Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
A multi-target, non-infectious and clonable artificial positive control for routine PCR-based assays

Donna Ria J. Caasi a,b,1, Mohammad Arif a,b, Mark Payton c, Ulrich Melcher d, Louise Winder e, Francisco M. Ochoa-Corona a,b,*

a National Institute for Microbial Forensics & Food and Agricultural Biosecurity, Oklahoma State University, Stillwater, OK 74078, USA
b Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA
c Department of Statistics, Oklahoma State University, Stillwater, OK 74078, USA
d Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA
e Lincoln University, Lincoln 7647, Canterbury, New Zealand

Abstract

Positive controls are essential for PCR reliability and are challenging to obtain for rare, exotic and/or emerging pathogens and pose biosafety risks if manufactured using infectious pathogens. Custom synthetic DNA inserts can be designed de novo in tandem of forward and reverse complement priming sequences to be inserted in circular plasmid vectors. To test this concept, artificial positive controls (APCs) for use in PCR were synthesized to contain primer sequences targeting four viruses (Barley yellow dwarf virus, Soilborne wheat mosaic virus, Triticum mosaic virus and Wheat streak mosaic virus) pathogenic to wheat and, as internal control, the plant mitochondrial nad5 gene. Thermodynamics and folding parameters of twenty-four APC inserts were assessed in silico. Two ther- modynamically different APCs, designated optimal and sub-optimal, were cloned and tested using end point PCR. The optimal APC had a 100% amplification rate, while only 92% of virus-infected plant tissues, commonly used as reference positive controls, amplified. An array of APC priming sequences from different organisms and/or previously tested primers can be accommodated in a large and flexible number of positive control targets. APCs will streamline and standardize routine PCR, improve reliability and biosafety, and create opportunities for development and commercialization of new synthetic positive control sequences.

Article Info

Article history:
Received 1 August 2013
Received in revised form 29 August 2013
Accepted 29 August 2013
Available online 5 September 2013

Keywords:
Synthetic DNA
Nucleic acids thermodynamics
Detection
Diagnostics

1. Introduction

The advent of synthetic biology offers new possibilities for designing, engineering and developing artificial molecules to reproduce natural biological processes, and for creating interchangeable biological parts to assemble new devices and systems (Louw et al., 2011; Schmidt, 2008; Stahler et al., 2006). The creation of synthetic artificial controls for a variety of microbial detection and disease diagnostic assays is a new alternative to the use of in vivo positive controls. Plasmid construct-based synthetic controls harboring a custom and de novo designed insert can enhance technologies such as polymerase chain reaction (PCR) to improve biosafety, speed of processing and overall detection without compromising sensitivity and specificity.

Examples of artificial plasmid constructs containing partial or complete sequences of a pathogen (or multiple pathogens) of interest are already in use or reported. Charrel et al. (2004) designed and constructed plasmids containing specific sequences of category A agents of bioterrorism, including exogenic sequences with inserted NotI sites and primers designed to amplify agent specific sequences and probes to detect specifically each sequence. Steps toward construction included: PCR amplification of targets and incubation to add restriction sites; column purification to discard residual small DNA fragments remnant from the restriction reaction; overnight incubation in the presence of T4-DNA ligase at 4 °C; and cloning of the final product into a plasmid. Similarly, Inoue et al. (2004) constructed a recombinant plasmid harboring fragments of the pag and cap genes of Bacillus anthracis distinguishable from the authentic sequences by the presence of restriction enzyme sites, the EcoRV for the pag gene and the BamHI for the cap gene, respectively. Siegling et al. (1994) reported the construction of a multispecific control fragment containing 14 different templates for use in competitive PCR quantification of rat gene expression. Primer pairs did not exhibit 3′-complementarity and spanned over one or more introns to facilitate distinguishing the amplicons obtained from cDNA from those from genomic DNA. The construction was made using complementary 40mer oligonucleotides cloned in a plasmid containing binding sites for both sense primers. Chomiec et al. (2010) reported the construction of synthetic positive controls for the detection of Luteoviridae through RT-PCR. The sequences of forward and reverse primers were arranged

