the unique RET mutations was not possible when the derivative curves overlapped. Although not all pathogenic RET mutations were available for analysis, a recent systematic study of high-resolution melting detection of heterozygous point mutations within a PCR amplicon found a sensitivity and specificity of 100% for amplicons <400 bp in size (15). High-resolution melting analysis for mutation scanning is a rapid (1–2 min after PCR), cost-effective assay that requires no processing or separation steps. As applied to RET mutation scanning, accuracy of heterozygote detection appears to be 100%, and some (but not all) sequence variations can be distinguished from each other. Because samples are immediately available for further processing after high-resolution melting analysis, the detected variant samples can be sequenced for confirmation of genotype.

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# Detection of biological threat agents by real-time PCR: comparison of assay performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler platforms, Clinical Chemistry 52:141 - 145

## Abstract

**Background:** Rapid detection of biological threat agents is critical for timely therapeutic administration. Fluorogenic PCR provides a rapid, sensitive, and specific tool for the molecular identification of these agents. A common chemistry that can be used on a variety of rapid, real-time PCR instruments provides the greatest flexibility for assay utilization. Methods: Real-time PCR primers and dual-labeled fluorogenic probes were designed to detect *Bacillus anthracis*, *Brucella* species, *Clostridium botulinum*, *Coxiella burnetii*, *Francisella tularensis*, *Staphylococcus aureus*, and *Yersinia pestis*. DNA amplification assays were optimized by using Idaho Technology, Inc. buffers and dNTPs supplemented with Invitrogen Platinum® Taq DNA polymerase, and were subsequently tested for sensitivity and specificity on the Idaho Technology, Inc. R.A.P.I.D.®, the Roche LightCycler®, and the Cepheid Smart Cycler®. Results: Limit of detection experiments indicated that assay performance was comparable among the platforms tested. Exclusivity and inclusivity testing with a general bacterial nucleic acid cross-reactivity panel containing 60 DNAs and agent-specific panels containing nearest neighbors for the organisms of interest indicated that all assays were specific for their intended targets. Conclusion: With minor supplementation, such as the addition of Smart Cycler Additive Reagent to the Idaho Technology buffers, a common chemistry could be used for DNA templates that resulted in similar performance, sensitivity, and specificity on all three platforms.

## Subject Terms

- methods, real-time PCR
- Idaho Technology, R.A.P.I.D.
- Roche LightCycler
- Cepheid Smart Cycler
- biological warfare agents
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18
Because disease-causing microorganisms can be used as aerobiological weapons, accurate and timely identification of these agents is necessary (1–4). Important agents include *Bacillus anthracis* (anthrax), *Brucella* species (brucellosis), *Clostridium botulinum* (botulism), *Coxiella burnetii* (Q fever), *Francisella tularensis* (tularemia), *Staphylococcus aureus*, and *Yersinia pestis* (plague) (5). Real-time PCR can rapidly detect the presence of nucleic acid markers with small reaction volumes and rapid cycling. Assay chemistry is increasingly important in this identification process because the choices and concentrations of enzymes, buffers, salts, primers, and probes affect assay detection limits (6–12).

Real-time PCR assays for detecting biological warfare agents have been developed on the R.A.P.I.D.® (Idaho Technology buffers, assays for DNA templates from biological threat agents demonstrated similar performance, sensitivity, and specificity on all 3 platforms.

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signal in 97% of runs (58 of 60 positive, 59 of 61 positive, or 60 of 62 positive, depending on number of replicates tested) was considered the assay limit of detection.

We analyzed a panel of 60 organisms (Table 2 in the online Data Supplement). Panels included threat organisms; nearest genetic neighbors to threat organisms; organisms sharing an environmental niche with a threat organism and thus likely to be found in environmental samples; organisms sharing a clinical niche with a threat organism, particularly respiratory pathogens, opportunists, and typical respiratory flora; and organisms observed repeatedly in clinical and environmental samples. In all cases, 100 pg of genomic DNA was used to determine whether the assays cross-reacted with nucleic acids from other organisms. Inclusivity and exclusivity tests were performed on the R.A.P.I.D., and qualitative results were obtained using R.A.P.I.D. Detector Software Ver. 1.2.14.

