IsoPCR: An Analytically Sensitive, Nested, Multiplex Nucleic Acid Amplification Method

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BACKGROUND: Nucleic acid tests that can simultaneously detect multiple targets with high sensitivity, specificity, and speed are highly desirable. To meet this need, we developed a new approach we call the isoPCR method.

METHODS: The isoPCR method is a 2-stage nested-like nucleic acid amplification method that combines a single multiplex preamplification PCR with subsequent distinct detection of specific targets by use of isothermal amplification. We compared isoPCR to nested quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), and nested LAMP (PCR followed by LAMP), for detection of DNA from Candida glabrata. We evaluated the method’s multiplex capability for detecting low copy numbers of pathogens commonly involved in sepsis.

RESULTS: IsoPCR provided detection of 1 copy of Candida glabrata, an LOD that was 5-fold lower than a nested qPCR assay (5 copies), while the amplification time was simultaneously halved. Similarly, the LOD for isoPCR was lower than that for a LAMP assay (1000 copies) and a nested LAMP assay (5 copies). IsoPCR required recognition of 6 regions for detection, thereby providing a theoretically higher specificity compared to nested qPCR (4 regions). The isoPCR multiplexing capability was demonstrated by simultaneous detection of 4 pathogens with individual LODs of 10 copies or fewer. Furthermore, the specificity of isoPCR was demonstrated by successful pathogen detection from samples with more than 1 pathogen present.

CONCLUSIONS: IsoPCR provides a molecular diagnostic tool for multiplex nucleic acid detection, with an LOD down to 1 copy, high theoretical specificity, and halving of the amplification time compared to a nested qPCR assay.

The same clinical symptom can be caused by infections from many different etiological agents, and hence nucleic acid tests (NATs)3 that can simultaneously detect multiple pathogens from a single sample are highly desirable (1). However, for a test to have diagnostic and therapeutic relevance, i.e., to provide actionable results, the limit of detection (LOD) is critical. Lehmann et al. argue that for sepsis the LOD for NATs must be <30 colony-forming units (cfu)/mL for each of 25 targeted pathogens (2).

A main hurdle in NATs relates to splitting the initial sample for parallel singleplex PCRs, a step that substantially reduces the detection capability and for statistical reasons impedes detection of targets with very low (<10) copy numbers (3). The need for initial sample splitting can be overcome with a multiplex PCR method that uses the entire sample as a template; however, uneven amplification efficiencies of the different primer sets can limit the method performance (4). A 2-stage nested multiplex PCR method can be used, but primer carryover from the first-stage PCR into the second-stage PCR has been shown to generate nonspecific amplification products (5).

As an alternative to PCR-based methods, considerable attention has been given to isothermal NATs because they do not require thermal cycling, making them ideal for point-of-care testing devices. Many different isothermal NATs have been developed, which require either complex primer design schemes or the simultaneous function of multiple enzymes (6). In particular, the loop-mediated isothermal amplification (LAMP) assay is highly specific because it requires the recognition of at least 6 conserved regions spanning more than 180 base pairs (7, 8). This high degree of specificity can limit the usability of LAMP to detection of specific strains if this method is not performed in a multiplex manner, i.e., with many singleplex LAMP reactions that compromise the LOD.

Hence, with the use of present-day methods, the benefits of simplified device design for real-time amplification/detection have not been achieved, and moreover, the drawbacks for multiplex PCR and nested multiplex PCR still exist.

Here we present the isoPCR method (isoPCR), a 2-stage, nested-like nucleic acid amplification method that allows for amplification and detection of multiple targets in samples with improved LOD, theoretically higher specificity, and shorter amplification time compared to state-of-the-art nucleic acid amplification methods (3).

3 Nonstandard abbreviations: NAT, nucleic acid test; LOD, limit of detection; cfu, colony-forming units; LAMP, loop-mediated isothermal amplification; isoPCR, isoPCR method; LF, forward loop primer; LB, backward loop primer; FIP, forward inner primer; BIP, backward inner primer; RT, reverse transcription.
methods. IsoPCR is highly specific because the recognition of 6 regions is required for detection (Fig. 1). These regions are recognized by 4 primers, which include a forward inner primer (FIP), a backward inner primer (BIP), a forward loop primer (LF), and a backward loop primer (LB). These 4 primers are identical to those used in LAMP (7, 8).