© 2013 Elsevier B.V. All rights reserved.
into two fragments, and inserted into a plasmid. The two fragments were 152 and 161 bp long; however, the synthesis of oligonucleotides greater than 100 bases was not possible. Therefore, each fragment was synthesized as two separate oligonucleotides overlapping by 20 bp. Smith et al. (2006) also described a labor intensive procedure to produce synthetic controls including probes for only two targets, SARS coronavirus and rodent glyceraldehyde-3-phosphate dehydrogenase (rGAPDH), for realtime RT-PCR (TaqMan). The method used the bacteriophage T7, RNA polymerase binding site included at the 5′ terminus of the oligonucleotide to allow RNA transcription of the oligonucleotide. The reported strategies in all of these examples provide a safe and reproducible positive-control DNA template and also allow the detection of possible cross contamination, but their development in vivo was laborious and the constructs have been limited to a small number of targets. The approach to be described here is unique in its combinatorial primer design, enlarged capacity for targets, flexibility to rearrange and update, and elimination of pathogen sequences (intron, overlapping, etc.) except for the primer sequences.

Nucleic acid-based molecular assays such PCR have become preferred diagnostic platforms, particularly due to recent enhancements (Alvarez, 2004; Henson and French, 1993). Although PCR has been adapted for routine testing for pathogens of interest (Miller et al., 2009), positive controls may be challenging to obtain (Smith et al., 2006) because not all proteins or pathogens are available as positive controls. Moreover, positive controls are especially difficult to obtain for exotic emerging pathogens, which are not commercially available in most cases, and require time-consuming obtaining of permits and clearances (Nechvatal et al., 2008; Smith et al., 2006). Infected tissue, in most cases, and require time-consuming obtaining of permits and clearances (Nechvatal et al., 2008; Smith et al., 2006). Infected tissue, purified or cultured pathogen, and/or purified or expressed proteins are positive controls commonly used. However, well-documented risks associated with the handling or shipping of infected sample controls, particularly those carrying highly pathogenic microorganisms that are efficiently transmitted (Charrel et al., 2004; Inoue et al., 2004; Siegling et al., 1994; Smith et al., 2006), threaten the biosafety of their use.

The aim of this research is to assess the suitability of a relatively new concept of customized synthetic DNA inserts consisting of linear arrays of primer sequences designed from a variety of genomes or targets of value for detection, diagnostics and research.

2. Materials and methods

2.1. Primer sequences

Sequences of five primer sets were selected to design and synthesize APC inserts. The five primer sets target four wheat viruses, Barley yellow dwarf virus (BYDV), Soilborne wheat mosaic virus (SBWMV), Triticum mosaic virus (TriMV), and Wheat streak mosaic virus (WSMV) and the putative plant internal control mitochondrial nad5 gene. Primers for BYDV and SBWMV were designed in this study. The Genbank accession numbers incorporated into consensus sequences for primer design of BYDV, SBWMV (RNA2), TriMV (Data not shown) and WSMV (Data not shown) are presented in Supplementary Table 2. Primer sequences targeting nad5 are from previously reported literature (Menzel et al., 2002). All consensus and primer design regions were obtained after complete alignment of multiple nucleotide sequences using ClustalX (Larkin et al., 2007). Forward and reverse primers were designed using Primer3 (Rozen and Skalaetsky, 2000) following reported parameters (Arif and Ochoa-Corona, 2012). The ΔG, secondary structures, self- and heterodimer formation of each set of primers were examined after matching and analyzing the outputs from the Primer3 ‘any’ self-complementarity score, which was taken as a measure of its tendency to anneal to itself or form secondary structure, and mfold (Rozen and Skalaetsky, 2000; Zuker, 2003). The specificity of each primer sequence was confirmed in silico by searching the NCBI nucleotide database using BLASTn (Altschul et al., 1990). The preliminary in vitro performance of all primer sets was tested by reverse transcription-PCR (RT-PCR) using virus infected plant tissue as positive controls for each targeted virus. Primers used for engineering APC inserts and performing PCR and RT-PCR are listed in Table 1.

