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References

Simple PCR-based Method for Synthesis of Molecular Calibrators and Controls, Steve Kaye (Medical Research Council Laboratories, Atlantic Road, PO Box 273, Banjul, The Gambia; fax 220-496513, e-mail skaye@mrc.gm)

The use of molecular methods such as PCR to quantify nucleic acids has become widely established for both research and diagnostic applications. Increasingly, such methodologies recognize the need for inclusion of internal standards and controls. These are used to define the efficiencies of nucleic acid extraction, reverse transcription, amplification, and detection and to quantify the effects of nonspecific inhibitors. Examples include the "Qs" controls included in Amplicor PCR assays (Roche Molecular Systems Inc.) (1) and the "Qa, Qb, Qc" controls used in Nuclisens assays (Organon Technica) (2). Control constructs (RNA or DNA) are derived from the nucleic acid sequence that the test method is designed to assay and span the primer binding sequences used in the assay. Thus, when added to the test sample, the control is amplified in the reaction with a single primer pair. To distinguish the control from the test sequence during the detection phase of the assay, a short sequence between the primer binding sites is replaced by a defined sequence to allow differential probing with labeled oligonucleotides.

Nucleic acid controls are usually 1 kb or longer, as shorter lengths may not by efficiently purified by the commonly used silica capture or precipitation methods. Previously, the construction of such controls required the identification or engineering of unique restriction sites between the primer binding sites to allow excision of the wild-type sequence and replacement with the probe sequence (1,2). The process also required ligation of the probe sequence into the target sequence backbone and cloning into a plasmid containing a transcription promoter in the case of RNA controls. The method described here simplifies the procedure to a two-stage PCR amplification and allows the probe sequence to be placed anywhere between the primer binding sites without the need to identify or engineer restriction sites. Similar approaches have been described previously (3–5), although none of these is as simple or universally applicable as the method described here. As an example, the method has been used to synthesize a molecular construct for use as an internal standard in a quantitative PCR method for measles virus (MV) RNA.

The principle of the method is shown in Fig. 1. In step 1, the target gene is PCR-amplified in two sections. The antisense primer (B) used in the PCR of the upstream target sequence and the sense primer (C) of the PCR for the downstream target are sited immediately either side of the desired probing site. These two primers are 5'-tailed with the required probing sequence. After amplification of the two sections, the PCR products are purified to remove the remaining primers (step 2). In step 3, the two amplicons are mixed and reamplified with the two outer primers (A and D) only. Melting of the amplicons from step 1 allows reannealing of the complementary

Fig. 1. Method for construction of molecular control (see text for details). A and C are sense primers; B and D are antisense primers. Thick lines indicate the probe binding site (antisense on primer B, sense on primer C). Long dashes indicate extension of annealed complementary probe sequences. Short dashes indicate T7 promoter site (only for synthesis of RNA constructs).
probe sequences. The annealed sequences prime extension to a full-length sequence that can then be PCR-amplified by primers A and D. If the control required is an RNA sequence, a T7-promoter site is included in primer A or D to allow transcription, followed by DNase treatment to remove the template. Finally (step 4), the DNA or RNA construct is purified and quantified by spectrophotometry (absorbance at 260 nm) before dilution and storage at −70 °C for use. The method was used for the construction of an RNA internal standard for use in a quantitative PCR method for the N-gene of MV (3).

The first-round PCR products (step 1) were amplified in a single-tube reverse transcription-PCR (RT-PCR) from ~10,000 RNA copies of the Edmonston–Zagreb vaccine strain of MV (6). The RT-PCR reaction volume was 50 μL when the Titan One-Tube PCR reagent set (Roche Biochemicals, Lewes, UK) was used according to the manufacturer’s instructions. Primers were 5′-ACC AAA CAA AGT TGG GTA AGG-3′ (bases 1–21 of the MV N-gene) and 5′-GCT CGT ACT CTT TGA TTG AAA GGA TAA TAT ACC TAT T-3′ (bases 346–327 plus a randomized tail) to synthesize the upstream portion of the construct. To synthesize the downstream portion, the primers were 5′-TTC AAT CAA AGA GTA GGA CTG CCC GCG CAA CAT GGA CAT AAT ACG ACT CAC TAT AGG GAG AAC TCA –3′ (N-gene bases 367–386 with randomized tail) and 5′-CTG CCC GCG CAA CAT GGA CAT AAT ACG ACT CAC TAT AGG GAG AAC TCA AGT GTG GAT AAC TCA-3′ (bases 1000–981 plus T7 promoter). Thermal cycling conditions were as follows: 1 cycle of 50 °C for 30 min; 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 68 °C for 1 min; and 1 cycle 68 °C for 7 min. Amplification was confirmed on a 2% agarose gel. RNA transcripts were generated in a 100-μL reaction containing 50 U of T7 polymerase (Life Technologies), 0.5 mM each nucleotide triphosphate, 0.01 mM dithiothreitol, and T7 reaction buffer (Life Technologies). The reaction was incubated at 37 °C for 2 h before 10 U of DNase I (Life Technologies) were added. DNA digestion was carried out at room temperature for 15 min and stopped by the addition of 0.2 mM EDTA; the DNase was inactivated by incubating at 65 °C for 10 min. Finally, the RNA transcripts were purified by the method of Boom et al. (7) and eluted into 100 μL of nuclease-free water. After quantification of the final product by spectrophotometry (absorbance at 260 nm), the internal standard was stored at −70 °C in Tris-EDTA buffer containing 1 g/L tRNA (Life Technologies). The performance of the control was assessed in a quantitative RT-PCR based on a previously described method (8). The sense and antisense quantitative PCR primers were targeted to bases 278–299 and 433–413, respectively. The MV-specific probe was complementary to bases 347–386, and the probe for the control sequence was complementary to the randomized sequence inserted into the control construct.

