Simultaneous control of DNA and RNA processing efficiency using a nucleic acid calibration set

Barbara Bartolini, Anna Rosa Garbuglia, Douglas Horejsh, Federico Martini, Fabrizio Carletti, and Maria Rosaria Capobianchi

The Italian National Institute for Infectious Diseases, "L. Spallanzani"—IRCCS Rome, Italy

BioTechniques 42:452-456 (April 2007)
doi 10.2144/000112450

PCR-based detection techniques enables reliable and sensitive nucleic acid target detection. However, quantitative determination methods often fail to control for the efficiency of nucleic acid extraction, reverse transcription, and PCR amplification. This problem is even more prominent when working with clinical samples due to target sequence loss during nucleic acid processing or the co-purification of PCR inhibitors (1,2). Handling processes are often assumed to approach 100% efficiency in the laboratory, even if practical experience shows that this efficiency can be much lower. This inability to ensure accuracy can lead to significant error in uncalibrated DNA sample quantitation. The additional need for reverse transcription of RNA may further increase the quantitative error rate, as yet another enzymatic process is involved.

Nucleic acid controls have been developed based upon known sequences to calibrate either DNA or RNA handling; DNA calibrators have been used to control for the amplification of target sequences using real-time PCR methods (3–8), while RNA calibrators have been developed to test reverse transcription and amplification efficiencies (9–11). A nonpathogenic viral particle carrying a sequence for use as an external positive control of extraction and amplification has also been described (12). Unfortunately, most of the established processing controls are only suitable for limited applications (i.e., either DNA or RNA detection). Cross-contamination of biological samples or minute detection from natural sources reveals the need for completely synthetic sequences, with no homology to sequences in the nucleic acid databases. It is, therefore, beneficial to design an internal, synthetic calibration system that can control for both DNA and RNA processing steps in a single tube. This set includes both RNA and DNA targets with identical primer binding sites and, thus, primer binding efficiency, but easily distinguishable sequence characteristics, allowing for simultaneous detection, quantitation, and calibration of nucleic acid processing efficiency.

A 150-bp randomly generated nucleic acid sequence was developed for use as a short control (SC). A GC-rich 75-bp sequence was inserted in the middle of the 150-bp sequence to generate a 225-bp sequence, long control (LC). Besides size, the two sequences were designed to have easily distinguishable probe binding sites with a predicted product melting temperature difference of 4°C. Calibrator sequences have been published as GenBank® accession nos. EF143258 (DNA control, LC) and EF143257 (RNA control, SC).

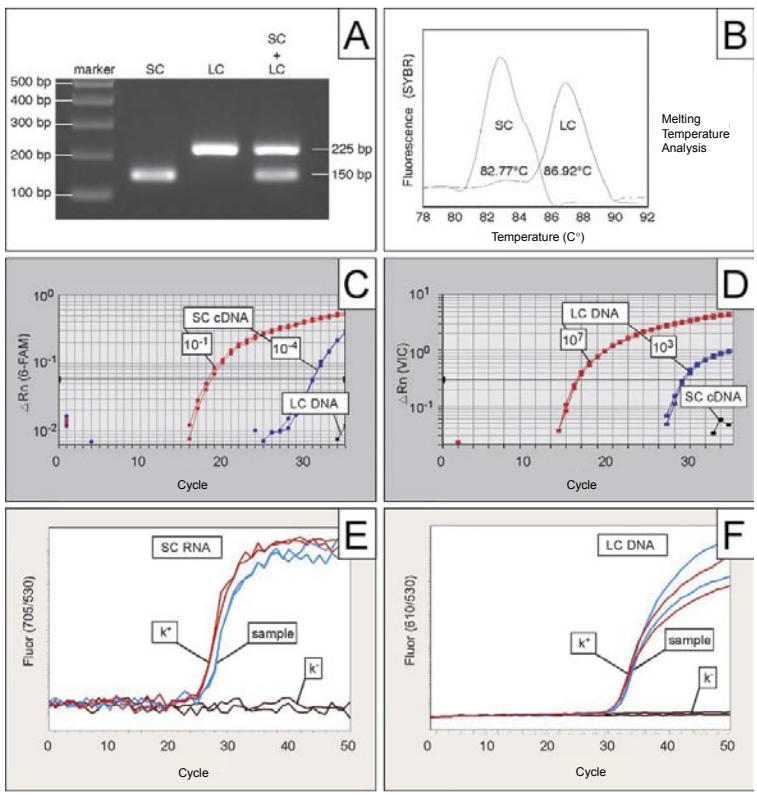

Figure 1. The nucleic acid control set in four different amplification systems. (A) The amplification of the controls by a normal PCR shows that the 75 bp difference is readily resolved on a 2% agarose gel. (B) The predicted 4°C difference of the product melting temperature for the two controls can be seen using SYBR real-time PCR. (C and D) Hydrolysis probe real-time PCR is specific for each control, with each duplicate showing a high level of precision in the assay. (E and F) Hybridization probe real-time PCR is also specific for each control (shown in blue), with a corresponding sample derived from a clinical specimen (shown in red) compared with the calibration control. SC, short control; LC, long control.