2.2. The APC concept and in silico assessment of APC inserts

The APC concept consists of a DNA insert that can be custom designed de novo and synthesized artificially. APC inserts contain primer sequences in series, an upstream component of 5′-3′ forward primers followed by a second (downstream) of 5′-3′ reverse complement primer sequences. The APC insert is ligated to a selected restriction enzyme site within a multiple cloning site of a chosen plasmid vector for circularization, yielding a functionally clonable APC construct (Fig. 1). The APC approach also allows the inclusion of a large number of targets arranged in groups or tandems of APC inserts (Fig. 1-B). APC inserts were designed combining a tandem of forward and reverse complement of primer sequences in different sequence orders for the nad5 internal control and the four targeted viruses (Table 2). Out of the 120 possible combinations, 24 with nad5 at the 5′ position were selected. Each combination was 203 bp long and all theoretically able to generate PCR products from 120 to 124 bp (Table 1 — size of target amplicons). All primer sequence combinations within the APC insert were determined using a SAS 9.2 permutation code in SAS 9.2 (SAS Institute Inc., Cary, NC). Because forward and reverse complement primer sequences were arranged in combinatorial tandems, the expected product of each primer set contains the amplifying primer sequences and other primer sequences within the APC insert, giving to each APC product a specific sequence identity useful for monitoring quality control and reaction specificity (Fig. 1-A).

The twenty-four APC inserts were assessed in silico. Plot ΔG, and optimal free energy of predicted secondary structures were determined using mFold at 60 °C. The Plot ΔG or energy dot plot contains the

### Table 1

| Target                  | Primer ID       | 5′-3′ sequence                                                                 | Size of target amplicons (bp) | Reference                  |
|-------------------------|-----------------|--------------------------------------------------------------------------------|-------------------------------|----------------------------|
| Barley yellow dwarf virus | BYDV-F1         | CAACACCGCAGCCGAAAC                                                               | 128                           | 121                        | 123                        |
|                         | BYDV-R1         | TGGACGTCGCTGATATCATCAAA                                                          | 92                            | 120                        | 122                        |
| Soilborne wheat mosaic virus | SBWMV-F1      | ATCAATTCCTCCGGTGTCTC                                                            | 218                           | 122                        | 122                        |
|                         | SBWMV-R1        | GACACCTCTCTTTTTTCA                                                               | 198                           | 122                        | 122                        |
| Wheat streak mosaic virus | WSMV-F2         | GAAATGCAAGAGCGACCTTCTT                                                         | 181                           | 124                        | 124                        |
|                         | WSMV-R2         | GCAAGACTTCTCTCTCC                                                                | 181                           | 124                        | 124                        |
| Triticum mosaic virus   | TriMV-F1        | GACCCAGGACAGACGTCTT                                                             | 181                           | 124                        | 124                        |
|                         | TriMV-R1        | GCAAGACTTCTCTCTCC                                                                | 181                           | 124                        | 124                        |
| NADH dehydrogenase subunit 5 | Nad5-s        | CATTCTGCTTGGGTCCTTGT                                                            | 181                           | 124                        | 124                        |
|                         | Nad5-as         | CTTGGACGTGCCTAACCAGTGCCCA                                                       | 181                           | 124                        | 124                        |

*Infected plant tissue.
superposition of all possible DNA folding and gives an overall visual of DNA folding facilitating the analysis.

