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Short communication

The use of Armored RNA as a multi-purpose internal control for RT-PCR

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ABSTRACT

Real time reverse transcriptase-PCR (RT-PCR) is now used commonly for the detection of viral pathogens in respiratory samples. However, due to potential inhibition of the RT-PCR or inefficient extraction, this sample type can present significant challenges to accurate patient testing. The goal of this study was to create an internal control to be multiplexed in a real time RT-PCR assay for detecting a viral target in respiratory samples. This report describes an Armored RNA (aRNA) internal control developed originally to be multiplexed in a real time RT-PCR assay for detecting SARS-associated Coronavirus, but can be incorporated into any RT-PCR assay. The internal control primers and probe target a region in the coat protein gene of the *E. coli* F-specific bacteriophage ms2, which is contained within the aRNA.

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The use of multiplexed internal controls to monitor sample extraction and inhibition of reverse transcriptase-PCR (RT-PCR) is becoming more routine in clinical molecular virology testing, providing an increased confidence in negative patient results (Bustin and Mueller, 2005; Bustin and Nolan, 2004). An RNA internal control is preferred because DNA internal controls typically used for PCR fail to monitor the reverse transcription reaction. Endogenous and exogenous RNA internal controls are both co-extracted and co-amplified with the viral target and have been described previously (Espy et al., 2006; Niesters, 2002; Tong et al., 1999). Endogenous controls are usually mRNAs present in the patient sample, encoded by housekeeping genes and typically expressed at relatively uniform levels. Exogenous controls are usually introduced into the sample prior to extraction. When possible, an internal control is included at a uniform copy number in each reaction. While simple to achieve using an exogenous internal control, strictly regulating copy number is more difficult with an endogenous RNA since the expression of mRNAs can vary significantly between samples, even for housekeeping genes used commonly (Huggett et al., 2005).

There are two general approaches to designing an exogenous internal control, each having specific advantages (Di Trani et al., 2006; Espy et al., 2006; Hoorfar et al., 2004; Pitcher et al., 2006). A 'competitive' internal control uses the same primers as the viral target sequence, but with an altered probe binding sequence. These controls most closely mimic amplification of the target sequence (i.e. similar PCR efficiencies), but can potentially compete with tar-

get amplification at low viral loads, decreasing assay sensitivity. Another limitation is that they normally require a different internal control for each assay. Finally, the process of generating a competitive internal control may be too complex for the typical clinical microbiology laboratory. A second approach or 'non-competitive' internal control uses a sequence entirely unrelated to the viral target, often either another RNA virus or an RNA transcript. However, transcripts suffer from the disadvantage of being vulnerable to degradation, a problem that viral particles are less sensitive to. Furthermore, amplification of a non-competitive internal control may not be an accurate reflection of target amplification. Nevertheless, this approach has been widely adopted, since in many laboratories its benefits outweigh the potential disadvantages.

The internal control target should be quantitated to ensure the appropriate copy number in each reaction. A quantitated internal control from a commercial vendor is an excellent solution for laboratories that either do not have the tools required to grow and quantitate virus, or do not find it cost effective. The potential to use a single internal control target for all RT-PCR reactions limits the number of different internal control designs necessary. This 'universal internal control' can be added to all samples prior to extraction and detected with a single set of primers and probe(s), irrespective of the virus ultimately being assayed. Furthermore, should sequence constraints dictate designing a new IC primer and probe set (e.g. interactions between the viral target and IC primers), the new design can still target this universal control.

