CRISPR-Cas12a based internal negative control for nonspecific products of exponential rolling circle amplification

Tian, Bo; Minero, Gabriel Antonio S; Fock, Jeppe; Dufva, Martin; Hansen, Mikkel Fougdt

Published in:
Nucleic acids research

Link to article, DOI:
10.1093/nar/gkaa017

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
CRISPR-Cas12a based internal negative control for nonspecific products of exponential rolling circle amplification

Bo Tian †, Gabriel Antonio S. Minero 1, Jeppe Fock 2, Martin Dufva 1 and Mikkel Fougt Hansen 1,*

1Department of Health Technology, Technical University of Denmark, DTU Health Tech, Building 345C, DK-2800 Kongens Lyngby, Denmark and 2Blusense Diagnostics ApS, Fruebjergvej 3, DK-2100 Copenhagen, Denmark

Received November 18, 2019; Revised December 16, 2019; Editorial Decision January 02, 2020; Accepted January 03, 2020

ABSTRACT

False-positive results cause a major problem in nucleic acid amplification, and require external blank/negative controls for every test. However, external controls usually have a simpler and lower background compared to the test sample, resulting in underestimation of false-positive risks. Internal negative controls, performed simultaneously with amplification to monitor the background level in real-time, are therefore appealing in both research and clinic. Herein, we describe a nonspecific product-activated single-stranded DNA-cutting approach based on CRISPR (clustered regularly interspaced short palindromic repeats) Cas12a (Cpf1) nuclease. The proposed approach, termed Cas12a-based internal referential indicator (CIRI), can indicate the onset of nonspecific amplification in an exponential rolling circle amplification strategy here combined with an optomagnetic readout. The capability of CIRI as an internal negative control can potentially be extended to other amplification strategies and sensors, improving the performance of nucleic acid amplification-based methodologies.

INTRODUCTION

Although nucleic acid amplification plays a vital and basic role in both research and clinic, its accuracy depends highly on the awareness of risk factors and the control of false-positive and false-negative results (1–3). The most sensitive nucleic acid amplification strategies are those employing exponential amplification formats in which amplicons (amplification products) are recycled as primers or templates (4). However, due to the exponential format, nonspecific background products (NBPs) that lead to false-positive results are inevitable after long reaction times and can be caused by, e.g., contaminants, off-template polymerase products, and secondary structures of primers or templates (4,5). Therefore, the reaction time of exponential amplification has to be evaluated and controlled in practice to avoid the influence of NBPs (5–7). Consequently, the sensitivity of exponential amplification based diagnostics is always determined by the resolution between true- and false-positive signals generated by diluted standard samples and blank/negative controls, respectively. Many efforts have been made to postpone the NBP generation. For example, Reid et al. applied single-stranded DNA-binding protein to reduce nonspecific template interactions in a nicking-based exponential amplification reaction and lowered the background by three orders of magnitude (8); Ding et al. utilized two pairing-competition primers in loop-mediated isothermal amplification to suppress the NBP generation during 2-h incubation (9).

In amplification strategies that recycle amplicons as templates (e.g. recombinase polymerase amplification), NBPs have different sequences compared to the desired specific products, suggesting that amplification results can be confirmed by electrophoresis and gene sequencing. However, in strategies that recycle amplicons as primers (e.g. primer generation-rolling circle amplification [PG-RCA]), NBPs are nearly identical to specific products, which means that the quality of the results depends highly on the performance of external negative controls. External negative controls, usually culture medium or samples from a control group of healthy individuals, are performed in parallel in nucleic acid diagnostics to indicate the generation of NBPs and hence the safe time range for a true-positive assessment (3). The application of external negative controls is based on the assumption that the control and test samples contain the same components (except for the specific target) and that differences, if any, have no influence on the final judgement. However, external negative controls usually have a simpler and lower background compared to the test sample. This

To whom correspondence should be addressed. Tel: +45 4525 6336; Email: botia@dtu.dk
Correspondence may also be addressed to Mikkel Fougt Hansen. Email: mfha@dtu.dk

© The Author(s) 2020. Published by Oxford University Press on behalf of Nucleic Acids Research. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
is particularly true for the common practice of using water or buffer solutions as blank controls, and results in underestimation of false-positive risks. Moreover, external negative controls are required in each relevant clinical diagnostic case for reliable results, which increases not only the cost of detection but also the complexity of designing automated nucleic acid analysis. Due to these inherent shortcomings of external controls, reliable internal negative controls are urgently needed in research and in clinic.

