Novel Internal Controls For Real-Time PCR Assays

In the 19 years since the first descriptions of the PCR *(1)*, nucleic acid amplification methods have made the transition from research to clinical laboratories. Molecular diagnostics are now firmly established as part of laboratory medicine, with applications in genetics, oncology, pharmacology, and infectious disease. Routine diagnostic applications of these methods have been made possible by the thoughtful use of controls coupled with laboratory practices intended to reduce false-positive and -negative results *(2–4)*.

Nucleic acid amplification assays are prone to inhibition by a variety of substances found in clinical samples. Although the identities and biochemical mechanisms of action of many inhibitors remain unclear, bile salts and complex polysaccharides in feces *(5)*, heme in blood *(6)*, and urea in urine *(7)* have all been shown to inhibit PCR, probably through interference with the binding and/or polymerization activity of DNA polymerases. Carryover of reagents used for isolation of nucleic acids from clinical specimens can also inhibit amplification reactions. Other causes of false-negative results include target nucleic acid degradation, sample processing errors, thermal cycler malfunction, and in reverse transcription-PCR, failure of the reverse transcription step.

A simple approach to detect inhibitors is to add the target nucleic acid to a separate aliquot of the sample after processing. Addition of the external control to a separate reaction, however, doubles the cost of the assay, and the approach may be unworkable if batch sizes are large. Moreover, addition of the control after sample processing is complete cannot detect sporadic false-negative results attributable to failed or inefficient nucleic acid extraction.

Internal amplification controls (IACs) that copurify and coamplify with the target nucleic acid are sensitive indicators of loss or degradation of the target during processing and inhibition of amplification and detection. IACs thus provide an accurate way to assess the integrity of all the steps in a nucleic acid amplification assay and are commonly used in in-laboratory-developed and commercially available tests *(8–10)*.

IACs should mimic the target sequences as closely as possible. Ideally an IAC contains primer binding sequences identical to the target but a unique internal sequence. This design minimizes the problems associated with primer multiplexing and facilitates differential detection of target and IAC amplicons. The IAC sequence is usually incorporated into a plasmid and cloned by standard techniques of molecular biology. The plasmid can be added directly to the clinical specimens to control for sample-to-sample variability in the subsequent steps in the assay. Internal control RNA can be packaged in MS2 coliphages to protect them against degradation of the RNA control by ubiquitous RNases found in clinical samples *(11)*. These armored RNA molecules have been used as effective IACs in a variety of nucleic acid amplification assays for RNA viruses. Normal cellular gene sequences, which are expected to be present in all specimens, have also been used as IACs *(12, 13)*. These endogenous sequences may not accurately reflect amplification of the primary target, however, because of differences in amplification efficiency and relative abundance of the two targets.

The scope of the problem is difficult to define because many nucleic acid amplification tests do not include IACs. Reported frequencies of false-negative results in tests that include IACs range widely, depending on the assay and type of specimen. The need to use IACs should be determined on a case-by-case basis, because development and implementation of such controls can be difficult and they can adversely affect assay sensitivity *(4)*. If the reaction failure rate is found to be ≤2% during test verification, it may not be necessary to routinely use an IAC unless the medical consequences of a false-negative result are severe.

Universal acceptance of IACs has been hindered by the technical complexity of their construction, the additional costs associated with the use of separate detection systems, and concerns about adverse affects on assay sensitivity as a result of competition with target template. In this issue of *Clinical Chemistry*, Burggraf and Olgemöller *(14)* describe a novel approach for the design and use of IACs in LightCycler (Roche Applied Science) real-time PCR assays. This approach does not require plasmid construction or use of a separate detection probe. Single-stranded oligonucleotides of ~120 nucleotides, containing the sequences for binding of primer and detection of probes, were chemically synthesized with several mismatches in the site for binding of a detection probe. Mismatches in the detection probe-binding region prevented hybridization to the internal control oligonucleotide (ICO) amplicon during fluorescence signal acquisition during real-time PCR. However, the ICO amplicon was detected and easily distinguished from the target amplicon in subsequent melting curve analysis. The authors demonstrated the feasibility of this new approach with LightCycler assays for *Mycobacterium tuberculosis* complex, hepatitis B virus, herpes simplex virus, and varicella zoster virus. Use of the ICOs did not change the detection limits of these assays.

In designing the ICOs, the authors exploited the ability of fluorescence resonance energy transfer (FRET) hybridization probes and the LightCycler to distinguish amplicons based on differences in melting temperature (T_m). Each PCR product–probe complex melts at a characteristic temperature, and with simple hybridization probes, the melting occurs at a characteristic temperature that can be used to distinguish the product from others. A decrease in fluorescence with heating follows the melting of the probe and indicates the T_m of the probe–target duplex. The base mismatches incorporated into the sequences of the IACs that hybridize with the detector probes dramatically reduce the T_m of the detector probe. The detector probe and

IAC produce no fluorescent signal during amplification because the T_m is lower than the temperature at which the fluorescence is measured. However, if the fluorescence is measured continuously over a wider temperature range after amplification, control- and target-specific melting curves are obtained.

Because the melting temperatures of the ICO products are much lower than the annealing temperatures used in the amplification reactions, it is not possible to monitor the accumulation of ICO product in real time. Consequently, ICOs cannot be used to assess partial inhibition in qualitative assays or as internal standards for quantitative assays. ICOs could be used to assess the integrity of cDNA amplification in reverse transcription-PCR, but they would not control the RNA isolation and reverse transcription steps.

The new amplification controls avoid the added expense of previously described approaches that used separate control-specific probes. Another advantage to using the same probe for detection of both target and control amplicons is that only one analysis channel on the LightCycler instrument is used. Conservation of analysis channels is most important in multiplex PCR applications.

The ICOs can be obtained easily by clinical laboratories that may not have the expertise and facilities for plasmid construction and cloning. The design of ICOs is simple, and their synthesis is within the capabilities of most microchemical facilities. Even at minimum synthesis scales, ICOs are inexpensive to produce, with typical microchemical facility fees of $1/base. If used at 1000 copies per reaction, a single synthesis run would produce enough ICO to perform billions of reactions.

The ICOs described by Burggraf and Olgemöller *(14)* provide a simple, inexpensive, and effective way to control for false-negative results in LightCycler PCR assays that use FRET hybridization probes for detection of amplicons. The authors addressed many of the concerns about complexity, cost, and decreases in sensitivity that have limited the use of IACs in diagnostic applications of this technology. ICOs could easily be included in existing assays that use this format and further increase the reliability of these important tests.

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