2.3. Construction of APCs and assessment by PCR

Two APC sequences from the pool of 24 theoretical APC inserts were categorized as suboptimal (SubOpt) and optimal (Opt) and were cloned into plasmid vector pUC57. The criteria for selecting the two inserts were: ΔG far from 0 kcal mol\(^{-1}\) and the presence of several secondary structures for the SubOpt insert, while the Opt APC insert had a ΔG value close to 0 kcal mol\(^{-1}\) and few or no secondary structures. The two selected SubOpt and Opt APC inserts (POS-2_1 and POS-2_7 in Table 1 respectively) were synthesized and inserted at the EcoRV site of plasmid vector pUC57 and subsequently cloned in *Escherichia coli* (DH5α) by Genscript USA Inc. (Piscataway, NJ). The APC construct plasmids were vector pUC57 and subsequently cloned in

\[
\Delta G = -RT \ln K
\]

structures for the SubOpt insert, while the Opt APC insert had a ΔG value close to 0 kcal mol\(^{-1}\) and few or no secondary structures. The two selected SubOpt and Opt APC inserts (POS-2_1 and POS-2_7 in Table 1 respectively) were synthesized and inserted at the EcoRV site of plasmid vector pUC57 and subsequently cloned in *Escherichia coli* (DH5α) by Genscript USA Inc. (Piscataway, NJ). The APC construct plasmids were vector pUC57 and subsequently cloned in

\[
\Delta G = -RT \ln K
\]

superposition of all possible DNA folding and gives an overall visual of DNA folding facilitating the analysis.

2.3. Construction of APCs and assessment by PCR

Two APC sequences from the pool of 24 theoretical APC inserts were categorized as suboptimal (SubOpt) and optimal (Opt) and were cloned into plasmid vector pUC57. The criteria for selecting the two inserts were: ΔG far from 0 kcal mol\(^{-1}\) and the presence of several secondary structures for the SubOpt insert, while the Opt APC insert had a ΔG value close to 0 kcal mol\(^{-1}\) and few or no secondary structures. The two selected SubOpt and Opt APC inserts (POS-2_1 and POS-2_7 in Table 1 respectively) were synthesized and inserted at the EcoRV site of plasmid vector pUC57 and subsequently cloned in *Escherichia coli* (DH5α) by Genscript USA Inc. (Piscataway, NJ). The APC construct plasmids were vector pUC57 and subsequently cloned in

\[
\Delta G = -RT \ln K
\]

structures for the SubOpt insert, while the Opt APC insert had a ΔG value close to 0 kcal mol\(^{-1}\) and few or no secondary structures. The two selected SubOpt and Opt APC inserts (POS-2_1 and POS-2_7 in Table 1 respectively) were synthesized and inserted at the EcoRV site of plasmid vector pUC57 and subsequently cloned in *Escherichia coli* (DH5α) by Genscript USA Inc. (Piscataway, NJ). The APC construct plasmids were vector pUC57 and subsequently cloned in

\[
\Delta G = -RT \ln K
\]
3. Results

3.1. Thermodynamics and folding of APC inserts

All designed APC inserts carried the same primer sequences but the order of these primers varied, leading to differences in thermodynamics and in the formation of predicted secondary structures. The plot $\Delta G$ values of the 24 APC synthetic inserts ranged from 0.2 to 1 kcal mol$^{-1}$, and the optimal energies of the secondary structure ranged from $-0.6$ to 1.0 kcal mol$^{-1}$. All APC inserts have one, two or three predicted folding regions. The predicted secondary structure and $\Delta G$ value of the Opt and SubOpt APCs are shown in Supplementary Table 1. The Opt APC has a $\Delta G$ of 0.2 kcal mol$^{-1}$, and one possible secondary structure with optimal energy of $-0.6$ kcal mol$^{-1}$ (Supplementary Fig. 1A). The SubOpt APC has a $\Delta G$ of 1.0 kcal mol$^{-1}$, and three possible secondary structures with optimal energies of 1.01, 1.21 and 1.31 kcal mol$^{-1}$ (Supplementary Fig. 1B, C and D). The synthetic inserts of both Opt and SubOpt APCs were 203 bp long with 49% GC as predicted and their sequences and primer organization are shown in Table 2. The expected size of PCR products from APC inserts ranged from 120 to 124 bp as predicted (Table 1, Fig. 2).