The synthetic sequences were constructed using a modified asymmetric PCR method (13). A more detailed description of the construction can be found in the supplementary material available online at www.BioTechniques.com. The final PCR products for both constructs were gel-purified and cloned into pCR®4 using the TOPO®-TA cloning system (Invitrogen, Carlsbad, CA, USA). Sequences were confirmed using BigDye™ chemistry on the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Plasmid DNA was prepared for the LC clone, a 10-fold dilution series was generated, and these aliquots were stored at -20°C. For the in vitro transcription reaction, SC plasmid DNA was prepared by restriction enzyme digestion using *Afl*III, the linear product (600 nucleotides) was resolved on an agarose gel, and then purified using the DNA gel extraction kit (Qiagen, Valencia, CA, USA). RNA was produced by transcription using the MEGAscript® T7 kit (Ambion, Austin, TX, USA) with 100 ng linear SC plasmid DNA input. At the termination of the incubation, 5 U DNase I (Ambion) were added to the reaction to limit plasmid DNA contamination. The RNA was purified using the RNeasy® RNA purification kit (Qiagen) with the supplemental kit for on-column RNase-free, DNase digestion. Dilutions and aliquots were stored at -80°C.

The DNA (LC) and RNA (SC) control set was tested using four different detection systems used by our molecular diagnostic laboratory. As shown in Figure 1A, the size difference between the two calibrators was evident even when performing PCR in a single tube containing both the LC and the SC cDNA. The 75-bp difference was easily resolved on a 2% agarose gel. The assay was sensitive to approximately 50 copies for both targets individually. This system could be used in end point assays such as traditional PCR or reverse transcription PCR (RT-PCR) as a qualitative control to eliminate false negative results.

SYBR® real-time PCR analysis yielded results with two distinct product melting temperature curves, as shown in Figure 1B. The control set had an actual difference in product melting temperature of 4.15°C, comparing favorably to the predicted difference of 4.0°C. Multiple target analysis is impractical in the SYBR system, since simultaneous detection is only possible when internal controls and unknowns are at similar levels. However, with this 4°C difference, it is possible to verify the specific detection of each control target as a distinct external control.

The control set could be readily detected using a pair of hydrolysis probes in a single tube. As shown in Figure 1C, the SC cDNA that was produced from the RNA control could be detected using the 6-FAM probe without cross-reaction with the DNA control. This probe binds across the junction site of the two conserved ends. Figure 1D shows that the LC probe with the VIC fluorophore is specific for the inserted 75-bp fragment that is only present in LC. The ability to use these probes is valuable, as this system works on plates, allowing high-throughput assays by automated robotic systems.

The hybridization probe technique (LightCycler®; Roche Applied Science, Indianapolis, IN, USA) was specific in control detection using two probes for each target. The system included the universal probe 1 (shown in the supplementary material) and a specific probe 2 with a unique fluorophore for each control, allowing the specific detection of the controls with the corresponding probe 2. Although the probe 2 binding site for SC is also present in LC, there are too many bases between the two probes to allow cross-reactivity. In fact, as shown in Figure 1E, the RNA control SC could be detected only when using the SC probe 2 (710 nm) and the One-Step RT-PCR kit (Roche Applied Science). Figure 1F shows that the DNA control was detected only in the LC probe 2 channel (610 nm), and the reverse transcription reaction failed to alter the results.

A series of clinical samples (whole blood, urine, and feces) were submitted to nucleic acid extraction with the control set added to the lysis mixture. The control set (40,000 copies of SC RNA and 20,000 copies of LC DNA) was added to each individual sample at the lysis step. The samples were analyzed using the LightCycler hybridization probes, and individual samples were compared with the positive control based upon the change in cycle threshold (ΔC_T) to derive the extraction efficiency. Figure 1, panels E and F, shows a representative set from the manual extraction, with a duplicate of the positive (unprocessed, in red) control set compared with a duplicate of an extracted sample (in blue). Since dilution analysis showed this reaction was linear across 5 orders of magnitude, 100% PCR efficiency was assumed in the relative calculations. Figure 2 shows an average extraction efficiency of 61% for RNA and 79% for DNA in experiments performed on three sample matrices (blood, urine, and feces). It is important to note that extraction efficiency varies according to sample type; therefore, for quality control assurance, each individual sample type must be calibrated.