The internal control target described here is the HCV-1b Armored RNA (aRNA) (Asuragen, formerly Ambion). This internal control is spiked into Qiagen's AVL lysis buffer prior to sample extraction at a concentration sufficient to yield 12,500 copies of IC per 50 µL of extracted sample (or 2500 copies per 10 µL RT-PCR

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Table 1
Primer and probe sequences (5'–3')

SARS forward	TTATCACCCGCGAAGAAGCT
SARS reverse	CTCTAGTTGCATGACAGCCCT
SARS probe ^a	FAM-TCGTGCGTGGATTGGCTTTGATGT-TAMRA
ms2 IC forward	GCGCAGAATCGCAAATACAC
ms2 IC reverse	CAACAGCTCTGGGTTGCCACTT
ms2 IC probe ^b	VIC-ATCAAAGTCGAGGTG CC-MGB

^a FAM: carboxyfluorescein, reporter; TAMRA: carboxytetramethylrhodamine, quencher.

^b VIC: reporter dye (Applied Biosystems); MGB: minor groove binder (Nanogen).

reaction). Lower concentrations of IC were also tested during assay development (see below).

True SARS positive clinical samples are highly infectious and difficult to obtain, so all positive samples in this study were spiked with inactivated SARS virus (generously provided by Matthias Niedrig at the Robert Koch Institute, Berlin). The concentration of this SARS stock was approximately 9.4e6 copies/mL. Samples were spun for 10 min at 1000 rpm prior to extraction. Supernatants were extracted using the QiaAmp Viral RNA mini kit (spin protocol), with the following modifications. At the final step, the columns were eluted with 50 μ L of molecular grade water that had been pre-warmed to 70 °C. Prior to centrifugation, the columns were incubated for 5 min at 70 °C.

This real time SARS test is based on a TaqMan assay published by Drosten et al. (2003) that employed an RT-PCR kit from Invitrogen using dTTP. This kit was replaced with the Qiagen QuantiTect kit that utilizes dUTP in place of dTTP, providing increased protection from amplicon contamination and making the assay better suited for clinical testing.

Ten microliters of extracted sample was added to 40 μ L of master mix, consisting of: 25 μ L 2 \times Qiagen QuantiTect Probe RT-PCR Master Mix with dNTPs (with UTP), MgCl₂ (4 mM final concentration), buffer and Qiagen HotStarTaq DNA polymerase, 0.5 μ L Qiagen QuantiTect RT Mix containing the RT enzymes (Omniscrypt and Sensiscript reverse transcriptases), SARS primers at 200 nM each, internal control primers at 400 nM each, SARS TaqMan probe at 240 nM, internal control TaqMan probe at 200 nM (Table 1), and 1.0 U of heat-labile UNG (Roche Diagnostics). The Applied Biosystems HT7900 was programmed with the following RT protocol: 30 min at 50 °C, 15 min at 95 °C, followed by 50 cycles of PCR (15 s at 94 °C, 60 s at 58 °C). Data analysis was done with the HT7900 SDS software from Applied Biosystems. Initial singleplex testing of the aRNA amplification was performed as described above, but with 25 μ L reactions and no SARS primers or probe. Heat denaturation of the aRNA was done at 70 °C for 3 min. The Norovirus assay has been described previously (W. Hymas et al., 2007).

An Armored RNA product from Asuragen (formerly Ambion) was evaluated to fulfill the need for a commercially available internal control. The ms2 coat protein gene was targeted since this sequence is included in the Asuragen aRNAs. Using an ms2 sequence from GenBank (gi:9626311), a TaqMan primer/probe combination for amplification and detection of a region within this gene was designed with Primer Express (Applied Biosystems). A central portion of the gene was targeted because sequences near the ends may have been altered during creation of the aRNA. Two different aRNAs, SARS (BNI-1) and HCV-1b were tested with design. The ms2 coat protein sequence was amplified from both targets (data not shown). The HCV-1b aRNA was chosen as a target because an accurate method for quantitating this material is available at ARUP (Roche HCV TaqMan Assay).

Multiplexing an internal control can affect amplification of the desired target sequence. There is often non-specific competition between the reactions that needs to be considered, and it is crucial

to maintain sensitivity for the target gene. This must be balanced by the need to minimize the number of internal control amplification failures in negative samples. A high rate of IC failures generates an unacceptable level of repeat testing for a clinical laboratory.

