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Notes & Tips

SPUD: A quantitative PCR assay for the detection of inhibitors in nucleic acid preparations

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Among the many factors that determine the sensitivity, accuracy, and reliability of a real-time quantitative reverse transcription polymerase chain reaction (qRT–PCR)[1] assay, template quality is one of the most important determinants of reproducibility and biological relevance [1]. This is a well-recognized problem [2], and there are numerous reports that describe the significant reduction in the sensitivity and kinetics of qPCR assays caused by inhibitory components frequently found in biological samples [3–8]. The inhibiting agents may be reagents used during nucleic acid extraction or copurified components from the biological sample such as bile salts, urea, haeme, heparin, and immunoglobulin G. At best, inhibitors can generate inaccurate quantitative results; at worst, a high degree of inhibition will create false-negative results. The most common procedure used to account for any differences in PCR efficiencies between samples is to amplify a reference gene in parallel with the reporter gene and to relate their expression levels. However, this approach assumes that the two assays are inhibited to the same degree. The problem is even more pronounced in absolute quantification, where an external calibration curve is used to calculate the number of transcripts in the test samples, an approach that is commonly adopted for quantification of pathogens. Some, or all, of the biological samples may contain inhibitors that are not present in the nucleic acid samples used to construct the calibration curve, leading to an underestimation of the mRNA levels in the test samples [9]. The increasing interest in extracting nucleic acids from formalin-fixed paraffin-embedded (FFPE) archival material undoubtedly will lead to an exacerbation of this problem. Obviously, such inhibitors are likely to distort any comparative quantitative data. However, a recent survey of practices revealed that only 6% of researchers test their nucleic acid samples for the presence of inhibitors [10].

Various methods can be used to assess the presence of inhibitors within biological samples. The PCR efficiency in a test sample can be assessed by serial dilution of the sample [11], although this is impossible when using very small amounts of RNA extracted, for example, from single cells or from laser capture microdissected sections. Furthermore, there are mathematical algorithms that provide a measure of PCR efficiency from analysis of the amplification response curves [12–14]. Internal amplification controls (IACs) that copurify and coamplify with the target nucleic acid can detect inhibitors as well as indicate template loss during processing. They can be packaged in phage coats [15], or they may be single-stranded oligonucleotides that contain the sequences for binding of primer and detection by probes. Several mismatches in probe binding site prevent hybridization to the IACs during fluorescence signal acquisition, but IACs and target amplicons can be distinguished in subsequent melting curve analysis [16].

IACs may well provide the best solution for assays designed to detect pathogens, but this approach is not practical for cellular mRNA quantification because it is unrealistic to contemplate designing mimics for every single target mRNA. Instead, the simplest approach is to mix a positive control nucleic acid with the sample nucleic acid after sample purification. This has the great virtue of simplicity and avoids the technical complexity of IACs, both in terms of the technical complexity of their construction and in terms

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[1] *Abbreviations used:* qRT–PCR, quantitative reverse transcription polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded; IAC, internal amplification controls.

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of the additional effort associated with their accurate detection following the assay. A recent report described an alternative approach that uses a whole bacterial genome to detect inhibition from clinical samples [17]. We have developed and refined a method, which we call the "SPUD" assay, that is more suitable for the detection of inhibitors in nucleic acids extracted from all tissue with the exception of potato.

We selected the *Solanum tuberosum* phyB gene (GenBank Y14572) for application in the SPUD assay because it encodes a species-specific regulatory photoreceptor that is involved in the pathway that results in the purple coloration of potato root. The total gene sequence of 4907 bp was divided into 500 bp overlapping lengths, and BLAST searches were performed to identify unique stretches of nucleotide sequence [18]. The chosen target sequences were located in the upstream nontranslated region of the *S. tuberosum* phyB gene between nucleotides 449 and 549. qPCR assays incorporating TaqMan probes were designed against the selected sequences using Beacon Designer (Premier BioSoft). The primers and probe producing the shortest amplicon and the highest predicted assay quality were selected (Table 1). Absence of primer–dimers was evaluated using melt curves. Amplification efficiency was assessed using 10-fold serial dilutions of the 101-bp amplicon (SPUD-A).

RNA was extracted by six individuals from the same batch of HeLa cells using Absolutely RNA extraction kits (Stratagene) according to the manufacturer's instructions.

The quantity and quality of the RNA were assessed using four different methods: RNA chips with the Bioanalyzer (Agilent) and Experion (Bio-Rad) systems, UV spectrophotometry, and NanoDrop A260/A280 ratios. In each case, there was no indication of inferior quality, although degradation was detected in 2 samples (as determined by Bioanalyzer RIN and Experion 28S/18S ratios). Each of the systems was also used to measure the RNA yield. There were large discrepancies among the concentrations attributed to identical samples by the different systems. In the absence of a "correct" system, the quantification from the NanoDrop was randomly selected as the basis of the input concentration of RNA to the cDNA synthesis.

cDNA synthesis was performed in 10-μl reactions containing 500 ng total RNA using a Brilliant qRT–PCR Core Reagent Kit (two-step, Stratagene) and random nonamer primers according to the manufacturer's recommendations. The reference SPUD assays consisted of the SPUD amplicon (SPUD-A) at approximately 20,000 copies (using NanoDrop concentration determination and an approximate mass of 330/base pair), forward and reverse primers (SPUD-R and SPUD-F) at 240 nM, and the TaqMan probe SPUD-P at 200 nM. All other assays also contained 10-fold serial dilutions of cDNA (at maximum 50 ng, RNA equivalent prepared from the HeLa cell-derived RNA). Duplicate amplifications were carried out in 25 μl using Brilliant qPCR master mix buffer on Mx3000p (Stratagene) qPCR systems.