Herein we report a CRISPR-Cas12a-based NBP-activated molecular strategy that digests all single-stranded DNAs (ssDNA) including the amplicons, and therefore suppresses the output signal upon NBP generation. This approach, named Cas12a-based internal referential indicator (CIRI), is performed together with PG-RCA (10) to indicate the level of nonspecific amplification for the tested samples. PG-RCA initiates by the hybridization between target sequences and circular templates (detection loops, DL). Target sequences are extended as primers on the DL by phi29 polymerase, generating amplicons that are subsequently nicked and strand-displaced. Since released single-stranded amplicons can hybridize with DL and serve as primers, PG-RCA achieves an exponential amplification process with time.

CIRI is applicable when nonspecific amplification products contain a Cas12a activation sequence. In addition to PG-RCA, CIRI can be implemented in (i) exponential amplification schemes that recycle amplicons as primers, e.g., bidirectional strand-displacement amplification (11) and exponential amplification reaction (EXPAR) (12); (ii) cascade amplification schemes containing a step utilizing ampiclons of the previous step as primers or triggers, e.g., hairpin-mediated quadratic enzymatic amplification (13); and (iii) cascade amplification schemes containing an RCA step, e.g., circle-to-circle amplification (14).

MATERIALS AND METHODS

Chemicals and DNA sequences

Phi29 polymerase, phi29 buffer, dNTP mix, bovine serum albumin (BSA), GeneRuler low range DNA ladder, SYBR Gold nucleic acid gel stain, Tris-HCl buffer (1 M, pH 8.0) and Tris-acetate-EDTA buffer (TAE, 50 ×) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). CircLigase II ssDNA ligase, together with other ligation reagents (buffer, MnCl2, betaine), was purchased from Biosearch Technologies (Novato, CA, USA). Nb.BtsI nickase, thermolabile exonuclease I, exonuclease III and loading buffer were purchased from New England BioLabs (Ipswich, MA, USA). Recombinant Acidaminococcus sp. BV3L6 Cas12a nuclease was purchased from Integrated DNA Technologies (Coralville, IA, USA). Fe(3+) bovine serum (FBS), salmon sperm DNA and agarose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Streptavidin-coated cross-linked starch iron oxide composite particles (100 nm size MNP) were purchased from Micromod Partikeltechnologie GmbH (Rostock, Germany). DNA and RNA sequences were synthesized by Integrated DNA Technologies and diluted in 50 mM Tris-HCl (pH 8.0).

Preparation of circular templates

Circular templates, DL (detection loop) and RL (reference loop) were prepared by self-ligation of linear detection template and linear reference template, respectively. The ligation mixture consisted of linear templates (0.5 μM), CircLigase II reaction buffer (1 ×), MnCl2 (2.5 mM), betaine (1 M) and CircLigase II ssDNA ligase (5 U/μl). The ligation mixture was incubated at 60°C on a Thermoshaker incubator (Grant-bio, Cambridge, UK) for 3 h to form circular templates, followed by heat inactivation at 80°C for 15 min.

Preassembly of MNP clusters

For PG-RCA reactions performed without CIRI, Cas12a (60 nM), crRNA (90 nM), circular templates (1 nM each of DL and RL) and detection probe-modified MNPs and DP-DL-II modified MNPs were prepared separately, mixed in a volumetric ratio of 1:1, and stored at 4°C prior to use. For the detection of RL amplicons, DP-DL-I modified MNPs and DP-DL-II modified MNPs were prepared, mixed and stored in the same way.

Primer generation-rolling circle amplification

For CIRI-controlled PG-RCA, the RCA reaction mixture consisted of phi29 polymerase (2×), BSA (0.4 μg/μl), dNTPs (0.45 mM), phi29 polymerase (0.67 U/μl), Nb.BtsI nickase (0.2 U/μl), Cas12a (60 nM), crRNA (90 nM), circular templates (1 nM each of DL and RL) and detection probe-modified MNPs (0.1 μg/μl). Upon detection, the RCA reaction mixture was mixed with the test sample in a volumetric ratio of 1:1, followed by isothermal incubation at 37°C on chips (for real-time optomagnetic measurement). For PG-RCA reactions performed without CIRI, Cas12a was excluded from the RCA reaction mixture. For preparing the blank control, 50 mM Tris-HCl (pH 8.0) free of target sequence was mixed with the RCA reaction mixture in a volumetric ratio of 1:1. In the utility test using cell DNA extractions, 50 mM Tris-HCl (pH 8.0) containing 0.1 μg/ml sheared salmon sperm DNA was used to prepare the blank control.