IsoPCR encompasses a first-stage multiplex PCR performed for a limited number of cycles. Eighteen cycles were used in this study, thus avoiding uneven amplification efficiency. The first-stage preamplification PCR reaction is performed with FIP/BIP primers, yielding an amplification product having the FIP and BIP primer sequences incorporated in the 5' ends. This first-stage reaction can be performed in a multiplex manner with multiple FIP/BIP primer sets recognizing different and specific nucleic acid targets. The product is then split into distinct chambers, tubes, or zones, in which second-stage isothermal amplification reactions are performed for specific and individualized target loci detection.

The first-stage product serves directly as a starting point for the second-stage singleplex nested-like isothermal amplification reactions, providing that the F1c or B1c regions, respectively, are present on the target DNA. The cycling reactions are identical to conventional LAMP cycling reactions. Additional loop primers, which recognize LFc and LBC regions, accelerate the isothermal amplification reaction, yielding a lower LOD.

Fig. 1. The IsoPCR method.
The first-stage PCR uses FIP and BIP primers each recognizing 2 specific regions. PCR thermocycling incorporates the 5' end (F1c or B1c) in the resulting amplification products. In the second-stage isothermal amplification, the amplification products from the first stage serve directly as starting points for isothermal cycling reactions, providing that the F1c or B1c regions, respectively, are present on the target DNA. The cycling reactions are identical to conventional LAMP cycling reactions. Additional loop primers, which recognize LFc and LBC regions, accelerate the isothermal amplification reaction, yielding a lower LOD.
Fluorescent detection using Sybr Green is used for real-time detection of amplification, the product of which is subsequently checked by using melting temperature analysis (9). Compared to PCR methods, isoPCR generates very high molecular DNA nanostructures, thereby providing many binding sites for double-stranded DNA–binding dyes such as Sybr Green. These DNA nanostructures represent an extra signal amplification step, making it possible to detect down to 1 copy using the time for detection or the CT (threshold cycle) method. In contrast, when the nested multiplex PCR method is used, a melting temperature analysis is necessary to detect such low copy numbers (5).

Initially, we demonstrated the LOD and time for detection for isoPCR by targeting the Candida glabrata pathogen. IsoPCR was compared to the conventional assays, nested qPCR, LAMP, and nested LAMP (PCR followed by LAMP), and various controls with different primer combinations. The LOD of isoPCR (1 copy) was 5-fold lower than a corresponding nested qPCR assay (5 copies), and the amplification time was simultaneously decreased to approximately one-half. Similarly, the LOD for isoPCR was 1000-fold lower than a conventional LAMP assay (1000 copies). The LOD for LAMP is even higher when multiple parallel reactions are required. Alternatively, a nested-LAMP approach combining a first-stage PCR using FP/BP primer sets followed by LAMP detection provided detection close to isoPCR, with an LOD of 5 copies. However, this method required the design of 4 additional primers (F3, B3, FP, and BP) compared to isoPCR.

Next, we demonstrated the multiplexing capability of isoPCR by targeting pathogens commonly involved in sepsis: Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, and the resistance marker, mecA. IsoPCR was performed with a first-stage 4-plex PCR preamplification consisting of primer sets targeting the oprL (peptidoglycan associated lipoprotein OprL precursor), femB (FemB, factor involved in methicillin resistance/glycine interpeptide bridge formation), and mecA (penicillin-binding protein 2') genes and Calb (26S rRNA sequence specific for Candida albicans) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue2). The amplification product was then used as the template in isothermal amplification reactions with individual primer sets for detection of specific targets.

