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A simple method for preparing synthetic controls for conventional and real-time PCR for the identification of endemic and exotic disease agents

Greg Smith [a,b,*], Ina Smith [a,b], Bruce Harrower [a], David Warrilow [a,c], Cheryl Bletchly [a]

[a] *Public Health Virology, Centre for Public Health Sciences, Queensland Health Scientific Services, 39 Kessels Road, Coopers Plains, Brisbane, Qld 4108, Australia*
[b] *Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital and Health Service District Back Road, Off Bramston Terrace, Herston, Qld 4029, Australia*
[c] *Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Herston, Qld 4029, Australia*

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Abstract

Medical and veterinary diagnostic and public health laboratories world-wide are increasingly being called upon to introduce molecular diagnostic tests for both endemic and exotic diseases. This demand has accelerated following increasing terrorism fears. Ironically these same concerns have lead to tightening of both import and export controls preventing many laboratories, particularly those outside of the United States, from gaining access to positive control material. This in turn has prevented many laboratories from introducing much needed molecular diagnostic tests. We describe here a generic approach for preparing synthetic DNA or RNA control material for use in either TaqMan or conventional PCR assays. The production of synthetic controls using this approach does not require cloning or special equipment or facilities beyond that found in any laboratory performing molecular diagnostics. The approach significantly reduces the possibility of contamination or erroneously reporting false-positive reactions due to contamination from positive control material. Synthetic controls produced using this approach have been employed in all molecular diagnostic tests performed in our laboratory and are used irrespective of whether we possess the organism or not.

Keywords: Synthetic control; TaqMan; PCR

1. Introduction

Most public health laboratories world-wide are expected to have an extensive repertoire of tests available for the rapid diagnosis of both endemic and exotic viral diseases (Frank, 2005; Rotz et al., 2000, 2002). Ready access to scientific journals has allowed the rapid introduction of molecular assays for a wide range of exotic viruses while access to genomic databases via the internet has enabled the development of in-house molecular assays in a variety of formats for a wide array of pathogens. The biggest stumbling block for laboratories introducing such assays is the lack of appropriate positive control material. Ironically the increasing concern over terrorism which has heightened demand for a broadly expanded diagnostic capability has also increased restrictions on the importation and export of exotic viruses or even extracted genetic material for use as controls for such assays. In Australia, laboratories face the dual obstacles of identifying a country prepared to release the material plus the difficulties of convincing Australian quarantine authorities to approve the importation of such material for diagnostic purposes.

Molecular diagnostics is now the cornerstone of routine viral diagnosis in many clinical and public health laboratories. The problems of false-positive results due to cross contamination in the molecular diagnostic laboratory are now well known and well documented (Aslanzadeh, 2004; Borst et al., 2004; Landry, 1995. Teles et al., 2005). The problem of false-positive results can be greatly reduced providing testing is conducted in appropriately designed facilities with dedicated rooms for reagent preparation, nucleic acid extraction, amplification and post PCR analysis and provided strict enforcement of directional movement of people and materials are observed (Borst

* Corresponding author. Tel.: +61 7 32749151; fax: +61 7 30009186.
E-mail address: greg_smith@health.qld.gov.au (G. Smith).

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et al., 2004; Millar et al., 2002). Even when dedicated rooms and directional movement is employed false-positives can still occur (Borst et al., 2004). In these circumstances the source of contamination can often be linked to the extraction of other positive samples in the same room at the same time. Since positive controls are included in every extraction procedure these can often represent the most common source of contaminating nucleic acids. Intact virus, extracted viral nucleic acid or cloned, unmodified viral segments all represent a potential source of contaminating nucleic acid when employed as positive controls.

Additionally, the use of live virus as a routine source of positive control material can also present a significant and unnecessary occupational health and safety concern for some laboratories. The requirement for positive controls may even preclude some laboratories performing molecular diagnostic tests for pathogens which they do not have the proper containment facilities to handle. For instance, in Australia, SARS coronavirus (and the preparation of viral RNA for controls) can only be handled in PC4 facilities while clinical material submitted for SARS exclusion testing using molecular detection can be handled at PC2 containment conditions providing additional precautions are followed.

