes paramount then, for purposes of harmonization and management of risk between buyers and sellers, to implement internationally-recognized standards that demonstrably provide accurate measurements when quantifying levels of biotechnology-derived traits in grains and oilseeds (ICH, 1996; Mihaliak & Berberich, 1995). For purposes of international trade, the most widely accepted method for detecting and quantifying levels of GM traits in grains and oilseeds is genomic DNA extraction and amplification of both trait- and taxon-specific targets using quantitative polymerase chain reaction (qPCR) (Anklam & Neumann, 2002; Garcia-Canas, Cifuentes, & Gonzalez, 2004; Lipp et al., 2005; Murray, Butter, Hardacer, & Timmerman-Vaughan, 2007; Rizzi, Panebianco, Giaccu, Sorini, & Daffonchio, 2003; Torsten, Hammes, & Hertel, 2004). Accurate and reliable detection and quantification of an analyte by qPCR is

* Corresponding author. Tel.: +1 816 891 0442; fax: +1 816 891 7314.
E-mail address: g.ronald.jenkins@usda.gov (G. Ronald Jenkins).

0956-7135/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.foodcont.2008.07.009

dependent upon factors including: specificity, sensitivity, amplification efficiency, and quality of genomic DNA used in the qPCR reaction (Namuth & Jenkins, 2005; Yoshimura et al., 2005). Extracting genomic DNA from grains is not trivial, and some amount of degradation during the extraction procedure is unavoidable (Ahmed, 2002; Holden et al., 2003; Kakihara, Matsufuji, Chino, & Yamagata, 2007). DNA degradation is more likely to be encountered in processed food than in grain. Once DNA is extracted, either the ultraviolet absorption (UV) (A_{260}) or the fluorescence response of intercalating dyes is used to measure the concentration of DNA prior to qPCR. Equivalent amounts of genomic DNA, set up either in a simplex or duplex format, are used for the analysis of both reference and unknown samples (Hohne, Santisi, & Meyer, 2002). Typically, the resulting amplification profile for a transgene target is normalized to the amplification profile of an endogenous control gene, and the final analytical result is reported as a % GM (Garcia-Canas et al., 2004; Lipp et al., 2005). Spectrophotometric methods of DNA quantification do not discern between intact DNA, RNA and single nucleotides. Fluorescent dyes on the other hand are highly specific for double-stranded DNA over RNA and single-stranded DNA. The picogreen fluorescent dye provides a linear response in the range of 0.2–200 ng DNA but factors such as % AT content, pH and contaminants including salts and organic solvents also contribute to errors in the calculated result on test samples (Rye, Dabora, Quesada, Mathies, & Glazer, 1993).

The degree of degradation between reference and unknown sample DNA can vary greatly (Hupfer, Sachse, & Engel, 1999). The final analytical value assigned to a particular sample should be independent of the degree of degradation of the DNA since the GM target sequence and the taxon-specific sequences should degrade in parallel. Some reports suggest that variation among replicate analyses increases as the degree of degradation increases (Ahmed, 2004). This trend can be countered by increasing the amount of sample DNA added to a PCR reaction. However, when DNA is highly degraded and the targets for the GM and reference genes differ significantly in size, the reference assay might not normalize the GM content correctly (Chen, Ge, & Xu, 2005). This study assessed the concordance between a UV spectrophotometric (A_{260}) and an intercalating dye method of quantifying identical DNA extracts that were: (1) degraded by sonication or heat compared with (2) intact controls. The genomic DNA of isogenic maize samples were analyzed for integrity via gel electrophoresis, quantified, and subjected to qPCR amplification for both an SSIIb-3 endogenous control gene and the genetically engineered NK603 trait-specific gene. The impact of differences in calculated DNA concentrations on qPCR analytical results was determined.

2. Materials and methods

2.1. Sample source

A set of finely ground, certified reference maize-flour obtained from the Institute for Reference Materials and Measurements (IRMM) (Sigma Aldrich, St. Louis, MO) consisted of six NK603 vials (ERMBF 415A to 415F) fortified at 0.0–5.0% (% w/w). The IRMM maize vials were stored at 4 °C until used in the DNA extraction procedure. Fortifications of IRMM at 0.1%, 1%, and 2% NK603 were either untreated, sonicated or heat denatured as described below. In addition, 0.0%, 0.1%, 1.0%, 2.0% and 5.0% IRMM fortification levels were extracted and used (untreated) for generating qPCR linear regression curves in a simplex format.