Preassembly of MNP clusters

The RCA reaction mixture (without CIRI, MNP concentration of 0.5 μg/μl) was mixed with a sample containing 2
pM of target DNA in a volumetric ratio of 1:1, followed by isothermal incubation at 37°C for 1 h. After PG-RCA reaction, MNP clusters formed in the suspension were collected by a magnetic stand, washed twice and resuspended to an MNP concentration of 0.1 µg/µl by Tris-HCl (50 mM, pH 8.0).

Real-time optomagnetic measurement with magnetic incubation

Optomagnetic detection chips (a minimum detection volume of 90 µl) containing RCA suspensions were sealed and thereafter mounted in the optomagnetic setup. Ten magnetic incubation cycles were automatically performed between two optomagnetic measurements, with each cycle consisting of 2 s of 2.6 mT field followed by 2 s of 0 mT. For each optomagnetic measurement, an optomagnetic spectrum consisting of 41 logarithmically equidistant frequencies between 1 and 2800 Hz was recorded in ~50 s.

Agarose gel electrophoresis

In the gel electrophoretic study, concentrations of circular templates, targets and Cas12a-crRNA used in the PG-RCA reaction were 0.5 nM, 1 pM and 30 nM, respectively. PG-RCA reactions were conducted at 37°C for 30 min. Products of PG-RCA were mixed with loading buffer as well as SYBR Gold gel stain, and analyzed by 2.5% (w/v) agarose gel electrophoresis in 1 × TAE buffer at a constant voltage of 100 V for 30 min at room temperature. Gel images were obtained by using a UVP BioSpectrum Imaging System (Analytik Jena, Jena, Germany).

RESULTS AND DISCUSSION

In this work, amplicons of PG-RCA hybridize with dual detection probe-modified magnetic nanoparticles (MNPs) such that MNP clusters form in the presence of DL amplicons (Figure 1, gray arrows). The clustering of MNPs is such that MNP clusters form in the presence of DL amplicons (Figure 1, red arrows). Therefore, the Cas12a-based signaling signal reduction due to the disassembly of MNP clusters, different concentrations of target B (as primers of RL) and Cas12a-crRNA complexes (30 nM). Figure 2C shows that activation of the Cas12a catalytic ability depends on the dose of target B that ranges from 100 fM to 100 aM. Optomagnetic signals remained constant at the beginning followed by an abrupt decrease due to the amplification of RL and the subsequent MNP cluster disassembly. Different concentrations of target B triggered signal reduction at different times, suggesting that CIRI can indicate amplification of RL during the PG-RCA reaction.

Assuming that NBPs are triggered by the presence of random nucleic acid sequences that can be contaminants, background nucleic acids and off-template products of polymerases, hybridization between those random sequences and circular templates can be regarded as a stochastic event influenced mainly by the temperature and their lengths (25). Therefore, we assume that nonspecific amplification reactions of both RL and DL in the same reaction suspension are stochastic events with very similar probabilities due to their identical length and GC content, thus the nonspecific activation of RL can be utilized to estimate the level of NBPs.

To demonstrate that RL can indicate the background amplification of DL on time, template mixtures containing 0.5 nM each of DL and RL were amplified without target, Cas12a or crRNA (Figure 3A). The nonspecific PG-RCA reactions were performed both in clean target-free buffer and in buffer containing 0.1 µg/ml sheared salmon...
sperm DNA (1 nM, with an average size of 150 bp). To detect the background amplification of DL, MNPs in the suspension were modified with DP-DL-I and DP-DL-II oligos that can hybridize with amplicons of DL. For the detection of RL amplification, we used MNPs modified with DP-RL-I and DP-RL-II that can hybridize with amplicons of RL. As shown in Figure 3A, coincident results of DL and RL were observed both in clean target-free buffer (black and red curves) and in buffer containing nonspecific DNA (blue and yellow curves), suggesting that the activation of RL can indicate the background amplification of DL.

To investigate the influences of RL length and GC content on its ability to simulate the background amplification of DL, several RL designs were tested in clean buffer (Figure 3B). Real-time optomagnetic measurements show that GC content changes of ±5% have no obvious influence on RL performance (blue, red and black curves in Figure 3B), which confirms the low sequence-dependent amplification bias of RCA (14) and suggests a certain level of universality for the proposed RL design. Compared to the three 123-nt-long RLs, the 93-nt-long RL (yellow curves) exhibited a ∼3 min delay of background amplification, which can be ascribed to the shorter binding sequence for nonspecific am-
Figures 3A and 4A are based on the same data. Other target concentrations are plotted in the same color. Black curves in independent replicates (n = 5 for 1 aM targets and blank controls, n = 3 for other target concentrations) are plotted in the same color. Black curves in Figures 3A and 4A are based on the same data.