We describe here two approaches for preparing synthetic positive control material for the molecular detection of virtually any virus, irrespective of whether the test is a published method or has been developed in-house based on published sequence information. Either method can be used for RNA (or DNA) viruses and both are suitable for adoption by any laboratory capable of performing PCR. Both methods are used routinely in our laboratory for all molecular assays irrespective of whether we hold the virus in our collection or not. The first method is applicable to real-time PCR (TaqMan) assays while the second method is suitable for conventional single round or nested PCRs. These methods have been used to establish 'controlled' assays for a variety of viral diseases which are exotic to Australia but which could be introduced into the country via international air travel or through intentional release. An in-house developed TaqMan assay and a reverse transcriptase (RT)-PCR test based on a published method for SARS coronavirus (Drosten et al., 2003) will be used for illustrative purposes to describe the construction of controls for both tests. This paper is intended to present a generic approach for the production of synthetic controls, not to describe in detail molecular diagnostic tests for SARS for which many have already been described and compared (Mahony et al., 2004; Drosten et al., 2003).

2. Material and methods

2.1. Virus isolates, plasmids

The SARS coronavirus used in this study was generously provided by Professor J.S.M. Peris, Department of Microbiology, The University of Hong Kong and was propagated by passage under PC4 containment conditions at 37 °C in Vero E6 cells (ATCC C1008) in OptiMEM containing 3% fetal bovine serum and antibiotics. SARS genomic RNA was provided as part of a National QC panel by Dr. Mike Catton of the Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia. The pUC18 plasmid used in this study was purchased from New England Biolabs, USA.

2.2. Preparation of synthetic controls for real-time RT-PCR (TaqMan)

Two separate synthetic controls (oligonucleotides) are routinely employed in this laboratory for the detection of any viral pathogen by TaqMan. In the case of SARS, the first, a probe control, was 85 nucleotides long, double stranded and consisted of the SARS coronavirus TaqMan probe sequence (bold) flanked by rodent glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) forward and reverse primer sequences (italics) as described for the rGAPDH TaqMan Assay (Perkin-Elmer, 1998) (Fig. 1). The bacteriophage T7

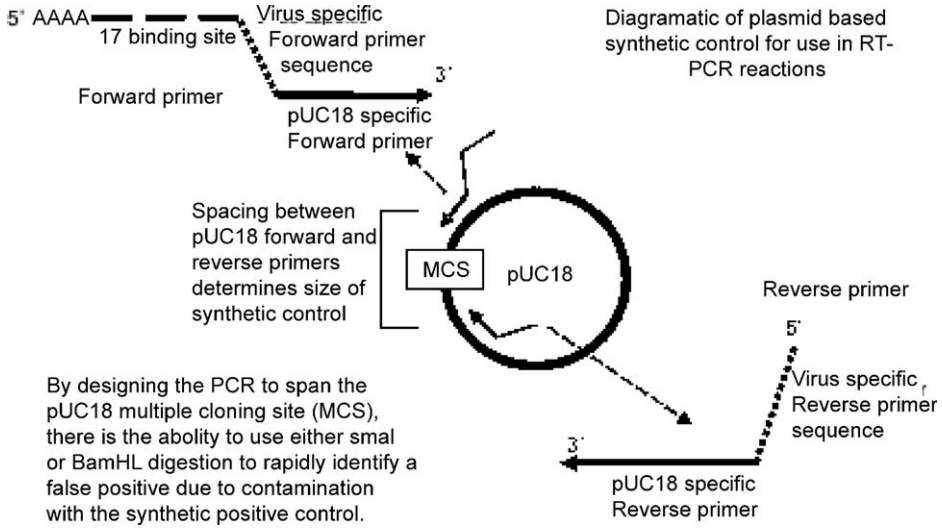

Fig. 1. Schematic showing the creation of a PCR amplicon for subsequent use as a template to generate a synthetic RNA control for use in first round RT-PCR reactions.