Initially, 50 copies of the ms2 internal control target were introduced into the SARS reaction. Heat denatured aRNA was added directly to the assay master mix. At this concentration of internal control, no apparent effect on amplification of the SARS target was observed (data not shown). Furthermore, 8/8 no template control (NTC) reactions (RNase free water) displayed amplification of the ms2 target, indicating an acceptable concentration of IC target.

The multiplexed assay was next evaluated to determine how it would perform on Qiagen extracted samples. Dilutions of inactivated SARS virus in water, as well as a number of NTCs, were extracted with IC added to the AVL lysis buffer at 50 copies per reaction. Amplification of the SARS target was satisfactory (efficiency=103%), but the IC failed to amplify in 5/12 of the NTC reactions. In order to achieve a 100% detection rate for the IC, its concentration was increased to approximately 500 copies per reaction. At this level, the IC was detected in 8/8 NTC reactions.

Lastly, the efficiency of IC detection was measured when extracted in respiratory samples from actual patients. Twenty-two respiratory samples (10 nasal swabs, 6 nasal washes, 2 BALs, 1 sputum and 3 of unknown respiratory source) that were presumed to be SARS negative, were extracted with IC and assayed. All samples were negative in the SARS assay, but a significant number of IC failures were observed (5/22 samples tested). These five samples were re-tested in duplicate, with the IC failing to amplify in two of the samples. Based on these results, the IC concentration was increased 5-fold, from 500 to 2500 copies per reaction. Eighteen of the SARS negative samples were repeated, with 2/18 failing to amplify the IC at this concentration, indicating some measure of inhibition. To determine the effect of this IC concentration on SARS target detection, the crossing thresholds for a series of SARS virus dilutions spanning three logs were compared, both with and without the IC, with no significant differences observed (Table 2). In addition, seven replicates of a sample near the limit of detection were tested (approximately three copies per reaction), both with and without IC amplification. In the SARS reaction alone, 7/7 replicates were detected, compared with 5/7 replicates in the multiplexed reaction (data not shown). It was concluded that this minor loss in sensitivity is an acceptable trade off for the benefits provided by the internal control. Each of the 22 SARS negative samples described above was spiked with inactivated SARS virus at a concentration of approximately 25 copies/reaction (about one log above the assay's limit of detection). All 22 samples were detected, indicating that the inhibition detected in the un-doped (negative) samples was insufficient to prevent detection of the SARS target, even at this low viral load.

Long-term stability was another desired feature for our universal internal control. Based on previous experience with the relative instability of frozen SARS aRNA used for a positive assay control, the IC aRNA was lyophilized. The SARS aRNA had been diluted in water, aliquoted and frozen at –20 °C. However, the crossing thresholds for this control became progressively later over time. To investigate this observation further, crossing thresholds of SARS aRNA dilu-

Table 2
Effect of aRNA internal control (IC) on SARS target amplification (C_t)

SARS dilution	Without IC	With IC
10 ⁻³	30.7	30.0
10 ⁻⁴	34.0	34.8
10 ⁻⁵	37.0	36.4

10⁻³ dilution is equivalent to approximately 260 copies per reaction; IC is included at approximately 2500 copies per reaction.

Table 3
Stability of ms2 internal control in the Norovirus assay

Norovirus assay	January/February 2006		September/October 2006		Total	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Group I	31.1	1.1	31.3	0.8	30.9	1.3
Group II	31.3	2.1	31.5	1.1	31.0	1.4

Mean and S.D. are calculated using internal control crossing threshold values.

tions that had been stored at -20°C for 5 weeks were compared with those generated using freshly diluted material. Over the period tested, the crossing threshold of a dilution containing 80 copies per reaction was delayed approximately 3 C_{t} s (from 32.2 up to 35.0), or a 7-fold decrease in detectable target. This result supports the decision to lyophilize the IC aRNA for enhanced stability over time.

