Inhibition Controls for Qualitative Real-Time PCR Assays: Are They Necessary for All Specimen Matrices?


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A retrospective analysis of 386,706 specimens representing a variety of matrix types used in qualitative real-time PCR assays determined the overall inhibition rate to be 0.87% when the inhibition control was added preextraction to 5,613 specimens and 0.01% when the inhibition control was added postextraction but preamplification in 381,093 specimens. Inhibition rates of ≤1% were found for all specimen matrix types except urine and formalin-fixed, paraffin-embedded tissue.

Inhibitors can be found in various specimen matrices, and these substances can interfere with PCRs by interacting directly with DNA and blocking the activity of the polymerase or other PCR mixture components (e.g., MgCl₂), thereby preventing target amplification. Examples of PCR inhibitors include bile salts in feces, heme in blood, and urea in urine. In addition, some components of common laboratory collection devices (e.g., viral transport medium, heparin, formalin, or swabs containing gel or charcoal) are known inhibitors of PCR. Inhibition (or internal) controls added directly to the specimen are often used in order to detect inhibition associated with the specimen matrix or the processing method (1, 2). Clinical and Laboratory Standards Institute document MM3-A2 recommends that the addition of an inhibition control be determined on a case-by-case basis by specimen type and with consideration of the potential consequences of a false-negative result (3). The College of American Pathologists recommends spiking an aliquot of the clinical specimen with target nucleic acid but indicates that “the practice can be discontinued once the laboratory accumulates sufficient data that the inhibition rate falls within acceptable limits” (MIC 63278; http://www.cap.org/apps/docs/laboratory_accreditation/checklists/new/microbiology_checklist.pdf). Some clinical laboratories are licensed by the New York State Department of Health, which indicates that an inhibition control is needed unless the inhibition rate is <1% for a specimen matrix (4). However, addition of inhibition controls by laboratory staff is not without issues, including added cost and labor.

In order to determine whether inhibition controls are necessary for each specimen matrix type, we performed a retrospective evaluation of 386,706 specimens used in validation studies or submitted to our laboratory for real-time PCR analysis across a variety of analytes in 28 qualitative real-time PCR assays using the LightCycler 1.2 and 2.0 platforms (Roche Applied Sciences, Indianapolis, IN). These assays consisted of 28 qualitative laboratory-developed tests (LDTs) that employ standardized specimen processing, extraction, and amplification methods. For each specimen matrix, we calculated the rate of inhibition observed when target DNA or a whole organism was used to spike an aliquot of a specimen prior to extraction and the rate of observed inhibition when a recovery template, target DNA, or a whole organism was added postextraction but before PCR amplification.

MATERIALS AND METHODS

Specimens. Inhibition rates were determined for specimens submitted for analysis or included in validation studies from 2004 to 2012 for LDTs. The specimen matrices examined were grouped according to similar physical or anatomic characteristics and included swabs (nasopharyngeal, nasal, throat, dermal/genital, anogenital, ocular, and perianal), EDTA-preserved whole blood and blood components, respiratory specimens (i.e., bronchoalveolar lavage fluid, bronchial wash samples, sputum samples, tracheal secretions, swabs submitted from respiratory sources), fresh tissue (organ tissue, bone, muscle, and connective tissue), body fluids (i.e., peritoneal/abdominal, pleural, abscess, and synovial fluids), cerebrospinal fluid (CSF), ocular fluid, stool samples, and urine. In addition, inhibition was determined by using formalin-fixed, paraffin-embedded (FFPE) tissue blocks.

Specimen processing. Standardized specimen processing protocols were used for all assays. A brief description of the preextraction processing steps used for each specimen matrix type is provided below; details can be found in the respective publications listed in Table 1. Specimens were extracted on the MagNA Pure LC platform and included whole blood, plasma, serum, ocular fluid, CSF, respiratory specimens, other body fluids (e.g., amniotic and synovial fluids), specimens collected on swabs (with the exceptions described below), stool samples, and fresh and FFPE tissue samples.

