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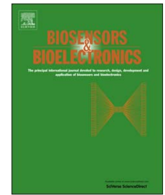

Microparticle-based RT-qPCR for highly selective rare mutation detection

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ABSTRACT

The quantitative reverse transcription polymerase chain reaction (RT-qPCR) has become one of the most widely used methods in the detection of disease-specific RNAs. The RT-qPCR involves two separate steps, RT and qPCR. In this study, we suggest a new RT-qPCR protocol with the particles of primer-immobilized networks (PINs), performing capture, RT and amplification of a target RNA in one particle. The production of undesired cDNAs was dramatically suppressed by the specific capture of the target RNA within the particle. Afterward, RT and amplification processes are performed without loss of cDNAs as exchanging the reaction solution. The biomarker gene of chronic myeloid leukemia, Bcr-Abl fusion transcript, is detected in the sensitivity of single mutant leukemic cell mixed in 10^4 normal cell using this protocol with the excellent restraint of non-specific signal. This protocol that whole processes are performed in the particle in a row is preferred for the highly specific detection of target RNAs in complex sample.

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1. Introduction

The reverse transcription polymerase chain reaction (RT-qPCR) procedure has become one of the most widely used methods for the detection of specific RNA biomarkers. This highly sensitive system allows the fast and accurate detection and quantification of target RNAs in clinical laboratories (Bernard and Wittwer, 2002; Bustin and Mueller, 2005). Many researchers have tried to diagnose infectious diseases (Ceroni et al., 2010; Bergin et al., 2010; Guerra et al., 2000) and various cancers (Bieche et al., 1998; Song et al., 2012; Schaefer et al., 2010) using the RT-qPCR technique. For example, Filippis detected and sequenced Zika virus RNA from amniotic fluid of fetuses (Calvet et al., 2016) and Filella group detected PCA3, the prostate cancer marker RNA, from the patients' urine samples using RT-qPCR method (Foj et al., 2014). However, clinical samples from patients are extremely complex. The non-specific binding of numerous interferants often overrides the signal from the small amount of target genes (Scheltinga et al., 2005). In an RT-qPCR system using the general DNA intercalating dyes such as SYBR® Green I, a false-positive signal may arise from the primer-dimer conformation and other undesired by-products, acutely deteriorating the data analysis (Kubista et al., 2006; Vandesompele et al., 2002).

For accurate analysis and diagnosis, a highly selective amplification system is required for the clinical usage of RT-qPCR. Thus, various approaches to enhance the selectivity of RT-qPCR have been reported. Most researchers have mainly focused on the molecular designing of primers and probes to distinguish partial mismatches. The most popular method is to add the specific TaqMan probes, molecular beacons and hybridization probes (Wong and Medrano, 2005; Postollec et al., 2011). Another approach utilizes dual priming oligonucleotides (Chun et al., 2007), which selectively reduces the production of non-specific amplicons. In addition to this molecular design, the platform development which can be commonly used for target gene capture and specific amplification is essential to improve the selecticity.

In this study, we present a new RT-qPCR method using primer-immobilized networks (PINs) and apply it to the detection of the rare mutation of Bcr-Abl fusion, which is the target of chronic myeloid leukemia (CML). The Bcr-Abl, which is a product of a reciprocal translocation between chromosomes 9 and 22 (Roh et al., 2015; Perego et al., 2000; Verma et al., 2009), has been a universal target for early diagnosis as well as assessing the prognosis of CML, leading to the development of various systems for detecting the mutation (Roh et al., 2015; Tkachuk et al., 1990; Emig et al., 1999). We made PIN particles using polyethylene target gene. In our previous work, the qPCR using PIN was multiplexed extensively for the quantitative profiling of miRNAs (Jung et al., 2016). The PIN qPCR showed high performance in amplification of gene with high

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signal to noise. However, the reverse transcription was conducted separately before the amplification step. We designed new protocol and PIN particles that combines capture, RT and amplification of target RNA in one particle. Capturing of target RNAs improved the selectivity of the assay and the synthesized cDNAs were conserved between RT and amplification without loss. Although it is reported that primer-immobilized beads were used for the preparation of DNA sample for sequencing (Klein et al., 2015), it is the first time to selectively capture the target gene from total RNA mixture and conduct the qPCR in same particle. With this PIN RT-qPCR system, sample contamination and loss which can be generated from separated two-step protocol (RT and qPCR) and low sensitivity from 'one-step RT-qPCR' technique (Sugita et al., 2001; Gregory et al., 2006) could be overcome. Moreover, the amplicons of the target gene can be released from the particle and collected for further analysis such as sequencing. We quantitated rare Bcr-Abl fusion transcripts among normal genes and confirmed the sequence conveniently with this concise work flow.

2. Material and methods

2.1. Cell culture and RNA extraction

The human chronic myelogenous leukemia cell line K562 was used as a Bcr-Abl positive cell line and the human promyelocytic leukemia cell line HL60 was used as a Bcr-Abl negative cell line. Both cell lines were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C under 5% CO_2. Total RNA was extracted from K562 and HL60 using an AccuZol™. Total RNA Extraction Solution (Bioneer, Korea) according to the manufacturer's instructions. The extracted RNA was confirmed using 1% agarose gel electrophoresis.