This aRNA approach to internal controls is now also being used with two assays offered recently at ARUP for the detection of Norovirus (separate assays for Groups I and II). Furthermore, two additional real time assays have been developed that utilize this strategy, one for Enterovirus (W.C. Hymas et al., 2007) and another capable of detecting Flu A, Flu B and RSV in a multiplex reaction (manuscript in preparation). All these assays use the HCV aRNA internal control and target the same region within the ms2 coat protein gene, but have different primer and probe sequences. These design changes were necessary because the SARS assay uses Taq-Man chemistry while the others listed above are hybridization probe assays. For all the assays, the aRNA is diluted in a BSA solution and lyophilized for enhanced long-term stability. The IC crossing thresholds of the negative control in the two Norovirus assays were examined to evaluate the stability of the aRNA over time. The average crossing thresholds during the first month of clinical testing (between January and February, 2006; $n = 11$) were compared with those observed approximately 8 months later (between September and October, 2006; $n = 13$) and no significant difference was detected over this time period (Table 3). These data provide clear evidence that this aRNA IC stock is stable over a period of at least 8 months and that lyophilization is a practical option when looking to stabilize control material for long-term storage.

As PCR assays became implemented more widely in the clinical laboratory and the need for internal controls achieved a greater appreciation, the quality of DNA control material steadily improved. However, the development of reagents for RNA targets has lagged behind. In recent years, the aRNA phage packaging system has been applied to commercial assays for HIV and HCV (Forman and Valsamakis, 2004). In addition, many laboratory developed assays have been described that utilize aRNA technology for controls (Beld et al., 2004; Bressler and Nolte, 2004; Eisler et al., 2004). An obstacle to assaying RNA targets in routine clinical testing is developing stable internal control material. Common approaches to this problem have been the use of either *in vitro* generated RNA transcripts or cultured viral particles, each having limitations. While the use of cultured ms2 phage as an internal control has been described previously (Dreier et al., 2005, 2006), commercially available products are often the first choice for clinical laboratories. This is the first report to describe targeting the ms2 sequence contained within an aRNA.

One advantage of the aRNA approach for internal controls over the use of transcripts is that they are resistant to the RNases often present in patient samples, while naked RNA transcripts are highly vulnerable. In addition, aRNAs mimic more closely true viral particles and therefore likely to be more sensitive to problems with viral decapsulation than would naked RNA transcripts. Besides their enhanced stability, aRNA particles are non-infectious, reducing the

potential risk to lab workers. The aRNA is also non-replicative, reducing the risk of laboratory contamination relative to viable ms2 phage. Since this product is available commercially, it eliminates the need for culturing the virus and therefore is an approach more appropriate for laboratories lacking these resources. When using the HCV aRNA described above, quantitation of the internal control material is straightforward, because it can be measured with common quantitative HCV assays being performed in many clinical laboratories. Since these assays are typically calibrated against a WHO secondary standard (and indirectly, by the primary standard), this IC quantitation can potentially be determined with a high degree of accuracy. Quantitation of the aRNA is not only useful when determining initially the number of copies included per reaction, but can also be a useful tool for quality control. Stored aliquots of IC can be assayed on a regular basis as part of the laboratory's quality assurance program to ensure that the material is not degrading over time. Results presented here using the aRNA IC in a Norovirus assay indicate that this material is stable for at least 8 months when lyophilized. Finally, the use of a commercial product instead of material generated in the lab or obtained from patient sources provides a well-characterized sequence that is less likely to contain variations.

Developing an assay with a multiplexed internal control requires careful optimization, using a low internal control copy number to minimize the potential for competitive effects between the two reactions. However, as demonstrated in this study, an IC level that is set too low can result in an unacceptable level of IC failures. Since samples that are negative for both the target and IC must be repeated, significant levels of IC failure are unacceptable due to the associated increased costs.

The choice of how to manage internal controls is complex and depends largely on a laboratory's needs and resources. For laboratories seeking a greater degree of assay standardization while retaining a measure of flexibility, targeting the ms2 coat protein sequence contained within aRNA particles can provide an excellent solution to many of the problems associated with internal controls for RT-PCR assays, and using Asuragen's HCV aRNA provides additional benefits.

