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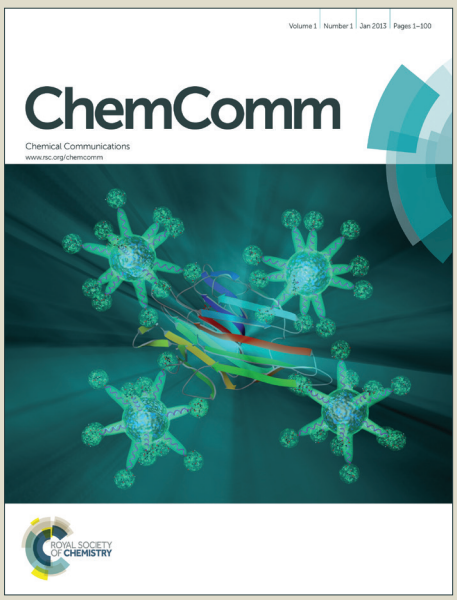

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ARTICLE TYPE

Invasive Reaction Assisted Strand-displacement Signal Amplification for Sensitive DNA Detection

Bingjie Zou[1,a], Qinxin Song[1,a,b], Jianping Wang[a,b], Yunlong Liu[a], and Guohua Zhou[*a,c]

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A novel DNA detection assay was proposed by invasive reaction coupled with molecular beacon assisted strand-displacement signal amplification (IRASA). Target DNAs are firstly hybridized to two probes to initiate invasive reaction to produce amplified flaps. Then these flaps are further amplified by strand-displacement signal amplification. The detection limit was around 0.2 pM.

With the progress in post-genome research, a growing number of genomic biomarkers have been identified. Thus, a sensitive and specific DNA detection method is required to detect those biomarkers, which are susceptible to diseases or related to drug responses. PCR[1] enables us to amplify DNA templates of interest by 10^9-10^{10} folds. Because amplification process of PCR needs a thermal cycler, many isothermal amplification strategies have been developed to replace PCR, such as rolling circle amplification (RCA),[2-6] loop-mediated isothermal amplification (LAMP),[7] invasive signal amplification,[8] nicking endonuclease signal amplification (NESA),[9-12] strand displacement amplification (SDA),[13, 14] beacon assisted detection amplification (BAD AMP),[15] and isothermal circular strand-displacement polymerization reaction.[16-18] Among them, invasive reaction is superior in specificity, because the signal corresponding to the target of interest is only produced when the invasive structure formed between upstream and downstream probes and target DNA. Invasive reaction is able to discriminate one base difference in a target, but the sensitivity is not enough. As the amplified flaps (signals) from invasive reaction are independent of target sequence, it should be possible to couple a second amplification reaction to further amplify the flaps. We have successfully combined nicking reaction and RCA with invasive reaction to increase the detection sensitivity.[6, 19] However, nicking enzyme is very expensive, and the use of a large amount of the enzyme for increasing the nicking velocity is much costly. Most importantly, both methods need a ligation step for bridging invasive reaction with the second amplification reaction. Therefore, three kinds of enzymatic reactions are used in the methods. As each enzyme has its own optimal condition for reaching a specific reaction, it is preferable to use enzymatic reactions as less as possible in an assay. Previous experience tells us that the ligation step makes the assay laborious and time-consuming. To escape the ligation step, herein, a novel strategy was proposed by coupling invasive reaction with modified beacon-assisted strand-displacement signal amplification (termed as IRASA). These two reactions are bridged by polymerase-based extension reaction instead of ligation reaction. As the polymerase could be shared by the strand-displacement reaction, only two enzymes are used in the new method. Compared to conversional beacon-assisted strand-displacement amplification (BASDA),[17] our method only employs a single kind of molecular beacon (MB) to detect different targets, significantly simplifying the experiment set-up. Another key to the method is the introduction of an extension-block base into the stem of MB to enable conventional BASDA to couple with the invasive reaction without any help of additional enzyme, such as ligase. This gives a new strategy for combining two different amplification methods for sensitive DNA detection.