For the R.A.P.I.D., each reaction capillary tube was read in channel 1 (F1) at a gain setting of 16, with data analyzed with the LightCycler Data Analysis software (Ver. 3.5.3). Qualitative calls were made using the Detector Software (Ver. 1.2.14). For the LightCycler, each reaction capillary tube was read in channel 1 (F1) at a gain setting of 8, with sample curves analyzed by use of the “Second Derivative Maximum” with the baseline adjustment set to “Arithmetic”. For the Smart Cycler, data analysis was performed with the Cepheid Smart Cycler software (Ver. 1.2d or 2.0b). Smart Cycler settings consisted of a primary curve analysis with a manual threshold setting of 10, background subtraction turned on, boxcar average set to 5 cycles, background minimum cycle set to 5, and background maximum cycle set to 5, and background maximum cycle set to 45.

Whenever possible, targets occurring in multiple copies within the agent genome were exploited for increased sensitivity. Assays were optimized with a standard protocol developed by the Diagnostic Systems Division at the United States Army Medical Research Institute of Infectious Diseases. All primer combinations giving PCR products smaller than 160 bp were tested for amplification efficiency. All probes designed to be used with the most favorable primer pairs were tested for fluorescent signal production. The optimum probe for each primer pair produced the highest EPF signal at a given template input (1 pg for most agents) and standardized probe background. The final primer and probe pairs and optimized assay conditions are listed in Table 1 in the online Data Supplement.

### Table 1. Assay sensitivities as established with at least 60 replicate tests at the limit of detection.\(^a\)

<table>
<thead>
<tr>
<th>Assay no.</th>
<th>Positive control</th>
<th>Probe</th>
<th>R.A.P.I.D.</th>
<th>Smart Cycler</th>
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<tbody>
<tr>
<td>1</td>
<td>B. anthracis Ames BAC008</td>
<td>BAPA3P2A</td>
<td>Sensitivity, fg (GE)</td>
<td>Hits</td>
</tr>
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<td>2</td>
<td>B. anthracis Ames BAC008</td>
<td>BALEF1P1S</td>
<td>50 (9)</td>
<td>61/62</td>
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<tr>
<td>3</td>
<td>B. anthracis Ames BAC008</td>
<td>BACAPBP2</td>
<td>50 (9)</td>
<td>60/62</td>
</tr>
<tr>
<td>4</td>
<td>B. anthracis Ames BAC008</td>
<td>BACAPB4P1S</td>
<td>50 (9)</td>
<td>60/62</td>
</tr>
<tr>
<td>5</td>
<td>Brucella melitensis BRUC013</td>
<td>OMP2aP1799-MGB</td>
<td>100 (30)</td>
<td>61/61</td>
</tr>
<tr>
<td>6</td>
<td>B. melitensis BRUC013</td>
<td>OMP2Bp39S-MGB</td>
<td>100 (30)</td>
<td>58/60</td>
</tr>
<tr>
<td>7</td>
<td>C. botulinum A CLOS001</td>
<td>CBOTA4P2A</td>
<td>100 (25)</td>
<td>60/62</td>
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<tr>
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<td>C. botulinum B CLOS023</td>
<td>CBOTTBP322F</td>
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<td>9</td>
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<td>CBOTTBP326-MGB</td>
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<td>62/62</td>
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<td>10</td>
<td>C. burnetii COX001</td>
<td>IS1111-p822S</td>
<td>1 (0.5)</td>
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<td>F. tularensis Schu4 FRAN016</td>
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<td>13</td>
<td>S. aureus STAP014</td>
<td>SEA318PF</td>
<td>50 (17)</td>
<td>58/60</td>
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<td>SEA882PF-MGB</td>
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<td>YPCAF1P1383S-MGB</td>
<td>100 (20)</td>
<td>61/62</td>
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</tbody>
</table>

\(^a\) Assay limits of detection were defined as the concentration of genomic DNA that produced a positive result in 97% of the replicates tested (58 of 60, 59 of 61, or 60 of 62 positives, depending on number of replicates tested). Molecular weights and genome-equivalents (GE) are provided.
were directly transferable among instruments without a loss in sensitivity. The only difference among the instruments was the need for a higher probe concentration and the addition of SCAR buffer on the Smart Cycler. Exceptions were assays that used MGB probes, for which SCAR buffer had a detrimental effect on assay performance.