RNA polymerase binding site (underlined) was included at the 5′ end of the oligonucleotide to allow RNA transcription of the oligonucleotide. An additional four nucleotides were included at the extreme 5′ end of the oligonucleotide. The target for the in-house developed SARS coronavirus TaqMan was in a highly stable region of ORF1b (nsp11). The probe control consisted of: 5′-AAAA-[T7 Promoter]-[rGAPDH forward]-[SARS Probe]-[rGAPDH reverse]-3′. The actual nucleotide sequence of the positive sense probe control was 5′-AAAATAATACGACTCACTATAGGG*TGCACCACCAACTG-CTTAG*^{18215}**CGTTCGTGCGTGGAT**^{18229}*GAACATCATCC-CTGCATCC*-3′. The relative nucleotide positions are based on the full genome length sequence of a SARS coronavirus (GenBank accession no. AY291451). A second HPLC purified oligonucleotide designed as a complimentary strand to that describe above was also obtained. The two separate oligonucleotides were diluted to a concentration of 200 μM and annealed by first heating to 94 °C for 1 min and then allowing to cool to room temperature. This double stranded oligonucleotide was then used to produce RNA control material as described in 2.4 below.

The second control, a primer control, was constructed in a similar manner with the GAPDH probe sequence (bold) replacing that of the SARS probe sequence while the SARS forward and reverse primer sequences (italics) were substituted for the GAPDH primer sequences (Fig. 1). The double stranded primer control, was 80 bp long and consisted of 5′-AAAA-[T7 Promoter]-[SARS forward]-[rGAPDH Probe]-[SARS reverse]-3′. The actual oligonucleotide sequence consisted of: 5′AAAA TAATACGACTCACTATAGGG ^{18193}*CCCGCGAAGAAGCTA-TTCG*^{18211}**CAGAAGACTGTGGATGGCCCCTC**^{18232}*GCT-TTGATGTAGAGGGCTGTCA*^{18253}-3′.

2.3. Design of synthetic controls for nested RT-PCR assays

First round primer pairs were chosen using the primer design software Primer Express™ Version 2.0.0 (Perkin-Elmer, Applied Biosystems, USA) to produce a product of approximately 220 bp in length from pUC18 DNA, a product 31 bp larger than the expected first round product of 189 bp. The first round primers were designed so that the *Sma*I containing multiple cloning site of the plasmid would be located approximately in the middle of the resulting amplicon to allow later digestion of the product by *Sma*I digestion (Fig. 2). The primers were designed to incorporate the addition of the first round forward primer sequence of the SARS coronavirus (5′-^{18153}ATGAATTACC AAGTCAATGGTTAC^{18176}-3′) to the 5′ end of a pUC18 specific sequence (5′-TGCTGCAAGGCGATTAAGTT-3′). The 5′ end of the pUC18 first round control forward primer was further extended by the inclusion of the T7 RNA polymerase promoter sequence and four additional A residues (5′-AAAATAATACGACTCACTATAGGG-3′) to the 5′ end of the 'forward control' primer in the order 5′-AAAA-[T7 RNA Polymerase]-[SARS Fwd Primer]-[pUC18 (Fwd) sequence]-3′ to produce a primer 68 bp in length.

The SARS coronavirus control first round reverse primer sequence (5′-^{18342}CATAACCAGTCGGTACAGCTAC^{18321}-3′) was 'added' to the 5′ end of a pUC18 specific sequence (5′-GGCTCGTATGTTGTGTGGAATT-3′) in the order 5′-[SARS Rev Primer]-[pUC18 (Rev) sequence]-3′ to produce a primer of 44 bp in length.