References

- Beld, M., Minnaar, R., Weel, J., Sol, C., Damen, M., van der Avoort, H., Wertheim-van Dillen, P., van Breda, A., Boom, R., 2004. Highly sensitive assay for detection of Enterovirus in clinical specimens by reverse transcription-PCR with an Armored RNA internal control. *J. Clin. Microbiol.* 42, 3059–3064.
- Bressler, A.M., Nolte, F.S., 2004. Preclinical evaluation of two real-time, reverse transcription-PCR assays for detection of the severe acute respiratory syndrome coronavirus. *J. Clin. Microbiol.* 42, 987–991.
- Bustin, S.A., Mueller, R., 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin. Sci. (Lond.)* 109, 365–379.
- Bustin, S.A., Nolan, T., 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.* 15, 155–166.
- Di Trani, L., Bedini, B., Donatelli, I., Campitelli, L., Chiappini, B., De Marco, M.A., Delogu, M., Buonavoglia, C., Vaccari, G., 2006. A sensitive one-step real-time PCR for detection of avian influenza viruses using a MGB probe and an internal positive control. *BMC Infect. Dis.* 6, 87.
- Dreier, J., Stormer, M., Kleesiek, K., 2005. Use of bacteriophage MS2 as an internal control in viral reverse transcription-PCR assays. *J. Clin. Microbiol.* 43, 4551–4557.
- Dreier, J., Stormer, M., Made, D., Burkhardt, S., Kleesiek, K., 2006. Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. *J. Clin. Microbiol.* 44, 2714–2720.
- Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H.R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R.A., Berger, A., Burguiere, A.M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J.C., Muller, S., Rickerts, V., Sturmer, M., Vieth, S., Klenk, H.D., Osterhaus, A.D., Schmitz, H., Doerr, H.W., 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* 348, 1967–1976.
- Eisler, D.L., McNabb, A., Jorgensen, D.R., Isaac-Renton, J.L., 2004. Use of an internal positive control in a multiplex reverse transcription-PCR to detect West Nile virus RNA in mosquito pools. *J. Clin. Microbiol.* 42, 841–843.
- Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D., Wengenack, N.L., Rosenblatt, J.E., Cockerill 3rd, F.R., Smith, T.F., 2006. Real-time

- PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev.* 19, 165–256.
- Forman, M.S., Valsamakis, A., 2004. Verification of an assay for quantification of hepatitis C virus RNA by use of an analyte-specific reagent and two different extraction methods. *J. Clin. Microbiol.* 42, 3581–3588.
- Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., Fach, P., 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *J. Clin. Microbiol.* 42, 1863–1868.
- Huggett, J., Dheda, K., Bustin, S., Zumla, A., 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* 6, 279–284.
- Hymas, W., Atkinson, A., Stevenson, J., Hillyard, D., 2007. Use of modified oligonucleotides to compensate for sequence polymorphisms in the real-time detection of norovirus. *J. Virol. Methods* 142, 10–14.
- Hymas, W.C., Aldous, W.K., Taggart, E.W., Stevenson, J.B., Hillyard, D.R., 2007. Description and validation of a novel real-time RT-PCR Enterovirus assay. *Clin. Chem.*
- Niesters, H.G., 2002. Clinical virology in real time. *J. Clin. Virol.* 25 (Suppl. 3), S3–S12.
- Pitcher, D., Chalker, V.J., Sheppard, C., George, R.C., Harrison, T.G., 2006. Real-time detection of *Mycoplasma pneumoniae* in respiratory samples with an internal processing control. *J. Med. Microbiol.* 55, 149–155.
- Tong, C.Y., Donnelly, C., Harvey, G., Sillis, M., 1999. Multiplex polymerase chain reaction for the simultaneous detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* in respiratory samples. *J. Clin. Pathol.* 52, 257–263.