3.2. Efficacy of APC as a positive control

During a preliminary screening, all infected plant positive controls were RT-PCR positive for their respective viruses, BYDV, SBWMV, TriMV or WSMV when tested with the appropriate primers as well as with the primers for the plant internal control mitochondrial nad5 gene (Fig. 2, lane 2). Also, all targets of both optimal and sub-optimal APCs were amplified, with no detectable difference in the intensity of the amplicons produced by the two APCs regardless of plasmid DNA concentration (10 to 0.1 ng $\mu$L$^{-1}$) (Fig. 2, Lanes 3–8). As expected, differences in APC product sizes were observed among four of the five reference positive controls due to differences in size of the expected products and different distances between priming sequences within the APC synthetic inserts (Fig. 2). No positive matches were found in the GenBank database after alignment using BLASTn (Altschul et al., 1990), which confirmed the uniqueness of the sequence identity of the Opt and SubOpt APC sequences.

PCR using Opt APC (144 repetitions) was compared with that done using infected plant tissue reference positive controls (116 repetitions) by Fisher’s exact test (Table 3). Nine false negatives occurred among the reference plant positive controls (92% success rate), while the Opt APC was 100% successful (Table 3).

### Table 3

| Target  | n    | Fisher’s exact (P-value) | Number of positive samples | % Opt APC, % reference success rates |
|---------|------|--------------------------|-----------------------------|-------------------------------------|
| | | | Total number of samples | Opt APC | Reference $^a$ | Total |
| BYDV    | 190  | 0.0225$^a$               | 100/100                     | 85/90                              | 100%, 94%            |
| SBWMV   | 16   | 0.2500$^a$               | 12/12                       | 3/4                                | 100%, 75%            |
| TriMV   | 9    | 0.2222$^a$               | 7/7                         | 1/2                                | 100%, 50%            |
| WSMV    | 45   | 0.1919$^a$               | 25/25                       | 18/20                              | 100%, 90%            |
| Total   | 250  | 0.0006$^a$               | 144/144                     | 107/116                            | 100%, 92%            |

$^a$ PCR tests from reference positive controls were from cDNA. All reference positive control tested positive during preliminary assays of this study.

$^a$ Significant at 0.05.

$^a$ Significant at 0.001.
DNA in the reaction is in equilibrium (Nelson and Cox, 2008). With this assumption, ΔG for unfolding was determined for all 24 APC inserts and the broadest possible ΔG range was selected empirically. An Mfold unfolding plot ΔG of 0.2 kcal mol⁻¹ and free energy of the major secondary structure — 0.6 kcal mol⁻¹ were considered to reflect weak interactions among nucleotide bases allowing a more relaxed secondary structure close to the optimal ΔG of 0 kcal mol⁻¹, while a plot ΔG of 1.0 kcal mol⁻¹ and free energy of the major secondary structure 1 kcal mol⁻¹ were considered to reflect less relaxed and stronger nucleotide sub-optimal interactions (Supplementary Table 1) (Jacobson and Zuker, 1993). In this experiment, PCR using Opt APC will be favored since it has a ΔG closer to the equilibrium than SubOpt APC does (Nelson and Cox, 2008). Our results are favorable considering that most APCs will be composed of previously validated primers, which are expected to have a balanced GC content (close to 50%), optimal ΔG (close to zero) and minimal secondary structure. These characteristics should reduce the risk of secondary structure formation over the APC insert. Secondary structure is to be considered during the APC design because it can decrease PCR sensitivity by limiting strand annealing (Jensen et al., 2010). Also, secondary structure in the form of stem-loop structures can lead to polymerase jumping and mispriming during PCR (Jensen et al., 2010; Weiss et al., 1994). The presence of secondary structures within the structure of APC inserts can be easily overlooked while designing by calculating the ΔG of the APC insert in silico, and the number of secondary structure and related free energy before the custom DNA synthesis. In general, the in silico assessment is precautionary because problems associated with folding among primers are not easily resolved by modifying annealing temperature, pH, and salt concentrations at the time of the reaction (Jacobson and Zuker, 1993; Fredman et al., 2004).