The second round control primer pairs were designed to yield a product of approximately 140 bp in length (32 bp larger than the expected second round product size of 108 bp),

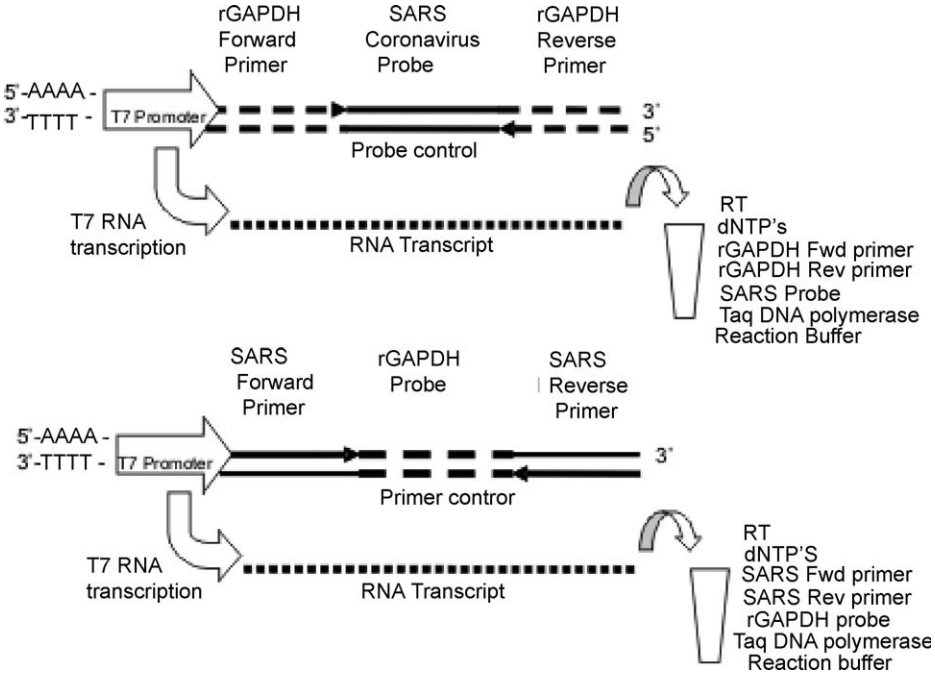

Fig. 2. Schematic of oligonucleotide and procedure for producing synthetic probe and primer controls for use in RT-TaqMan assays.

again spanning the plasmid's multiple cloning site. The second round control forward primer was 39 bases in length and consisted of the SARS coronavirus forward primer 5′-^{18201}GAAGCTATTCGTCACGTTCG^{18220}-3′ joined to the pUC18 specific sequence 5′-CCTGCAGGTCGACTCTAGA-3′. In contrast to the first round control forward primer no. T7 RNA polymerase binding site was included. The second round control reverse primer was 40 bp in length and comprised the SARS coronavirus reverse primer 5′-^{18309}CTGTAGAAA ATCCTAGCTGGAG^{18288}-3′ linked to a pUC18 specific sequence 5′-TGGAATTGTGAGCGGATA-3′.

A standard AmpliTaq Gold 'hot start' PCR cycling reaction was employed and consisted of a single activation cycle at 95 °C for 12 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min followed by a single extension cycle of 72 °C for 7 min. The volume of the PCR mixture was 50 μl. All the PCR mixtures contained 0.5 μM (each) primer and 0.2 mM (each) deoxyribonucleoside triphosphates, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.75 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus) and 1 ng of undigested, circular pUC18 DNA.

The products were analysed by electrophoresis on a 2% agarose gel at 8 V/cm, the bands excised and recovered using the Qiagen Gel Purification Kit (Qiagen) according to manufacturers instructions. The recovered 195 bp 'first round' PCR product was used for the synthesis of RNA as described below.

2.4. Synthesis of RNA control material

The Promega T7 Riboprobe kit was used according to manufacture's (Promega, USA) instructions to produce RNA transcripts from either oligonucleotide DNA or PCR amplified products. Following DNase digestion the RNA synthesis reactions were phenol: chloroform extracted and ethanol precipitated. The precipitated RNA was resuspended in RNA Secure Buffer (Ambion, USA) and analysed for the presence of residual plasmid DNA or oligonucleotide DNA by diluting the samples in a serial 10-fold series and performing either PCR or TaqMan assays in parallel with and without reverse transcriptase. The dilution used as a control was that dilution which produced a distinct product with RT but failed to do so when RT was left out of the reaction mix.