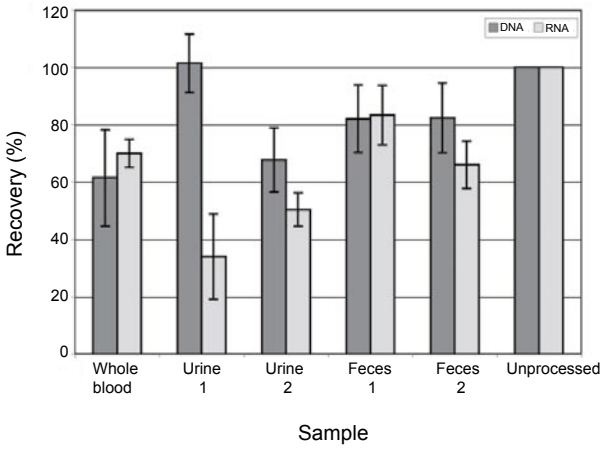

Figure 2. Various clinical sample extraction efficiencies compared with the calibration control set. The efficiencies are calibrated compared with the positive control set after automated extraction of nucleic acids by BioRobot® MDx Workstation (Qiagen) and amplification using the probe hybridization set. The overall average for the efficiency using this system was 79% for the DNA and 61% for the RNA in this clinical sample set.

Finally, the strategy described here may be easily adapted to design further nucleic acid processing control sets suitable for monitoring multiple steps of nucleic acid handling, such as extraction, reverse transcription, and amplification, with different molecular amplification platforms.

ACKNOWLEDGMENTS

The authors wish to thank Loredana Aleo and Stefania Neri for technical assistance. This work was supported by grants "Ricerca Finalizzata" and "Ricerca Corrente," nos. 528/C3 and 28C5, respectively, from the Italian Health Ministry.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Poon, L.L., B.W. Wong, K.H. Chan, C.S. Leung, K.Y. Yuen, Y. Guan, and J.S. Peiris.** 2004. A one step quantitative RT-PCR for detection of SARS coronavirus with an internal control for PCR inhibitors. J. Clin. Virol. *30*:214-217.
2. **Ursi, J.P., D. Ursi, M. Ieven, and S.R. Pattyn.** 1992. Utility of an internal control for the polymerase chain reaction. Application to detection of *Mycoplasma pneumoniae* in clinical specimens. APMIS *100*:635-639.
3. **Bennett, J.M., S. Kaye, N. Berry, and R.S. Tedder.** 1999. A quantitative PCR method for the assay of HIV-1 provirus load in peripheral blood mononuclear cells. J. Virol. Methods *83*:11-20.
4. **Broccolo, F., G. Locatelli, L. Sarmati, S. Piergiovanni, F. Veglia, M. Andreoni, S. Butto, B. Ensoli, et al.** 2002. Calibrated real-time PCR assay for quantitation of human herpesvirus 8 DNA in biological fluids. J. Clin. Microbiol. *40*:4652-4658.
5. **Johansson, M., E.K. Pisa, V. Tormanen, K. Arstrand, and B. Kagedal.** 2000. Quantitative analysis of tyrosinase transcripts in blood. Clin. Chem. *46*:921-927.
6. **Meijerink, J., C. Mandigers, L. van de Locht, E. Tonnissen, F. Goodsaid, and J. Raemaekers.** 2001. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J. Mol. Diagn. *3*:55-61.
7. **Najioullah, F., D. Thouvenot, and B. Lina.** 2001. Development of a real-time PCR procedure including an internal control for the measurement of HCMV viral load. J. Virol. Methods *92*:55-64.
8. **Stranska, R., R. Schuurman, M. de Vos, and A.M. van Loon.** 2004. Routine use of a highly automated and internally controlled real-time PCR assay for the diagnosis of herpes simplex and varicella-zoster virus infections. J. Clin. Virol. *30*:39-44.
9. **Dumur, C.I., C. Dechsukhum, D.S. Wilkinson, C.T. Garrett, J.L. Ware, and A. Ferreira-Gonzalez.** 2002. Analytical validation of a real-time reverse transcription-polymerase chain reaction quantitation of different transcripts of the Wilms' tumor suppressor gene (WT1). Anal. Biochem. *309*:127-136.
10. **Kielar, D., W. Dietmaier, T. Langmann, C. Aslanidis, M. Probst, M. Naruszewicz, and G. Schmitz.** 2001. Rapid quantification of human ABCA1 mRNA in various cell types and tissues by real-time reverse transcription-PCR. Clin. Chem. *47*:2089-2097.
11. **Witt, D.J. and M. Kemper.** 1999. Techniques for the evaluation of nucleic acid amplification technology performance with specimens containing interfering substances: efficacy of boom methodology for extraction of HIV-1 RNA. J. Virol. Methods *79*:97-111.
12. **Pasloske, B.L., C.R. Walkerpeach, R.D. Obermoeller, M. Winkler, and D.B. DuBois.** 1998. Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. J. Clin. Microbiol. *36*:3590-3594.
13. **Sandhu, G.S., R.A. Aleff, and B.C. Kline.** 1992. Dual asymmetric PCR: one-step construction of synthetic genes. BioTechniques *12*:14-16.

Received 22 December 2006; accepted 27 February 2007.

Address corresponding to Maria R. Capobianchi, The Italian National Institute for Infectious Diseases, "L. Spallanzani"—IRCCS, Via Portuense, 292, 00149 Rome, Italy. e-mail: capobianchi@inmi.it