Internal controls are crucial for reliable detection of pathogens in nucleic acid amplification assays. Without an internal control, a negative amplification result may mean that the sample contains no target or that the reaction failed.

We recently described (1) the use of internal controls that consist of internal control oligonucleotides (ICOs) that contain little more than primer sites and a probe-binding site that contains mismatches to the actual target. ICOs can be obtained commercially and do not require a separate control-specific detection probe. Because of the mismatches, the control does not hybridize to the single detection probe during the fluorescence-acquisition step, and thus the probe binds and detects only nucleic acid derived from the pathogen. However, amplification of the ICO can be verified during melting analysis after PCR.

Many important viral pathogens contain an RNA genome that must be transcribed to cDNA before amplification. To provide a control for both reverse transcription and amplification, an internal control must be an RNA molecule. The standard method for generating such a control involves directional cloning of a double-stranded DNA template into a plasmid vector carrying bacteriophage RNA polymerase promoter sequences. The RNA is then transcribed in vitro from the DNA template (2, 3).

We have extended the ICO technique to RNA by introducing an RNA polymerase promoter and adding 2 simple preparation steps. We demonstrate the application of this control technique to the reverse transcription-PCR (RT-PCR) step of a LightCycler (Roche Molecular Biochemicals) real-time hybridization probe assay for respiratory syncytial virus (RSV).

The sequences of the primers and probes for the RT-PCR assay were 5′-GCCAAAAAATTTGTTCACACAATA-3′, 5′-TCCTTATCCACCATCTTTCATGTTA-3′, 5′-CY5.5-AGACTCCAGACATGTGAGA-3′, 5′-ATTACATGGTCTACATCTACTGACTGT-phosphate, and 5′-GGTGATCTAAGCTTCTATAGCATATGCA-fluorescein-3′ (4). The 2-step LightCycler (Roche) RT-PCR was performed as follows. For the reverse transcription step (total volume of 10 μL), the reaction mixture consisted of 1.5 mM MgCl2, 25 μM random hexamers, 25 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems), 10 units of RNase inhibitor, 1 mM 1,4-dithiothreitol, and 0.1 M each of the deoxyribonucleoside triphosphates in 1× GeneAmp PCR buffer II (Applied Biosystems). After the RNA template (5 μL) was added to the mixture, the reverse transcription reaction mixture was incubated for 10 min at 25 °C, followed by 30 min at 42 °C and 5 min at 99 °C. PCR and melting analysis were performed on a LightCycler (Roche). Each glass capillary contained 20 μL of the reaction mixture, which included 10 pmol of each primer, 4 pmol each of the anchor and detection probes, 4 μL of LightCycler-FastStart DNA Master Hybridization ProbesPLUS (Roche), 0.2 μL of LightCycler uracil DNA glycosylase (Roche), and 5 μL of cDNA. An initial 10-min incubation at 40 °C allowed uracil DNA glycosylase to remove any traces of remaining double-stranded DNA control oligonucleotide (if not totally removed by the DNase step during RICO production; see below) and any carryover PCR contaminant. The next incubation, at 95 °C for 10 min, denatured the DNA and activated the Taq polymerase. Amplification was done in 45 thermal cycles (95 °C for 0 s, 55 °C for 10 s, and 72 °C for 20 s), with a ramping rate of 20 °C/s. Fluorescence was measured during each 55 °C stage. After amplification, the instrument performed a melting analysis by heating the capillary at 95 °C for 0 s, incubating it at 45 °C for 30 s, and then slowly (0.2 °C/s) heating it to 75 °C. Fluorescence was measured continuously during the melting experiment. To convert melting curves to melting peaks, the LightCycler software (Ver. 4.0; Roche) calculated the negative derivative of each fluorescence measurement with respect to the temperature (−dF/dT), then plotted −dF/dT against temperature for the entire melting experiment.

Production of an RNA internal control oligonucleotide (RICO) is outlined in Fig. 1A. The initial synthetic oligonucleotide basically contained only the primer- and probe-binding regions of the target. It was designed as described for the ICO technique (1), the only difference being the addition of a T7 RNA polymerase promoter sequence to the 5′ end of the oligonucleotide (Fig. 1A, PR). The sequence of the oligonucleotide, synthesized and HPLC-purified (Metabion), was 5′-CCAGACTTCATAATAGACTATAGAGGAGGCCAAAAATTTGTTCACACAATA-3′ (Fig. 1A, PR). The sequence of the oligonucleotide, synthesized and HPLC-purified (Metabion), was 5′-CCAGACTTCATAATAGACTATAGAGGAGGCCAAAAATTTGTTCACACAATA-3′ (Fig. 1A, PR). The sequence of the oligonucleotide, synthesized and HPLC-purified (Metabion), was 5′-CCAGACTTCATAATAGACTATAGAGGAGGCCAAAAATTTGTTCACACAATA-3′ (Fig. 1A, PR). The sequence of the oligonucleotide, synthesized and HPLC-purified (Metabion), was 5′-CCAGACTTCATAATAGACTATAGAGGAGGCCAAAAATTTGTTCACACAATA-3′ (Fig. 1A, PR).

The PCR product was purified (QIAquick PCR Purification; Qiagen), and ~50 ng of the PCR product (estimated by comparison with the size marker used in the agarose gel electrophoresis) was transcribed with T7 RNA polymerase. The 20-μL transcription mixture included 2 μL each of the ribonucleoside triphosphates (containing 10 mmol/L each of ATP, CTP, TTP, and GTP), transcription buffer, and T7 RNA polymerase (20 U/μL; Roche), along with 1 μL of RNase inhibitor (20 units/μL; Applied Biosystems). The mixture was incubated at 37 °C for 2 h. The transcript was diluted 10−2 in RNase-free water, and
10 μL of diluted transcript was digested with 5 μL of DNase I (3 U/μL; RNase-Free DNase Set; Qiagen) for 30 min at 25 °C in a total volume of 100 μL that included 10 μL of RDD buffer (RNase-Free DNase Set; Qiagen) and 5 μL of RNase inhibitor (Applied Biosystems). After the digestion, the mixture was incubated for 3 min at 90 °C and stored in aliquots at −20 °C as RICO stock solution.