All PCR products generated in the synthesis of the control construct were of the predicted size on an agarose gel. After purification of the final product, the RNA yield was 10 μg in a volume of 100 μL. For a 1000-base sequence, this represented 1011 RNA copies/μL. The control construct was quantified by end-point dilution in the chemiluminescence assay described. The results are shown in Table 1 and are given as ratios of test signals to the mean signal generated by negative (water) controls.

### Table 1. Quantitative PCR assay results.

<table>
<thead>
<tr>
<th>Construct dilution</th>
<th>RNA copy number/reaction*</th>
<th>Signal ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻²</td>
<td>10⁹</td>
<td>1.33</td>
</tr>
<tr>
<td>10⁻³</td>
<td>10⁸</td>
<td>1.37</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>10⁵</td>
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<td>10⁻¹⁰</td>
<td>10</td>
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<td>10⁻¹¹</td>
<td>1</td>
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</tr>
<tr>
<td>10⁻¹²</td>
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<tr>
<td>DNA control</td>
<td>0</td>
<td>73.55</td>
</tr>
<tr>
<td>Negative control</td>
<td>0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Based on spectrophotometry (absorbance at 260 nm). Concentration range, undiluted to 10¹¹ RNA copies/μL. PCR input volume was 1 μL.

** Signals are the means of four replicates. Signal ratios are between test and negative-control signals.

† DNA control was a RT-PCR product of the MV N-gene diluted to input 100 copies/PCR.
Signal ratios generated by wild-type probe reacted with PCR products from the control construct were all close to unity (i.e., there was no cross-reaction), as were those from wild-type PCR products reacted with the control sequence probe. End-point dilution of the control construct confirmed the spectrophotometric estimate of 10^{11} RNA copies/µL. Assay of the final construct for DNA copies showed that this contained ~10^9 DNA copies/mL (~10^{-6} of the RNA copies). It was intended that the RNA construct be added to the final assay at a concentrations of 100 RNA copies/reaction. At this dilution, the DNA contaminants would be diluted to <0.001 copies/reaction; thus it was decided not to risk the integrity of the RNA construct by the use of more vigorous DNase treatment.

Quantitative molecular assays, particularly quantitative PCR, have become an important tool in medical research and routine clinical investigation, particularly for the assay of microbial genomes in clinical samples and routine clinical investigation, particularly for the quantification of gene expression in mammalian cells (10). In such assay systems, accurate quantification depends on standardization and control of all steps in the protocol. In the case of quantitative PCR, this includes controls for nucleic acid extraction efficiency, amplification efficiency, and nonspecific inhibition of the reaction (11). Additionally, there is a need for accurately quantified internal standards with which to compare unknowns to obtain absolute nucleic acid copy numbers in the sample.

The method described here allows molecular constructs for use as internal controls in quantitative molecular assays to be synthesized in a simple two-step PCR protocol. The simplicity of the procedure allows controls to be synthesized in laboratories that do not have facilities available for molecular biology procedures beyond PCR, such as diagnostic laboratories that use molecular detection methods. The method has been applied to the control of a quantitative PCR for MV RNA but could also be applied to the generation of DNA targets. Controls may be synthesized that are appropriate to any source of nucleic acid (e.g., mRNA in the quantitation of microbial or mammalian gene expression) that is being detected or quantified by PCR or any other molecular method.

### References


### Protein Glutathionylation in Erythrocytes, Daniela Giustarini,1 Isabella Dalle-Donne,2 Roberto Colomba,2 Salvatore Petralia,3 Simonetta Giampaletti,3 Aldo Milzani,7 and Ranieri Rossi1 (1) Department of Neuroscience, Pharmacology Section, University of Siena, Via A. Moro 4, 53100 Siena, Italy; 2 Department of Biology, University of Milan, Via Celoria 26, 20133 Milan, Italy; 3 Department of Laboratory Medicine, Policlinico “Le Scotte”, Azienda Ospedaliera Senese, Viale Mario Bracci 16, 53100 Siena, Italy; *author for correspondence: fax 39-0577-234208, e-mail ranieri@unisi.it

Analysis of antioxidant molecules is potentially important to understanding the role of oxidative stress (1–6) in disease, as oxidative damage is accompanied or preceded by their depletion.

Reduced glutathione (GSH) is ubiquitous and abundant; it can be oxidized to its disulfide form (GSSG) in response to an oxidative perturbation. Usually, however, this species is rapidly reduced by the action of glutathione reductase (7). If GSSG accumulates within the cell, it can create protein-glutathione adducts via thiol-disulfide exchange reactions. Thus, in addition to the ratio of GSH to GSSG, the content of glutathionylated proteins (GSSPs) can indicate oxidative stress. The analysis of GSSPs has potential advantages over measurements of GSH and GSSG because GSSPs are more stable than GSSG, being less prone to enzymatic reduction by glutathione reductase (8).

Because blood can be studied as an indicator of the overall body oxidative status, GSSPs and, particularly, glutathionyl hemoglobin (Hb-SSG) could represent useful markers of oxidative stress. The use of Hb-SSG as a clinical marker has been proposed (9, 10). Significant increases in GSSP concentrations have been found in diabetes mellitus, hyperlipidemia, Friedreich ataxia, and chronic renal failure (10–13).

The technology commonly used for the assays of GSSPs, electrospray ionization mass spectrometry (ESI-MS), is not widely available. In addition, we have recently reported (14) that oxygenated Hb is able to artificially produce large amounts of GSSG and GSSP if not adequately manipulated. We have therefore developed a