IsoPCR showed an LOD of 10 copies or fewer for all targets (see online Supplemental Table 2), corresponding to below 23 cfu/mL (see online Supplemental Table 3). This LOD is comparable to the SeptiFast multiplex real-time PCR assay (3–100 cfu/mL) for

<table>
<thead>
<tr>
<th>Method</th>
<th>Regions recognized</th>
<th>First-stage PCR</th>
<th>Second-stage amplification/detection</th>
<th>Time for LOD</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsoPCR</td>
<td>6</td>
<td>FIP, BIP</td>
<td>Isothermal FIP, BIP, LF, LB</td>
<td>00:45:49 00:14:42</td>
<td>1</td>
</tr>
<tr>
<td>Nested qPCR</td>
<td>4</td>
<td>FP, BP</td>
<td>qPCR F3, B3</td>
<td>00:45:49 01:16:00 (Ct: 23,70)</td>
<td>5</td>
</tr>
<tr>
<td>LAMP</td>
<td>6</td>
<td>— b</td>
<td>LAMP F3, B3 FIP, BIP, LF, LB</td>
<td>— 00:22:30 00:22:30</td>
<td>1000</td>
</tr>
<tr>
<td>Nested LAMP</td>
<td>8</td>
<td>FP, BP</td>
<td>LAMP F3, B3 FIP, BIP, LF, LB</td>
<td>00:45:49 00:13:11</td>
<td>5</td>
</tr>
<tr>
<td>Control 1 c</td>
<td>2</td>
<td>—</td>
<td>Isothermal FIP, BIP</td>
<td>— ND ND ND</td>
<td>100</td>
</tr>
<tr>
<td>Control 2</td>
<td>4</td>
<td>—</td>
<td>Isothermal FIP, BIP, LF, LB</td>
<td>— 00:34:06 00:34:06</td>
<td>1000</td>
</tr>
<tr>
<td>Control 3</td>
<td>6</td>
<td>F2, B2</td>
<td>Isothermal FIP, BIP, LF, LB</td>
<td>00:45:49 00:16:14</td>
<td>100</td>
</tr>
<tr>
<td>Control 4</td>
<td>2</td>
<td>FIP, BIP</td>
<td>Isothermal FIP, BIP</td>
<td>00:45:49 00:29:39</td>
<td>100</td>
</tr>
</tbody>
</table>

a LOD values are the lowest copy number that was positively detected in 5 of 5 replicates. qPCR results were obtained as Ct values, which were converted to time for detection.

b —, not performed; ND, negative detection.

c Control 1 shows that FIP and BIP primers cannot alone amplify the target DNA. Control 2 shows that a first-stage PCR with FIP and BIP primers is required to obtain an LOD <1000 when using an isothermal detection reaction with FIP, BIP, LF, and LB primers. Control 3 shows that the whole FIP and BIP primers, not just the F2 or B2 parts, are necessary in the first-stage PCR to obtain an LOD <100. Control 4 shows that LF and LB in the second-stage isothermal reaction speed up the reaction and lower the LOD.

Genes: oprL, peptidoglycan associated lipoprotein OprL precursor; femB, FemB, factor involved in methicillin resistance/glycine interpeptide bridge formation; mecA, penicillin-binding protein 2'.
the detection of pathogens involved in sepsis (Roche Molecular Diagnostics) (2). However, the time for amplification/detection is decreased from approximately 2 h using SeptiFast to 1 h using isoPCR.

The specificity of isoPCR was investigated using samples containing DNA from 1 or more pathogens (see online Supplemental Table 4). The results successfully demonstrated the method’s ability to detect very small copy numbers of pathogens, even when more than 1 pathogen was present, without any cross-reactivity. Moreover, this investigation illustrated that isoPCR was highly specific because it did not produce false positives due to primer carryover from the first-stage multiplex PCR, a problem encountered when using nested multiplex PCR (5).

This method could theoretically be used for multiplex detection of mRNA expression levels. This detection could be achieved by performing a reverse transcription (RT) step in combination with the first-stage PCR, providing an RT-isoPCR method capable of detecting mRNA signatures specific for patients with diseases such as obstructive coronary artery disease (10) and colorectal cancer (11).

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References


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