The amount of RICO to be used in an assay should be as low as possible to minimize competition with target, particularly in low-titer samples, and to detect even weak inhibition. The optimal amount of RICO for the RSV assay was determined in a 2-step LightCycler RT-PCR with different dilutions (10<sup>-3</sup>–10<sup>-7</sup>) of the RICO stock solution. Reverse transcription and PCR were performed as described above. Because the RICO is designed to be undetectable under the PCR conditions for the RSV assay (see above), the annealing temperature for this experiment had to be decreased to 45 °C. To verify that the control was pure RNA, free of residual control DNA, the 10<sup>-3</sup> dilution of the RICO stock solution was tested both with and without reverse transcriptase. No increase of fluorescence was seen in the absence of reverse transcriptase (data not shown). We therefore used the 10<sup>-6</sup> dilution to ensure a stable RICO signal. The diluted RICO was stored in small portions, which were used only once.

To determine the influence of the RICO on assay sensitivity, we assayed a dilution series of RSV RNA with and without RICO. There were no substantial differences in the crossing points observed (data not shown).

To mimic inhibition of the reverse transcriptase step, we used decreasing concentrations of reverse transcriptase to assay the same amount of RNA (2.5 μL of RSV RNA and 2.5 μL of the RICO). With undiluted reverse transcriptase, the RT-PCR produced a real-time fluorescence signal (Fig. 1B, upper panel, line 1) as well as RSV-specific and RICO-specific melting peaks (Fig. 1B, lower panel, line 1). With a 1:10 dilution, it produced a fluorescence signal with a markedly higher crossing point (Fig. 1B, upper panel, line 2), demonstrating that partial inhibition of reverse transcription can strongly influence quantification of RNA templates. This assay did not produce a RICO-specific melting peak (Fig. 1B, lower panel, line 2), indicating inhibition. No real-time fluorescence signal was obtained in reaction 3, which contained a 1:50 dilution of the reverse transcriptase (Fig. 1B, upper panel, line 3). The absence of a RICO peak in the melting
analysis (Fig. 1B, lower panel, line 3) confirmed that the result obtained in reaction 3 was a false negative.

In the experiments of this study, the RICO was added to the reverse transcription mixture. It is possible to co-extract the RICO with the sample RNA to control for the extraction procedure. In this case, however, careful validation is necessary: because of the small size of the RICO, its extraction efficiency might be low or variable depending on the method used. We have noticed these effects when using Qiagen columns for RNA extraction.

In summary, we have developed a simple, inexpensive method that allows laboratories to produce their own RNA controls. Sufficient RICO for millions of RT-PCR assays can be produced from a synthetic oligonucleotide in <5 h with little hands-on time. The RICO technique could be applied to many real-time amplification protocols that use a probe format and an instrument that can perform a melting-point analysis after amplification. Because the method does not require a second, control-specific probe, it conserves detection channels on the real-time amplification instrument and reduces reagent costs. Because of some difficulty in titrating the amount of RICO needed for reliable detection of partial inhibition of the reverse transcription step, however, a separate probe should still be used to detect the RICO during amplification in quantitative RT-PCR assays.

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References


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Isovaleric acidemia (IVA) is an autosomal recessive genetic disorder of the enzyme isovaleryl-CoA dehydrogenase, which is involved in leucine metabolism (1). Clinical symptoms include poor feeding, tachypnea, vomiting (2), listlessness, lethargy, coma (2,3), and dehydration (4). Individuals homozygous for this defect are characterized primarily by the excretion of isovalerylglucose (2). The metabolic profile may be further complicated by intermediate metabolites such as 3- and 4-hydroxyisovaleric acid (5), methylsuccinic acid (6), methylfumaric acid (7), isovalerylglucuronide (8), iso-valerylglyc alcohol (9), N-isovalerylamine and N-isovalerylarscinate (10), isovalerylaminine (11), 3-hydroxyisooctan acid (12), and alloisoleucine (4). Diagnosis of IVA is based on clinical symptoms and the presence of isovalerylglucose and 3-hydroxyisovaleric acid (13), with some of the above-mentioned metabolites also occurring to a greater or lesser extent (4). Despite the large number of already identified excreted metabolites, their occurrence still does not entirely explain all of the clinical symptoms experienced by these patients. The statement made in 1982 by Duran et al. (14) that “[t]he continuing search for ‘new’ metabolites may eventually lead to a better understanding of the relationship between the clinical conditions and their biochemical abnormalities” is the motivation for our search for previously unidentified metabolites in IVA. In this study, we analyzed the urine of IVA patients for the presence of induced amino acid metabolites not previously identified in IVA.

Most of the amino acid conjugates occurring in the urine of patients with IVA have been detected via gas chromatography–mass spectrometry (GC-MS). Because this technique is not suitable for detection of acetyl and isovaleryl conjugates of tryptophan, lysine, histidine, and asparagine because of their polar characteristics, we identified and quantified these metabolites by electrospray tandem mass spectrometry (ESI-MS-MS). Mass spectra (GC-MS) and daughter-ion profiles (ESI-MS-MS) were obtained by analyses of the chemically synthesized calibrators. These spectra were then used to identify the metabolites in the urine of these patients.

Six IVA patients were investigated for the presence of new urinary metabolites. The concentrations of the diagnostic metabolites found in urine samples from these patients are summarized in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/.

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