2.5. Real-time and conventional RT-PCR

Amplification for the detection of SARS RNA and synthetic control material was performed in an ABI Prism 7700 sequence detector system using a single-tube one step RT-TaqMan approach. A 50 μL reaction mix contained (Perkin-Elmer, Applied Biosystems, USA), TaqMan buffer A (50 mM KCl, 10 mM Tris–HCl, 0.01 mM EDTA, 60 nM ROX passive reference (pH 8.3), 5.5 mM $MgCl_2$, dNTPs (300 μM dATP, dCTP, dGTP) with dUTP (600 μM), 150 nM FAM labelled TaqMan probe, 300 nM of each primer, 12.5 U multiscribe reverse transcriptase, 20 U RNase inhibitor, 1.25 U Taq Gold polymerase). Cycling consisted of: one cycle of 48 °C for 30 min for reverse transcription of RNA to cDNA followed by one cycle at 95 °C for 10 min to inactivate the reverse transcriptase. The cDNA was amplified by cycling for 40 cycles between 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (C_t) was determined. A negative result was considered where no amplification occurred (a reaction where the C_t was equal to or greater than 40 cycles).

Cycling conditions for a touchdown nested RT-PCR for the detection of SARS coronavirus was performed as described previously (Drosten et al., 2003). The superscript one step RT-PCR system (Roche) was employed for first round amplification. Primer pairs for the first round reaction were BNIoutS2 (5′-ATGAATTACCAAGTCAATGGTTAC-3′) and BNIoutAs (5′-CATAACCAGTCGGTACAGCTAC-3′). Amplification was performed in a Perkin-Elmer, Applied Biosystems 9700 thermocycler using 50 μL reactions comprising 1× RT-PCR reaction buffer containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 mM DTT, 20 U of RNasin, 0.2 μM of each primer and 1 μL of Titan enzyme mix (Roche). Cycling conditions were as recommended and included an initial reverse transcriptase cycle of heating to 45 °C for 30 min followed by inactivation at 95 °C for 3 min. This was followed by 10 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s (the annealing temperature was decreased by 1 °C per cycle over the 10 cycles) and extension at 72 °C for 20 s. This was followed by a further 40 cycles consisting of denaturation at 95 °C for 10 s, annealing at 56 °C for 10 s and extension at 72 °C for 20 s.

The PCR product from the first round reaction was diluted 1:100 in sterile water and 2 μL added to a 50 μL, second round PCR reaction consisting of 1× PCR buffer II (10 mM Tris–HCl pH 8.3, 50 mM KCl) (Applied Biosystems), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each of second round primer and 2 U AmpliTaq Gold (PE Applied Biosystems). The two second round primers were BNIinS 5′-GAAGCTATTCGTCACGTTCG-3′ and BNIinAs 5′-CTGTAGAAAATCCTAGCTGGAG-3′. Following the initial denaturation at 95 °C for 12 min reactions were submitted to 10 cycles of 95 °C for 10 s, 60 °C for 10 s (the annealing temperature was decreased by 1 °C per cycle over the 10 cycles) and 72 °C for 20 s followed by a further 25 cycles consisting at 95 °C for 10 s, 56 °C for 10 s and 72 °C for 20 s.

2.6. Restriction enzyme digestion and agarose gel analysis

To identify amplified synthetic control material from authentic viral amplicons PCR products were digested in a 10 μL reaction which consisted of 9 μL of the PCR product and 1 μL of *Sma*I (10 U/μL) (Roche, Germany). The reaction was incubated at 25 °C for 1 h, 2 μL of 5× gel loading buffer (Promega) added and the reaction analysed by electrophoretic separation on a 2% (SARS) or agarose gel at 10 V/cm.