The selected range of plasmid concentration assures the presence of sufficient template for consistent amplification while plasmid dilutions under 0.1 ng μL⁻¹ may lead to variable PCR yields. The concentration of a microbial target in a sample is usually unknown, but in an artificial positive control this concentration is known, and has to be sufficient to allow monitoring the performance of the APC during the reaction.

PCR products from APCs may differ in size from those generated by traditionally-used positive controls (Fig. 2) due to the discrepancy in the lengths between the annealing sites of the target sequences in vivo and the arrangement of primer sequences in the APC template (Table 1). Such discrepancy should not be a problem. Certainly the operator knows what APC:sample size product to look for. The generation of products of different sizes by the infected positive control and the APC is appealing to some operators who like the ability to discern the APC:sample size product to look for. The generation of products of different sizes by the infected positive control and the APC is appealing to some operators who like the ability to discern the two types of controls as well as to be able to recognize false positives resulting from cross-contamination of APC batches (Charrel et al., 2004; Tao and Zhang, 1998). However, the option for reducing or eliminating the size discrepancy is available.

The APC can be designed to have product sizes identical or similar to those expected with reference primers (Fig. 2A) by reducing or adding non-complementary nucleotides between primer sets until the desired product size is obtained. Confirmation and quality control can be verified by direct sequencing because each product corresponds to a specific APC sequence identity. Also, the restriction sites flanking the APC could be used to confirm that the correct primer pairs were used in a determination. Alternatively, further monitoring can be performed by high resolution melting (HRM) analysis which allows characterizing each DNA product or APC according to its dissociation behavior (Charrel et al., 2004).

The APC approach described here allows mimicking any kind of target from diverse groups of organisms, including viruses, as well as the inclusion of a large number of targets in one APC or in groups or tandems of APCs (Fig. 1-8). The design in groups or tandems of APCs also brings flexibility to maintain and update a particular group of primers after certain period of time. For example, we can speculate upon a 200 bp long APC insert that harbors one linear array of five different targets with an average of 20 nt per primer (ten primers) (see Fig. 1), from which 120 bp PCR products are amplified (see Table 1). Because the APC sequence is expected to be designed with multiple primer inserts it is unlikely to expect no specific amplifications. Assuming that DNA segments up to 10 kb can be synthesized and cloned into pUC57 using sub-cloning and short DNA oligos as building blocks (Genscript, 2012), an APC insert of 10 kb would carry 50 different sub-APCs in tandem containing 5 primer sets each, which is equivalent to 250 predetermined microbe targets. Such construct would allow a broad and diverse positive control to include a large number of pre-determined targets for numerous pathogens or genes, eliminating biosafety risks associated to the handling of high consequence pathogens.

When the use of APCs and infected plant tissue was compared in PCR, the former yielded maximum accuracy (100%), while the latter provided only 92% accuracy (Table 3) over all targets (p = 0.0006). For just the BYDV target, the comparison of APCs vs. PCR yielded a difference of 100% to 94% (p = 0.0225). This difference is difficult to attribute to a single factor, and is primarily due to small sample sizes associated with the SBWMV, TriMV and WSMV targets. Actually, the biological significance for SBWMV, TriMV and WSMV is greater than that of BYDV (as evidenced by the greater differences seen in the percentages). This is why the significance for the total is much greater than that of just BYDV (p = 0.0006 vs. p = 0.0225). Loss of accuracy with traditional positive controls is normally associated with RNA or cDNA degradation, human errors occurring during the multi-step extraction, or during reverse transcription, PCR or one step RT-PCR (Chomczynski and Sacchi, 1987; Garber and Yoder, 1983; Park, 2007). Furthermore, in the case of plants, plant-associated PCR inhibitors (polysaccharides, tannins and polysaccharides) can cause anomalous association among PCR components during storage (Li et al., 2008; Murray and Thompson, 1980) and interfere with subsequent assays. This kind of interference was not observed within the APCs PCR amplification because APCs were tested on two-step RT-PCR, which freed APC reactions from host inhibitors making the reaction highly accurate.