For most assays, 97% detection (sometimes called sensitivity) was <100 genomic copies on each instrument (Table 1) and was identical among instruments, with 2 exceptions: the outer membrane protein 2a gene in Brucella spp. and the assay for the multiple-copy insertion element (IS1111) of Coxiella burnetii were less sensitive on the Smart Cycler (Table 1). Primer sets compatible with both TaqMan and TaqMan-MGB probes produced identical results; however, the fluorescent signals of TaqMan-MGB probes generated more robust EPF signals on the LightCycler and R.A.P.I.D. instruments. Interestingly, TaqMan-MGB probes produced weaker fluorescent signals than TaqMan probes on the Smart Cycler, but sensitivity was not compromised. All assays were inclusive to organisms known to have the gene target of interest and exclusive to organisms lacking the corresponding gene targets.

We conclude that the tested assays have comparable sensitivity and specificity on these rapid cycling instruments. The data provide evidence for the easy transfer of assays from one platform to another. TaqMan-MGB probes are attractive because they contain a nonfluorescent quencher and an MGB protein at the 3’ end. The nonfluorescent quencher is more effective in the quenching of reporter dyes than its TAMRA counterpart on the Smart Cycler, but sensitivity was not compromised. All assays were inclusive to organisms known to have the gene target of interest and exclusive to organisms lacking the corresponding gene targets.

We recommend further investigation of the performance of TaqMan-MGB probes with SCAR buffer on the Smart Cycler.

Further testing of the assays presented here could expand data on specificity and could demonstrate utility in clinical and environmental matrices. Although the data in this study were developed on purified nucleic acids, preliminary data with a subset of these assays (assays no. 1 and 20 in Table 1 in the online Data Supplement) indicate that they should perform well in any matrix, as long as sample processing removes inhibitors of PCR (28, 32). This collection of assays provides a repertoire of molecular diagnostic tools that can serve as a foundation for identifying biologic threat agents on multiple, rapid-cycling, real-time, PCR platforms.

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Effects of Folic Acid Before and After Vitamin B₁₂ on Plasma Homocysteine Concentrations in Hemodialysis Patients with Known MTHFR Genotypes

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Background: Treatment with folic acid and vitamin B₁₂ appears to be effective in lowering total plasma homocysteine (tHcy) concentrations, but whether vitamin B₁₂ alone lowers tHcy in patients with normal vitamin B₁₂ status is unknown. The aims of the present study were to explore the effect of individual supplementation with folic acid or vitamin B₁₂ on tHcy concentrations in hemodialysis (HD) patients and to compare changes in tHcy concentrations with MTHFR genotype.

Methods: We recruited 200 HD patients (119 men) from the “Umberto I” Hospital (Frosinone, Italy) and the Dialysis Unit of University Hospital “Tor Vergata”. These patients were randomized blindly into 2 groups of 100 each. Unfortunately, during the study, 36 patients in the first group and 16 in the second group died. The first group was treated initially with vitamin B₁₂ for 2 months and with folic acid for a following 2 months. The second group was treated initially with folic acid and then with vitamin B₁₂. Samples were drawn before administration of either, after the first and second periods, and again 2 months after treatment.

Results: The concentrations of tHcy decreased in both groups after the consecutive vitamin therapies, and the decrease was genotype-dependent. The decrease was greater for the T/T genotype (P < 0.05) and was more significant when the treatment was started with folic acid (P < 0.01).

Conclusion: The alternating vitamin treatment demonstrated for the first time the importance of folate therapy and the secondary contribution of vitamin B₁₂ in lowering tHcy in HD patients.

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