3. Results

3.1. Synthetic TaqMan controls

The synthetic RNA probe and primer controls produced typical amplification plots across the range of serial 10-fold dilutions

Table 1
Typical C_t values obtained when TaqMan reactions were performed on serial 10-fold dilutions of synthetic probe control with (column 2) and without (column 3) reverse transcriptase in the reaction

Dilution	Probe control with RT	Probe control no RT	SARS RNA
10^{-2}	14.24	21.33	22.53
10^{-3}	14.81	24.65	26.08
10^{-4}	16.22	28.09	28.71
10^{-5}	20.46	30.97	32.34
10^{-6}	23.04	37.45	36.17
10^{-7}	26.34	40	40
10^{-8}	30.41	40	40
10^{-9}	34.10	40	40
10^{-10}	40	40	40
NTC	40	40	40

Column 4 shows the SARS coronavirus RNA with reverse transcriptase reaction. NTC refers to non-template control.

from 10^{-2} to 10^{-9} (Table 1). The shape of the amplification plots obtained for the synthetic probe and primer controls were virtually indistinguishable from those obtained with SARS coronavirus RNA (plots not shown). As indicated in Table 1 residual DNA template was present in the TaqMan control as is evidenced by the positive signal at a dilutions of 10^{-6} (C_t 37.45) or lower in the no RT control (column 3).

3.2. Synthetic RT-PCR controls

The first round synthetic RNA control produced a positive, easily discernable band of approximately 245 bp in length in RT-PCR (Fig. 3, lane 4) while the second round DNA control produced a product of an estimated size of approximately 140 bp (Fig. 3, lane 8). These amplicons were discernibly larger than the 189 bp product produced from SARS RNA in the first round (Fig. 3, lane 2) and second round reactions (108 bp, Fig. 3, lane 6). *Sma*I digestion resulted in cleavage of the synthetic controls for both first (Fig. 3, lane 5) and second round products (Fig. 3, lane 9). In contrast, *Sma*I failed to cleave either the first or second round RT-PCR products from SARS coronavirus RNA (Fig. 3, lanes 3 and 7).

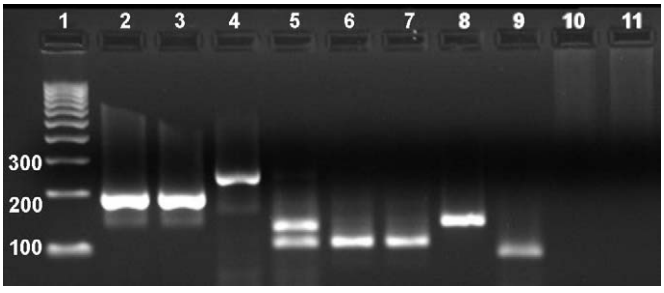

Fig. 3. RT-PCR Amplification products. Lane 1, 100 bp ladder with 100, 200 and 300 bp bands marked. Lane 2, SARS RNA first round product. Lane 3, SARS first round product following *Sma*I digest. Lane 4, synthetic RNA control first round product. Lane 5, first round synthetic control following *Sma*I digest. Lane 6, SARS RNA second round product. Lane 7, SARS second round product following *Sma*I digestion. Lane 8, synthetic control second round product. Lane 9, synthetic control second round product following *Sma*I digestion. Lane 10, no template control; Lane 11, negative extraction control.

3.3. TaqMan and conventional nested RT-PCR assay for SARS coronavirus

The TaqMan assay described here performed equally well with synthetic control material and extracted viral RNA (Table 1). The assay showed no cross reactivity with other respiratory viruses including influenza A, influenza B, respiratory syncytial virus, adenovirus or parainfluenza virus 1 (data not shown). SARS RNA produced products of the predicted sizes for the first (Fig. 3, lane 2) and second round (Fig. 3, lane 6) RT-PCR reactions.

4. Discussion

The use of fully synthesized oligonucleotide or PCR generated DNA templates as positive control material in its own right (DNA viruses) or as a template for generation of single-stranded RNA controls (RNA viruses) is an approach that is accessible to any laboratory that performs in-house generated PCR tests. The approach does not require cloning of products, generation of genetically modified organisms, specialised containment facilities or specialised skills or knowledge. This approach allows the development of TaqMan or conventional PCR assays for virtually any virus or organism for which a published method or sequence information exists. The two approaches described here, an in-house designed TaqMan test and a nested RT-PCR test based on a published method (Drosten et al., 2003), allowed us to introduce molecular testing for SARS as soon as sequence information became available on the internet. We believe this approach will be particularly useful for those laboratories which lack appropriate physical containment facilities for propagating a particular virus or have difficulty gaining access to the organism in question due to international tightening of both import and export controls. This laboratory currently performs a large number of both TaqMan and conventional PCR tests for a broad range of endemic and exotic viral diseases for which we have actual viral stocks. Despite this we use synthetic controls, produced as described above, for all of our assays in order to reduce the risk of false-positive reactions.