The use of plasmid-based PCR controls to mimic pathogens has been reported, but their development is laborious and their impact has been limited to a small number of targets (Caruthers, 1985, 1991; Charrel et al., 2004; Chomić et al., 2010; Inoue et al., 2004; Siegling et al., 1994; Stähler et al., 2006). Advances in their development have included the linear arrangement of several genes linked through restriction and hybridization sites, and the use of a complete gene cloned into a plasmid vector (Charrel et al., 2004; Inoue et al., 2004; Siegling et al., 1994; Smith et al., 2006).

This study described the concept, development and functional validation of fully synthetic APCs, designed and constructed using a novel design involving cloning of custom and de novo synthetic DNA inserts constructed of either primer sequences from multiple genomic targets or existing priming sequences of value for detection and diagnostics. This approach offers the possibility of engineering single controls for a large number of predetermined targets. If adopted, this concept will find applications in many areas of microbiology. Moreover, APC constructs would be used in conjunction with EICD or alternative dry or paper based storage technology to facilitate preservation, shipping and commercialization. The implementation of APCs can eliminate biosafety risks associated with in vivo positive controls, can streamline and improve reliability of PCR detection and diagnostics, and offer new opportunities for development of synthetic positive control combinations to fulfill the demand of different health, forensics or agricultural markets.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2013.08.017.

Funding

This study was supported by Oklahoma State University, Oklahoma Agricultural Experiment Station Project Number OKL02773. The
mention of trade names or commercial products in this publication does not imply recommendation or endorsement by Oklahoma State University.

Conflict of interest statement

None declared.

Acknowledgments

The authors acknowledge Drs. Jacqueline Fletcher, Ulrich Melcher and Hassan Melouk, committee members of the doctoral research program of Dr. Donna Caasi at Oklahoma State University, for their mentoring, review and supervision. Jennifer Olson and Dr. Robert Hunger for providing reference plant positive controls, and to Drs. Jacqueline Fletcher and Ulrich Melcher for reviewing this manuscript. The authors also extend their acknowledgment to Oklahoma State University, Oklahoma Agricultural Experiment Station for funding and support.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Alvarez, A.M., 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. Annu. Rev. Phytopathol. 42, 339–366.

Arief, M., Ochoa-Corona, F.M., 2012. Comparative assessment of 5′-A/T-rich overhang sequences with optimal and suboptimal primers to increase PCR yields and sensitivity. Mol. Biotechnol. http://dx.doi.org/10.1007/s12033-012-9617-5 (First published online on 02 November, 2012.).

Caruthers, M.H., 1985. Gene synthesis machines: DNA chemistry and its uses. Science 230, 281–285.

Caruthers, M.H., 1991. Chemical synthesis of DNA and DNA analogs. Acc. Chem. Res. 24, 278–284.

Charvol, R., La Scala, B., Ranolt, D., 2004. Multi-pathogens sequence containing plasmids as positive controls for universal detection of potential agents of bioterrorism. BMC Microbiol. 4, 21.

Chromczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Chomil, A., Pearson, M.N., Clover, G.R.G., Fareyrol, K., Saul, D., Hampton, J.G., Armstrong, K.F., 2010. A generic RT-PCR assay for the detection of Luteoviridae. Plant Pathol. 59, 429–442.

Fleming, D., Job, M., Strömqvist, L., Brooks, A.J., 2004. DFold: PCR design that minimizes secondary structure and optimizes downstream genotyping applications. Hum. Mutat. 24, 1–8.

Garviel, G.C., Yoder, O.C., 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. Anal. Biochem. 135, 416–422.