Figures 3A and 4A are based on the same data. Other target concentrations are plotted in the same color. Black curves in independent replicates (n = 5 for 1 aM targets and blank controls, n = 3 for other target concentrations) are plotted in the same color. Black curves in Figures 3A and 4A are based on the same data.

Thereafter, we moved on to the RL amplicon-activated Cas12a system. PG-RCA reactions without and with CIRI were performed and monitored optomagnetically to demonstrate the capability of CIRI for indicating NBP s during nucleic acid amplification. Conventional PG-RCA reactions (without Cas12a) were performed to amplify target DNA of different concentrations. Real-time changes of \( \phi \) (1–10 Hz) exhibited a time- and dose-dependence similar to some variation in the GC content and length of the RL (Figure 3B), similar GC contents and lengths between RL and DL are preferred. Third, RL should contain secondary structures similar to DL, to ensure loop templates contain similar amounts of single-stranded areas that can bind freely to NBP triggers. In this work, secondary structures were investigated using NUPACK (www.nupack.org).

The stability of PG-RCA and CIRI against matrix effects was evaluated using diluted FBS (fetal bovine serum) samples. PG-RCA worked well in 2% FBS (final concentration) but was nearly inhibited in 10% FBS (Figure 5A). Stability of Cas12a and crRNA against FBS was evaluated by adding 30 nM of Cas12a ternary complexes into diluted FBS suspensions containing MNP clusters. The cleavage activity of Cas12a-based ternary complexes was lost within 20 min in 2% FBS (black curve in Figure 5B). This loss of activity was ascribed to the crRNA hydrolysis by serum nuclease. Although it was reported that chemical modification could make crRNAs stable at 37°C in 5% FBS for 30 min (27), the chemically modified crRNAs do not make our requirement since CIRI needs Cas12a-crRNA to stay activatable for at least 1.5 h. Therefore, additional reagents (RNase inhibitor) or processes (extraction or purification) have to be considered when testing serum samples using CIRI-controlled PG-RCA.
The utility of CIRI was evaluated through PG-RCA reactions performed in the presence of 0.1 µg/ml sheared salmon sperm DNA (1 nM, with an average size of 150 bp) simulating the background of total DNA extraction samples (Figure 6). Due to the high DNA background, PG-RCA of blank controls was initiated earlier than in clean buffer (Figure 3A). Therefore, target concentrations <100 aM could hardly be distinguished from blank controls (cf. red and black curves in Figure 6). As expected, reactions performed with CIRI showed signal reductions after ~50 min, demonstrating the capability of CIRI to indicate the background DNA level as an internal negative control.

Although the capability of CIRI was demonstrated only in a PG-RCA strategy combined with an optomagnetic readout, it can be extended to other nucleic acid amplification strategies and readout methods. However, limitations have to be considered for applying CIRI. First, the reaction temperature has to be controlled near the optimal temperature of Cas12a. Second, similar to most of exponential amplification-based methodologies, CIRI requires or at least prefers a real-time (or time-resolved) readout not only for a wide dynamic detection range but also to avoid false-negative judgments (converted from false-positive judgments by CIRI). Third, the signal transducer (e.g. the MNP cluster) should be designed responsive to activated Cas12a. Fourth, the reference template has to be carefully designed to simulate the probability of detection template-based nonspecific amplification. Finally, it should be noted that there are two kinds of false-positive situations that cannot be detected by CIRI, (i) self-amplification of primers or primer dimers, which produces NBP without the crRNA recognition sequence and (ii) amplification caused by carry-over contamination containing amplicons from previous reactions.

CONCLUSION

In summary, we have developed an NBP-activated ssDNA-cutting approach that contains a reference template (RL), Cas12a, and its corresponding crRNA. The proposed approach, termed CIRI, can indicate the onset of nonspecific amplification when performed simultaneously with PG-RCA. Therefore, it can be utilized as an internal negative control to monitor the background level of amplification reactions in real-time, lowering the cost and simplifying the design of these methodologies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

Dr Wei Hu at the Clinical Lab, the First People’s Hospital of Yichang and the People’s Hospital of China Three Gorges University is gratefully acknowledged for discussions and suggestions.

FUNDING

H2020 Marie Skłodowska-Curie Actions [713683, CO-FUNDfellowsDTU], Environmental Technology Development Program [MUDP, MST-141-01415]; Independent Research Fund Denmark [DFF, 4184-00121B]. Funding for open access charge: H2020 Marie Skłodowska-Curie Actions. Conflict of interest statement. None declared.

REFERENCES