2.2. Primer design

For the detection of Bcr-Abl gene, forward and reverse primers were designed as follows; forward: 5′-TGG GTT TCT GAA TGT CAT CGT CCA CTC A-3′, reverse; 5′-AGT TCC AAC GAG CGG CTT CAC TCA-3′. For the construction of PIN particle and the purification of target gene, the acrydite and restriction enzyme EcoRI site were added at 5′ terminus of the reverse primer. The forward primer of Abl is designed as 5′-AAG CTG GTG GGC TGC AAA TCC AAG-3′.

2.3. Preparation of PIN particles

The pre-polymer solution for PIN particle preparation was made by mixing 20% v/v of UV-phorocrosslinkable Poly(ethylene glycol) diacrylate (PEGDA, Sigma-Aldrich, MW700), 40% v/v of Poly (ethylene glycol) (PEG, Sigma-Aldrich, MW600), 5% v/v of photoinitiator 2-hydroxy-2-methyl propiophenone (Sigma-Aldrich) and 35% v/v of 3X Tris-EDTA buffer (TE, Sigma-Aldrich) with 0.15% Tween-20 (Sigma-Aldrich). The PEG600 was used to increase the porosity of PIN particle for the facile mass transfer during reverse transcription and PCR steps. The 200 μM of the acrydited-reverse primer of Bcr-Abl was added to the pre-polymer solution to make a final concentration of 20 μM. The PIN particles were produced by spotting of the pre-polymer solution onto the PDMS surface using a jetting system (Arrayer 2000, Advanced Technology Inc., Korea) followed by UV exposure (360 nm wavelength, 35 mJ/cm^2) for 1 min. The photocrosslinked PIN particles were released from the PDMS surface by mild agitation and the PIN particles were washed with TE buffer containing 0.05% Tween-20 to remove the uncrosslinked compounds such as reverse primer, PEGDA, and PEG.

2.4. cDNA synthesis

Reverse transcription step was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, USA). Initially, PIN particles were incubated with 1 μg of total RNA extracted from the K562 or HL60 cells at 65 °C for 5 min and chilled on the ice. Then, the particles were washed with TE buffer containing 0.15% Tween-20 to remove the nonspecifically bound-RNA. After the washing step, reaction buffer, RNase inhibitor, dNTP and reverse transcriptase were added and incubated at 42 °C for 60 min. The reaction was terminated by heating at 70 °C for 5 min. The conventional reverse transcription was also conducted as a reference for comparison. The PIN particles carrying the first synthesized cDNA were stored at −20 °C for the next experiment.

2.5. Real-time quantitative PCR using PIN particles

The PIN RT-qPCR was conducted using the UltraFast LabChip Real-time PCR G2-3 System (Nanobiosys, Seoul, Korea). For the qPCR using PIN particles, 8 μL of 2X SYBR Green master mix (Nanobiosys, Korea), 0.8 μL of 10 μM forward primer, and deionized water were mixed to make a final volume of 16 μL. In the conventional RT-qPCR for the comparison, 0.8 μL of 10 μM reverse primer and cDNA template were also added to the solution. The single particle was captured in the PCR chamber and the mixed solution was injected into the chamber. The PCR condition was one cycle of pre-denaturation at 95 °C for 8 s, 40–50 cycles of denaturation at 95 °C for 3 s and annealing/elongation at 60 °C for 11 s. The fluorescence images of each cycle were captured and the fluorescence intensities were calculated by software. The intensity value was normalized by software and plotted by line graph.

2.6. Sensitivity test

To evaluate the sensitivity of PIN particle-based qPCR, the increasing number of K562 cells were mixed with the fixed number of HL60 cells. 10^2 to 10^6 K562 cells were mixed with 10^6 HL60 cells and total RNAs were extracted from the cell mixture. 1 μg of the total RNA of each cell mixture was used for the sensitivity test.

2.7. Purification of target gene using enzyme digestion

For the purification of the amplified target gene, the restriction enzyme EcoRI (TaKaRa Bio Inc., Japan) was used to digest the specific sites in front of the target gene. To visualize the amplified target gene on the agarose gel, 50 PIN particles were used for the amplification. After amplification of target gene, the PIN particles were washed with TE buffer containing 0.15% Tween-20 three times to remove the PCR reagent and non-bound primers. Then the buffer was changed to deionized water for EcoRI treatment. The enzyme digestion was conducted at 37 °C for 2 h. The amplified gene was detected in 3% agarose gel by electrophoresis.

3. Results and discussion

3.1. Primer-immobilized network(PIN) RT-qPCR

We quantitated rare Bcr-Abl fusion transcripts among normal genes and confirmed the sequence conveniently. As shown in Fig. 1(A), the process is as follows. i) We immobilized the reverse primers for reverse transcription (RT) to PIN particles. ii) The target RNAs selectively bound to the RT primers along with nonspecific adsorption of total RNA to the PIN particles. iii) The nonspecifically-bound RNA was rinsed off with agitated buffer. iv) The target RNA was reverse transcribed to the cDNA template fixed in

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