The principle of IRASA was shown in Figure 1. At first, conventional invasive reaction is carried out by employing two target-specific probes (**Up** and **Dp**) to form an invasive structure, in which the 3'-termini of **Up** invades into **Dp**-target duplex with one base. Flap endonuclease (FEN) recognizes this structure and cuts the **Dp** to release a flap, which is a fragment non-complimentary to the target in the 5' region of **Dp**. As the reaction temperature is close to the melting temperature (Tm) of **Dp**, an intact **Dp** will hybridize to the target again, producing a cleavage cycle. The flaps are accumulated. Then, use a DNA polymerase (typically Klenow fragment) to extend the cleaved flaps, which are captured by the loop region of a molecular beacon. As a result, the stem of the beacon is opened. To achieve the amplification of the flaps, a primer, which is complementary to the stem region of the beacon, is employed to displace the extended flap by extension reaction. Once the extended flap is released from the beacon, a new beacon will soon hybridize to it,

[1] These authors contribute equally to this work.

[a]*Department of Pharmacology, Jinling Hospital, Medical School of Nanjing University, Nanjing, 210002, China.Fax: +86 25 80860196; Tel: +86 25 80860195; E-mail: ghzhou@nju.edu.cn*

[b]*School of Life Science and Technology, China Pharmaceutical University, Nanjing, 210009, China*

[c]*State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University, Nanjing, 210009, China*

† Electronic Supplementary Information (ESI) available: Experimental section, the illustration of IRASA using four dNTPs, the comparison of IRASA for detecting flaps withfour dNTPs and three dNTPs, optimization of extension conditions, IRASA for flaps detection, and pyrograms of amplicons from biological specimensfor EGFR gene c.2573T>G mutation analysis. See DOI: 10.1039/b000000x/

forming a displacement cycle. The signals corresponding to the flaps are hence amplified by consuming the primer and the beacon during the cycle. The amount of opened beacons can be readily measured by a real-time fluorescence detector. If the target DNA is absent, no flap would be generated from the cleavage cycle, and the stem of the beacon would not be opened.

However, the displacement cycle would be blocked if the stem region of the beacon was extended by the flaps. This is because the primer for triggering the strand-displacement is complementary to the stem region, and the displaced strand would be extended by the primer if containing the sequence of the stem region (Figure S1). To form an efficient displacement cycle, the displaced strand should be single-stranded; otherwise, it is difficult to open the molecular beacon. To stop the extension of the flap at an expected position, the sequence of the loop in the beacon was artificially designed with just three kinds of base types (A, G and C), and the base T is used as the "stop" base. The extension reaction will stop at this base when adding dNTPs complementary to the bases A, G, and C. Therefore, dTTP, dCTP and dGTP were employed for extending the flaps, and the extension reaction should terminate at the stem region (base T) marked with blue circle in Figure 1.

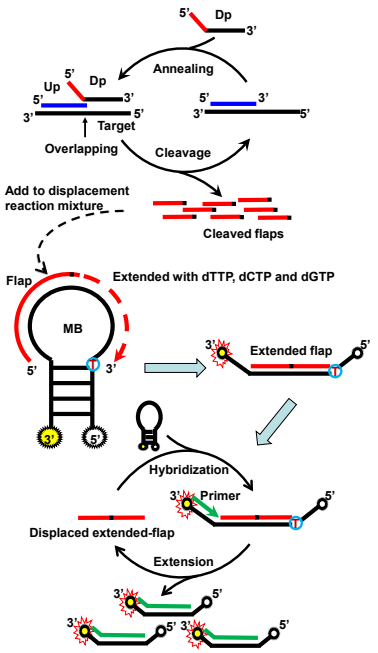

Figure 1.The principle of IRASA. The target DNA is firstly converted into amplified flaps by invasive reaction. Then the strand-displacement reaction mixture is added, and the amplified flaps are extended along the loop of molecular beacon by polymerase with dTTP, dCTP and dGTP to trigger the beacon-assisted strand-displacement reaction.