Swab processing. Specimens collected on swabs, with the exception of swab specimens for group A Streptococcus, Bordetella pertussis/Bordetella parapertussis, Staphylococcus aureus, and vanA/vanB assays, were extracted with the MagNA Pure LC instrument. Twenty-five percent of the swabs tested were supplied in M5 viral transport medium. These assays used a simple lysis procedure in which a stainless steel wire cutter with a flat bottom was used to cut the swab shaft. The swab was then placed into a methicillin-resistant Staphylococcus aureus lysis tube (Roche Molecular Diagnostics, Indianapolis, IN). The capped tube was placed on a Thermomixer R (Eppendorf AG) for 6 min at 99°C and 1,400 rpm and then centrifuged at 20,800 × g for 20 s. Five microliters of the supernatant was used in the assay.

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Stool sample processing. Stool samples were processed by transferring a pea-sized amount of material with a sterile cotton swab into a 2-ml microcentrifuge tube containing 50% stool transport and recovery buffer (stool sample dilution of approximately 1:10; Roche Applied Sciences). The suspension was vortexed and allowed to settle for 1 min. Two hundred microliters of the supernatant was placed into a specimen cartridge for extraction with a total nucleic acid isolation kit on the MagNA Pure LC system.

Respiratory specimen processing. Respiratory specimens (bronchoalveolar lavage fluid, bronchial washings, sputum, and tracheal secretions) were processed as previously described (6) by pipetting 500 μl of raw specimen and 100 μl of proteinase K (Roche Applied Sciences) into a 1.5-ml tube containing 0.1-mm silica glass beads and 2.4-mm zirconia beads (BioSpec Products, Bartlesville, OK). Specimens were incubated at 55°C for 15 min on a Thermomixer R (Eppendorf) at 1,400 rpm and subsequently placed on a 95°C heat block for 5 min. To facilitate complete lysis and nucleic acid liberation, specimens were placed on a Disruptor Genie for 2 min and then centrifuged briefly at 20,800 × g to collect the sediment at the bottom of the tube (6). Two hundred microliters of solution was placed into the MagNA Pure LC cartridge for extraction.

Tissue processing. Fresh tissue specimens were processed for PCR analysis by placing a small piece of tissue, approximately 0.5 cm³, into a 1.5-ml tube containing 0.1-mm silica glass beads and 2.4-mm zirconia beads (BioSpec Products, Bartlesville, OK). Specimens were incubated at 55°C for 15 min on a Thermomixer R (Eppendorf) at 1,400 rpm and subsequently placed on a 95°C heat block for 5 min. To facilitate complete lysis and nucleic acid liberation, specimens were placed on a Disruptor Genie for 2 min and then centrifuged briefly at 20,800 × g to collect the sediment at the bottom of the tube (6). Two hundred microliters of solution was placed into the MagNA Pure LC cartridge for extraction.
Inhibition in Real-Time PCR

TABLE 2 Inhibition percentages grouped according to specimen matrix

<table>
<thead>
<tr>
<th>Sample(s)</th>
<th>Inhibition control added preextraction</th>
<th>Inhibition control added postextraction, preamplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of specimens tested</td>
<td>No. (%) of specimens inhibited</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>63</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Body fluids</td>
<td>319</td>
<td>3 (0.94)</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>547</td>
<td>1 (0.18)</td>
</tr>
<tr>
<td>Blood and components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>482</td>
<td>1 (0.21)</td>
</tr>
<tr>
<td>Plasma</td>
<td>149</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>Serum</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Whole blood (EDTA)</td>
<td>936</td>
<td>9 (0.96)</td>
</tr>
<tr>
<td>CSF/ocular fluid</td>
<td>603</td>
<td>4 (0.66)</td>
</tr>
<tr>
<td>Upper respiratory tract swab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Throat</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nasal</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nasopharyngeal</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Perianal swab</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Anogenital swab</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Respiratory specimens (nonswab)</td>
<td>581</td>
<td>6 (1.03)</td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
<td></td>
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<tr>
<td>Fresh</td>
<td>790</td>
<td>4 (0.51)</td>
</tr>
<tr>
<td>FFPE</td>
<td>210</td>
<td>14 (6.67)</td>
</tr>
<tr>
<td>Stool</td>
<td>551</td>
<td>3 (0.54)</td>
</tr>
<tr>
<td>Urine</td>
<td>382</td>
<td>4 (1.05)</td>
</tr>
<tr>
<td>Total</td>
<td>5,613</td>
<td>49 (0.87)</td>
</tr>
</tbody>
</table>

a ND, not determined.
b NA, not applicable; nonextracted specimen matrix.
c Includes sputum, induced sputum, bronchial washes, tracheal secretion, and bronchoalveolar lavage fluid.

with the MagNA Pure total nucleic acid isolation kit (small volume) and the Blood, Plasma, Serum program. All specimens were eluted into a final elution volume of 100 μL.