Genescript, 2012. Gene synthesis. http://www.genescript.com/index.html.

Henson, J.M., French, R., 1993. The polymerase chain reaction and plant disease diagnosis. Annu. Rev. Phytopathol. 31, 81–109.

Inoue, S., Noguchi, A., Tanabayashi, K., Yamada, A., 2004. Preparation of a positive control DNA for molecular diagnosis of Bacillus anthracis. Jpn. J. Infect. Dis. 57, 29–32.

Jacobson, A.B., Zuker, M., 1993. Structural analysis by energy dot plot of a large mRNA. J. Mol. Biol. 233, 261–269.

Jensen, M.A., Fukushima, M., Davis, R.W., 2010. DMSO and betaine greatly improve amplification of GC-rich constructs in de novo synthesis. PLuS One 5, e11024.

Josse-Caasi, D.R., 2012. Assessment of Soluble Biomaterials and Innovation of an Eutin-independent Collection Device for Rapid Collection, Detection, and storage of Plant Pathogens. (Doctoral dissertation) Oklahoma State University 3524481.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Li, R., Mock, R., Huang, Q., Abad, J., Hartung, J., Kinard, G., 2008. A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens. J. Virol. Methods 154, 48–55.

Louv, T.M., Whitney, S.E., TerMaat, J.R., Pienaar, E., Viljoen, H.J., 2011. Oligonucleotide optimization for DNA synthesis. AICHE 57, 1912–1918.

Menzel, W., Jeckmann, W., Maiss, E., 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99, 81–92.

Miller, S.A., Beed, F.D., Harmon, C.L., 2000. Plant disease diagnostic capabilities and networks. Annu. Rev. Phytopathol. 38, 15–34.

Murray, M.G., Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321–4325.

Nechvatal, J.L., Ram, J.L., Basson, M.D., Namprachan, P., Niec, S.R., Badsha, K.Z., Matherly, L.H., Majumdar, A.P.N., Kato, I., 2008. Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. J. Microbiol. Methods 72, 124–132.

Nelson, P.E., Cox, M.M., 2008. Lehninger Principles of Biochemistry. W.H. Freeman and Company, New York, NY.

Ochoa-Corona, F.M., 2012. United States Patent Application US2012/0202211 A1.

Park, D., 2007. Genomic DNA isolation from different biological materials protocols for nucleic acid analysis by nonradioactive probes. In: Hilarius, E., Mackay, J. (Eds.), Protocols for Nucleic Acid Analysis by Nonradioactive Probes, Second edition. Methods in Molecular Biology, 353. Humana Press, New York, NY, pp. 3–13.

Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Kravetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, pp. 365–386.

Schmitt, M., 2008. Diffusion of synthetic biology: a challenge to biosafety. Syst. Synth. Biol. 2, 1–6.

Siegring, A., Lehmann, M., Platzer, C., Emmrich, F., Volk, H.-D., 1994. A novel multispecific competitor format for quantitative PCF analysis of cytokine gene expression in rats. J. Immunol. Methods 173, 23–28.

Smith, G., Smith, I., Harrower, B., Warrillow, D., Betchley, C., 2006. A simple method for preparing synthetic controls for conventional and real-time PCR for the identification of endemic and exotic disease agents. J. Virol. Methods 135, 229–234.

Sthaler, P., Beier, M., Gao, X., Hoheisel, J.D., 2006. Another side of genomics: synthetic biology as a means for the exploitation of whole-genome sequence information. J. Biotechnol. 124, 206–212.

Tao, Q., Zhang, H.-B., 1998. Cloning and stable maintenance of DNA fragments over 300 kb in Escherichia coli with conventional plasmid-based vectors. Nucleic Acids Res. 26, 4901–4909.

Weiss, J., Zucht, H.D., Forssmann, W.G., 1994. Amplification of gene fragments with very high G+C content: c7dGTP and the problem of visualizing the amplification products. Genome Res. 4, 124–125.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3465–3475.