The pathway of IRASA was verified by electrophoresis. As shown in Figure S3 A, band intensities of the flaps increased with target concentration, indicating that invasive reaction is target-specific. As expected, the amount of double-stranded MB produced by extension reaction increased along with the reaction time (Figure S3 B), but no double-stranded MB was observed if Klenow or flaps were absent (see arrows in Figure S3 B), suggesting that the strand-displacement reaction is flap-specific.

To investigate the effectiveness of this strategy, extension reaction with all four dNTPs and three dNTPs (excepting for dATP) were individually carried out for extending 2 nM and 20 nM flaps. As shown in Figure 2S, the fluorescent intensity profile (time-course) of 2 nM flap was almost the same as that of the blank control if four dNTPs were added; however, the signal intensities significantly increased when adding three dNTPs for the extension reaction. Comparison between Figure 2S (A) and Figure 2S (B) indicated that extension with three kinds of dNTPs is key to sensitive detection by IRASA.

As the sensitivity of IRASA dependents on the strand displacement rate, it is necessary to investigate the reaction conditions, including the concentrations of polymerase, the beacon and the primer, as well as reaction temperature. To look for optimal conditions, we performed strand displacement reaction to detect 2 nM flaps with different amounts of Klenow fragment polymerase, different concentrations of the molecular beacon and the primer, and different reaction temperature. For comparison, the fluorescence intensities from 2 nM flaps and a blank control (without flap) at various conditions were measured after 20-min reaction. We found that the positive signal and background signal were increased with the concentration-increase of Klenow fragment (Figure S4 A), the primer and the beacon (Figure S4 B). The maximum difference between the positive signal and the background was achieved at 2.5 U of Klenow fragment and 100 nM of both the primers and the beacons. The optimal reaction temperature was 37°C (Figure S4 C), which is the recommended polymerization temperature of Klenow by the manufacturer. At the optimal conditions, various concentrations of flaps were detected by the strand displacement reaction, and as low as 0.2 nM flaps was successfully detected (Figure S5).

To improve the sensitivity, invasive reaction is combined with beacon-assisted strand displacement reaction. A series of target DNAs with different concentrations (20, 10, 2, 1, 0.2, 0.1, and 0 pM) were firstly amplified by invasive reaction, yielding cleaved flaps. Then the flaps were further amplified by beacon-assisted strand-displacement reaction. The time-courses of IRASA (Figure 2A) showed that the detection limit of IRASA is around 0.2 pM, which is 1000-fold higher than that of beacon-assisted strand displacement amplification (0.2 nM flaps in Figure S5). In addition, the plot between the initial reaction rate and target concentrations exhibited a linear relationship (Figure 2B), indicating that IRASA method proposed here can be used for quantitative DNA detection. Because the beacon-assisted strand displacement in IRASA is independent of target DNA sequences, any target can be detected just by changing the invasive probes specific to the target.

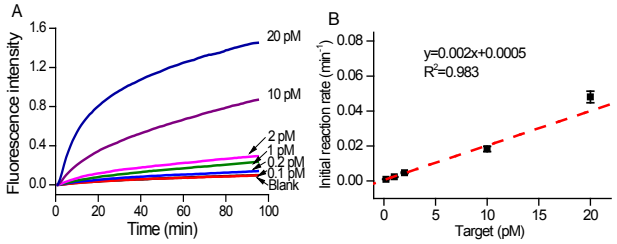

Figure 2. The time-course of IRASA for detecting targets with various concentrations (A) and the relationship between the target concentrations and the initial reaction rate (B). The sequence of the target and IRASA condition is described in Supplementary Information.