LightCycler PCR conditions. Fifteen microliters of the “hot start” reaction mixture containing the LightCycler FastStart DNA master mixture (including Taq polymerase, PCR buffer, a deoxynucleoside triphosphate mixture with dUTP, and 10 mM MgCl₂), additional MgCl₂, primer pairs, fluorescence resonance energy transfer hybridization probes, and a recovery template (if applicable) was added to the LightCycler cuvette. Five microliters of lysed or extracted specimen was added, and the reaction mixture was placed into the LightCycler. Two cycling/melting profiles were used on the LightCycler. The principal profile was as follows: 95°C for 10 min; 45 amplification cycles of 10 s at 95°C, 15 s at 55°C (single annealing step), and 15 s at 60°C; melting curve analysis for 1 s at 85°C, 20 s at 95°C, 70°C, 85°C, and 95°C (ramp rate of 0.2°C/s, and 15 s at 72°C; melting curve analysis for 0 s at 95°C, 20 s at 59°C, 20 s at 45°C (ramp rate of 0.2°C/s), and 0 s at 85°C (ramp rate of 0.2°C/s and continuous acquisition); and finally cooling for 30 s at 40°C. The M. tuberculosis complex, Mycobacterium genus screen, Coccidioides species, and Histoplasma capsulatum/Blastomyces dermatitidis assays used the same cycling parameters except that a 15-s annealing step at 60°C rather than 55°C was used to improve the specificity of the assays.

PCR assay information. The LDT assays used for this study targeted adenovirus, Babesia species, Bartonella species, BK virus, Bordeella pertussis/Bordetella parapertussis, Borrelia species, Campylobacter jejuni/Campylobacter coli, Clostridium difficile, Coccidioides species, Coxiella burnetii, cytomegalovirus (CMV), Ehrlichia species, Enterococcus vanA/vanB, Epstein-Barr virus (EBV), group A Streptococcus, herpes simplex virus 1/2, Histoplasma capsulatum/Blastomyces dermatitidis, JC virus, human herpesvirus 6 (HHV6), Legionella species, Microsporidia species, the genus Mycobacterium, the M. tuberculosis complex, parvovirus, Plasmodium species, Pneumocystis jirovecii, Salmonella species, Shigella species, Staphylococcus aureus, Shiga toxin 1/2, Tropheryma whippelii, varicella-zoster virus (VZV), and Yersinia species. For many of the assays listed above, primers and probes are commercially available from TIB MolBiol (Adelphia, NJ) or as analyte-specific reagents (ASR) from Roche Molecular Diagnostics (Table 1). The majority of the assays (24/28) have been published or presented as abstracts at national meetings. References are provided in Table 1.

Determination of inhibition. Inhibition was determined at two points in the testing process. Inhibition controls were added preextraction by spiking an aliquot of the specimen matrix with a whole organism or target DNA incorporated into a plasmid at a level of 100 target copies/μl (approximately 10 times the limit of detection) and then subjecting it to processing and extraction as described above. In addition, inhibition controls were added postextraction but preamplification by incorporation of an ASR recovery template into the PCR master mixture or by spiking an aliquot of specimen extract with a whole organism or a target plasmid when recovery templates were unavailable. The recovery template, whole organism, or plasmid containing the target was for spiking at a level of 100 target copies/μl. Inhibition rates were calculated on the basis of lack of detection of the recovery template, whole organism, or target DNA-containing plasmid. Confidence intervals for the inhibition rates were determined by the modified Wald method.

RESULTS
Of 386,706 specimens examined, 95 (0.02%) had evidence of inhibitors present (Table 2). The inhibition rate when inhibition
controls were added preextraction was 49/5,613 specimens, for an overall rate of 0.87% (95% confidence interval, 0.69 to 1.1%). FFPE tissue had the highest rate of inhibition of any individual specimen source, at 6.67%, with urine having the second highest rate (1.05%). When the inhibition control was added postextraction but preamplification, inhibition was observed in 46/381,093 specimens, for an overall rate of 0.01%. FFPE tissue (1.72%) was the only matrix with an inhibition rate above 1%.

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**REFERENCES**