By using T7 RNA polymerase and commercially available RNA transcription kits it is possible to produce controls to monitor the performance of either a RT-TaqMan or RT-PCR for detection of RNA viruses at all stages of the reaction including the reverse transcription, primer (and probe) binding and Taq DNA polymerase extension steps. Because of the small size of the synthetic TaqMan DNA template, residual DNA is frequently present following many commercial kit-based RNA transcription reactions. Although it is not necessary for the RNA preparations to be totally free of DNA template it is important that the dilution chosen as an RT control does not produce a product following a PCR reaction which contains no RT. A simple dilution series similar to that depicted in Table 1 will allow selection of a dilution that is free of DNA template and thus suitable for use as an RT control.

The use of synthetic controls for TaqMan assays allows all steps of the reaction to be controlled (including, reverse transcription, Taq DNA polymerase activity, primers and fluorescent

probe). Their use also effectively eliminates the risk of contamination from either the positive control itself or from the resulting amplicon since neither the probe nor primer control can lead to a false-positive reaction as neither the rGAPDH probe or rGAPDH primers are present in the viral TaqMan reaction mix. When using this approach for producing RNA control material for use in RT-PCR or TaqMan assays it is also important to note that double stranded templates are required for effective RNA transcription by T7 RNA polymerase. This approach requires two separate control reactions (a primer control and a probe control) to be set up for each diagnostic assay. This inconvenience is easily offset by the ability to ensure that all components of the assay are working while also ensuring that there is no possibility of producing a false-positive reaction due to contamination with either control. False-negative reactions due to inhibitors and the efficacy of the extraction process can be controlled by whatever strategy a particular laboratory finds appropriate for their purposes.

The production of a synthetic RT-PCR control based on pUC18 (or similar plasmid) does not require cloning—the DNA which is commercially available is all that is needed. Although this approach has the potential to produce a false-positive product of the appropriate size it is easy to rule out the inadvertent amplification of the synthetic control by restriction endonuclease digestion. By using a plasmid template with a *Sma*I or *Bam*HI containing multiple cloning site the product can be digested directly in the PCR buffer (without buffer exchange) with either enzyme and visualised by electrophoresis thus allowing confirmation of a positive result within 2 h. Digestion of the product to produce bands of the expected size provides rapid confirmation of a false-positive reaction. This of course is provided that the target organism does not possess a *Sma*I or *Bam*HI restriction endonuclease recognition site in nearly identical locations as the synthetic control. A simple in silico analysis of the target sequence would allow this unlikely eventuality to be ruled out. The failure of *Sma*I or *Bam*HI to cleave the product may warrant sequence confirmation of the product before a positive report is released depending upon the clinical or public health implications of doing so. An additional safeguard, employed here, is to design a synthetic control which is slightly smaller or larger than the expected product which thus allows immediate visual identification of a false-positive due to contamination with the synthetic control.

In contrast to the RT-PCR synthetic controls described here it would be much more difficult or even impossible to distinguish contamination from cloned viral material (which some laboratories use as positive controls) from 'real virus' as the nucleotide sequence of the amplified contaminant would be indistinguishable from actual virus.

Although synthetic controls are used in this laboratory for a wide variety of in-house developed assays, we are careful to only use this approach when we have fully validated the assay (Smith et al., 2001, 2002; Warrilow et al., 2002) or have based the assay on a published paper from a respected research group which contains detailed procedures for performance of the assay. Few laboratories would be comfortable issuing a negative or 'not detected' result for an exotic agent with potentially far reaching public health or political implications without some assurance that all components of the reaction had performed as expected. Synthetic controls produced as described here could help address such a need.

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