The specificity of IRASA was investigated by detecting a series of target DNAs each with one base difference at various positions, which were specified as bold and italic font in the targets from **a** to **g** in Figure 3A. As shown in the figure, the time-course of targets **a**, **b**, **c**, and **e** are the same as that of the blank control (N), suggesting that IRASA is able to discriminate one base difference in the targets. Although the fluorescence intensities of targets **d**, **f**, and **g** were a little higher than that of the blank control, we are very satisfied with this specificity, because we can put the base (e.g. the mutant base) of interest at the overlapping position (**a**) or near the overlapping position (**b**, **c** and **e**). This is readily to be achieved by designing an upstream probe and a downstream probe for invasive reaction.

To further evaluate the feasibility of the proposed IRASA in the detection of real biological samples, four FFPE (Formalin-fixed, paraffin-embedded) specimens from non-small cell lung cancer patients were employed as detection targets. The c.2573T>G mutation in the EGFR gene was analysed by IRASA. As shown in Figure 3B, the samplesS3 and S4 gave positive signals significantly higher than the negative control (genomic DNA from a healthy person) as well as samples S1 and S2 (marked with NC, S1 and S2 in Figure 3 B). While no difference in the detection profile was observed between S1, S2 and NC. Therefore the samplesS3 and S4 have mutated EGFR gene at c.2573T>G, which is sensitive to the targeted therapy by gefinitib. However, no EGFR gene mutation occurred at c.2573T>G for samples S1 and S2; thus these two patients would not benefit from the gefinitib-based therapy. The results are consistent with that by pyrosequencing (Figure S6), indicating that IRASA is feasible to pick up a low level of mutant in tumour tissues. Although pyrosequencing is widely used for mutation detection by quantifying the peaks corresponding to mutants in pyrograms, it is obvious that IRASA is superior to pyrosequencing in the strong intensities of positive signals.

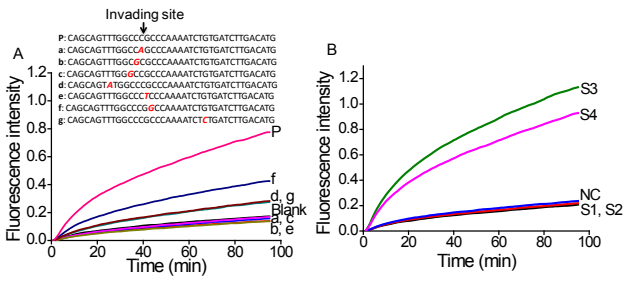

Figure 3. The time-courses of IRASA for detecting artificial targets with one base mutation at various positions (A) and real biological samples (B).The sequences of mutant targets from **a** to **g** are listed as the inset, and the mutant bases are in red-bold and italic font. **P** is perfect complementary target. Samples from S1 to S4 are from FFPE specimens suffered from non-small cell lung cancer (from Jinling Hospital). NC is from genomic DNA of a healthy person. FFPE samples were extracted using QIAamp DNA FFPE Tissue Kit (Qiagen). The starting material for IRASA was 1000-fold dilution of PCR products.

In summary, an isothermal DNA detection by coupling invasive reaction with strand displacement amplification was proposed. A target DNA was initially amplified by invasive reaction using two sequence-specific probes, and then further amplified by a universal molecular beacon-assisted strand displacement. This gave the sensitivity as low as 0.2 pM targets. As the second amplification is universal to any target, the proposed method is cost-effective. The specificity is high enough to discriminate one base difference between targets, the same as invasive reaction. A proof-of-concept application of IRASA is demonstrated by the successful discrimination of mutated EGFR gene from cancer tissues. As IRASA is based on signal amplification, the risk of false-positive results due to amplicon-contamination should be much low. We believed that IRASA would be a good tool for clinical diagnosis, for example, gene-guided personalized medicine.

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An extension-block base in molecular beacon enables beacon-assisted strand-displacement amplification to couple with invasive reaction efficiently by flap extension.