Control qPCR assays were performed in the presence of water, with the SPUD-A amplicon being the only amplifiable target. Under these conditions, a reference C_t value of between 23 and 24, characteristic of an uninhibited assay, was generated. A midrange C_t ensures a highly reproducible and reliable control reaction, such that there is sensitive detection of inhibitors. However, it is possible to use higher copy numbers of the amplicon, as shown in Fig. 1B. Because C_t values are specific to particular instrument and reagent combinations, determination of inhibition by C_t shift is valid only when these are made as a single experiment and with constant threshold settings [1].

Each sample was included in a SPUD assay, and an additional sample containing phenol was run as a positive control indicating inhibition. Most assays containing HeLa cell RNA generated the same C_t values as the water controls. However, there was an obvious shift to a higher C_t and reduced amplification efficiency for two samples containing phenol (Fig. 1A). There was no indication from either the NanoDrop or the A260/A280 ratios that any of these samples contained materials that would be inhibitors of the qPCR assay. These inhibitors most likely are to be components of the extraction procedure and to be present as a result of operator procedure. This suggests that current quality assessment procedures are unable to detect reliably the presence of inhibitors in RNA samples and demonstrates the need for this additional quality assessment parameter. We also applied this assay to the assessment of RNA extracted from FFPE tissue and recorded higher C_t values (>1) in up to 30% of samples analyzed (Fig. 1B).

There is a concerted effort under way to standardize the parameters associated with qPCR experiments. One of these concerns the assessment and publication of template-quality data for all samples included in a study. This is particularly important when quantitative data are sought for comparative applications involving samples extracted from in vivo biopsies. In addition, for genotyping, the theoretical

Table 1
Nucleic acid sequences for SPUD assays

Oligo	DNA sequence
SPUD-T	5′-FAM-TGCACAAGCTATGGAACACCACGT-TAMRA-3′
SPUD-R	5′-ACATTCATCCTTACATGGCACCA-3′
SPUD-F	5′-AACTTGGCTTTAATGGACCTCCA-3′
SPUD-A	5′- AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTGCACAAGCTATGGAACACCACGT AAGACATAAAACGGCCACATATGGTGCCATGTAAGGATGAATGT-3′

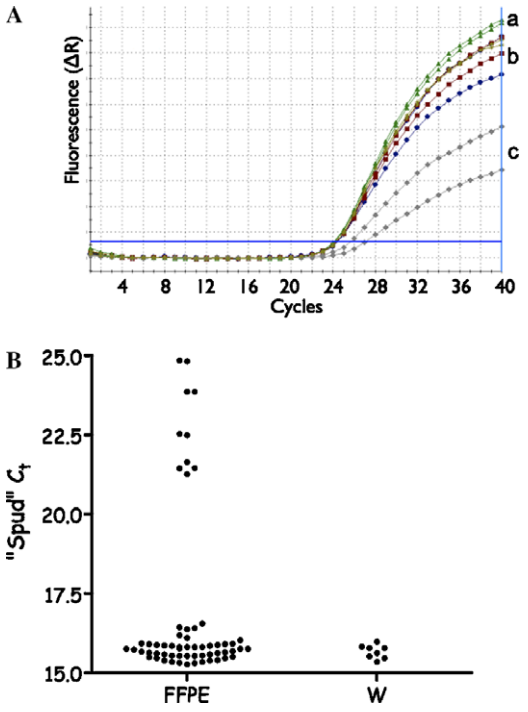

Fig. 1. Detection of inhibition by the SPUD assay. (A) Amplification plots of HeLa cell-derived RNA showing the control assay carried out in the presence of water (a), several assays carried out in the presence of template RNA (b), and inhibition of the assay in the presence of trace amounts of phenol (c). (B) The SPUD assay was carried out in the presence of RNA obtained from formalin-fixed, paraffin-embedded samples (FFPE) or of water (W). Of 30 samples, 5 showed significant inhibition.

quantitative difference between a homozygotous gene sequence and a heterozygotous one is represented by a single C_t value. When experimental data deviate from this, ambiguous results are produced and the sample must be tested again. Such differences could be the result of one or more samples containing inhibitors. There is a clear requirement for a simple, rapid, and universal test to identify samples that contain inhibitors and therefore will cause spurious data if included in experiments. The basis of the SPUD assay is a single qPCR test using oligos that are routinely produced by oligo manufacturers. The lack of homology with any other known sequence ensures that these sequences can be used in the presence of all samples with the exception of those from potato (*S. tuberosum*), allowing a single quality control protocol to be used for validation of all samples regardless of organism and by all research groups. The simplicity of the test allows transfer among all qPCR platforms, and the simple analysis is carried out using the platform-specific software.

In conclusion, all scientists using qPCR could use the SPUD assay. Application is free from constraints such as downstream application, the organism from which the experimental sample originated, the source or storage history of the experimental sample, the number and variety of samples to be processed, and the instrumentation used. The SPUD assay is a universal system for rapid quality control of nucleic acid samples.

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