2.2. DNA extraction

DNA was isolated from 200 mg of finely ground IRMM NK603 reference material using a hexadecyltrimethylammonium bromide (CTAB) (Sigma Aldrich)[1] method with minor modifications as described (Majchrzyk, 2002; Murray & Thompson, 1980). Briefly, maize-flour was incubated at 65 °C with 700 μL of CTAB extraction buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris, 20 mM Na_2 EDTA, pH 8.0) and 300 μL water to lyse the cells. Following cellular lysis, an extended 60 min treatment with 20 μL RNase A (10 mg/mL) at 37 °C (Fermentas, Hanover, MD) followed by a 20 μL proteinase K (Sigma Aldrich) digestion were performed to divest the DNA product of contaminating RNA and proteins. The resulting digests were extracted with chloroform twice to eliminate PCR-inhibiting polysaccharides and polyphenols, and incubated in CTAB precipitation buffer (5 g/L CTAB, 0.04 M NaCl, pH 8.0) at 25 °C overnight to allow selective precipitation of DNA. After precipitation, the samples were re-solubilized into 175 μL of 0.5× TE (5 mM Tris and 0.5 mM EDTA) buffer and treated with 10 μL RNase A at 37 °C for 1 h (Freese et al., 2007). An equal volume (175 μL) of 2.4 M NaCl was added to each sample followed by a chloroform extraction, and then ethanol-precipitated overnight at −20 °C using twice volume (700 μL) of 100% ethanol (Sigma Aldrich). After overnight precipitation, the samples were washed with 500 μL of 70% ethanol and dried in a vacuum microfuge (Eppendorf, Westbury, NY). The DNA pellets were then dissolved in 100 μL 0.5× TE, pH 8.0 buffer. The yield of maize-flour sample was typically 20–50 μg DNA from 200 mg starting material of flour. DNA stock samples were solubilized in 100 μL of 0.5× TE buffer, pH 8.0, at a concentration of 200–300 ng/μL, and stored at 4 °C until further use.

2.3. DNA quantification

2.3.1. Fluorescent dye assay

DNA was quantified using a fluorescent dye assay with a TD-700 fluorometer (Turner Biosystems Inc., Sunnyvale, CA) in conjunction with Quant-iT PicoGreen (PG) reagent kit (Invitrogen/Molecular Probes, Eugene, OR). The PG reagent binds double-stranded DNA with high specificity (Singer, Jones, Yue, & Haugland, 1997). Stock DNA samples were diluted either 1:500 or 1:1000 with 0.5× TE buffer to a target concentration of 30–500 pg/μL. The PG reagent was prepared according to the manufacturer's protocol; the diluted stock DNA unknown samples were mixed 1:1 with PG reagent to a final volume of 200 μL to produce either 1:1000 or 1:2000 final dilutions, and compared with fluorometric measurements from a standard curve. Identical samples were quantified using a Hoescht dye (Fisher Scientific, Pittsburg, PA) method. With the Hoescht dye method, diluted stock DNA samples (as described above) were mixed 1:1 with 2× Hoescht dye assay solution that was prepared from 0.2 ug/mL Hoescht dye dissolved in TNE buffer (0.2 M NaCl, 20 mM Tris–Cl, 1 mM EDTA, pH 7.4) prior to quantification. A calibration curve was generated from λ-phage DNA, supplied by the manufacturer at a stock concentration of 100 ng/μL, and diluted to 500 pg/μL, 250 pg/μL, 125 pg/μL, 62.5 pg/μL, and 0.0 pg/μl with 0.5× TE buffer. The calibration slope error generally ranged from 5% to 9% and was within the tolerance limit of 25% recommended by the instrument's manufacturer. Spectral processing was conducted using the "TD-700 Hyperterminal Software Package." Experimental samples had greater than 85% concordance compared to PG prior to "accepting" an empirically determined concentration. The mean of DNA quantification values from both the PG and Hoescht dye methods provided an "accepted" concentration for a specified sample. Stock DNA samples were diluted to a working concentration of 10 ng/μL. Five microliters (50 ng) of sample was loaded onto a 0.8% agarose gel and size-

[1] Identification of commercial products in this paper was done in order to specify the experimental procedure. In no case does this imply endorsement or recommendation by the National Institute of Standards and Technology or the United States Department of Agriculture.