Enriched high-throughput reverse transcription-quantitative PCR template preparation without pre-amplification

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Abstract. A cDNA template with a high concentration is required to generate a high number of copies for accurate downstream high-throughput reverse transcription-quantitative PCR screening. However, with the traditional method, pre-amplification is not widely available. In the present study, a novel strategy to resolve the pre-amplification limitation has been developed. Total RNA was extracted using a commercially available RNeasy Micro kit then, the cDNA was synthesized using SuperScript® III First-Strand Synthesis system. PCR inhibitors (proteins and soluble salt ions) in the enriched cDNA were removed using saturated phenol-chloroform extraction. Finally, genes were evaluated using PCR amplification and the BioMark™ HD system. The positive detection rate of individual target gene expression reached 70.18%; however, it markedly decreased to 35.42% using PCR amplification without prior dilution. Next, the reverse transcription product was purified using saturated phenol-chloroform extraction, and the positive detection rate increased to 97.04%. Notably, the positive detection rate of cDNA prepared using this method of high-throughput and traditional PCR (97.04 vs. 96.6%) was not significantly different. In conclusion, the results demonstrate the novel method was an easy and reproducible method for performing robust and highly accurate targeted amplification.

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Introduction

At present, high-throughput reverse transcription-quantitative PCR (RT-qPCR) allows for the detection and quantification of small amounts of DNA, even individual molecules, in an accurate and quantitative manner (1). However, limited sample sizes of rare tissues, liquid biopsies, fine-needle aspirates, and single cells have been the bottleneck of research studies and clinical assessments based on DNA and RNA analyses (2-4). The amplification reaction fails for limited samples and poor cDNA templates. Thus, researchers urgently require an easy and reproducible method to prepare available cDNA for high-throughput qPCR screening.

Pre-amplification is the most common strategy for the enrichment of target cDNA templates (5). Pre-amplification, multiplex PCR with specific primer pairs (6), can target all DNA in an unselective manner (7) and specifically target only genes of interest (8-12). The formation of non-specific PCR products and the competition of reagents between the parallel reactions limit the application of pre-amplification during template enrichment (13). Fewer cycles and lower primer concentration will reduce the limitation of pre-amplification. However, despite its wide application, targeted pre-amplification during DNA template quantification, particularly its properties and characteristics, such as templates and dNTP mix concentrations, is poorly understood (14). The process is still a time-consuming and it is expensive to amplify specific primer pairs during multiplex PCR. Furthermore, the whole process is poorly repeatable (15).

Heterogeneity of all types of cancer leads to differences in the sensitivity of patients to chemotherapy drugs (16). As the cost decreases, to achieve precision medicine, RNA-sequencing in individual patients is possible in the future. The results of sequencing require further verification using conventional PCR. However, it is impossible to verify thousands of differentially expressed genes in individual patients using traditional PCR. Therefore, high-throughput PCR, with enough cDNA template, may provide a suitable method to be used for precision medicine of tumors.

In the present study, a commercially available RNeasy Micro kit (Qiagen GmbH) was used to improve the quality of total RNA extracted from cultured cells. Saturated phenol-chloroform extraction was also used to remove PCR

inhibitors in the samples. The high-throughput qPCR was performed using the BioMark™ HD system. Notably, the aforementioned workflow was used to verify peripheral blood mononuclear cells (PBMC) separated from blood cells in patients infected with Hepatitis B virus. Using the novel method, an easy and reproducible strategy was developed to prepare cDNA templates for high-throughput qPCR screening using the BioMark™ HD system.

Materials and methods

Cell lines. All cell lines, which were purchased from the China Center for Type Culture Collection, were cultured at 37˚C in a humidified incubator with 5% CO_2. Liver cancer cell lines, HepG2 and Hep3B, were authenticated using STR profiling and cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Thermo Fisher Scientific, Inc.).

Total RNA extraction using TRIzol®. Cells were washed three times with cold PBS. To avoid fragmenting DNA, harvested cells were directly lysed with 800 μl TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and homogenized gently using a pipette. The lysate was added to 160 μl chloroform and mixed thoroughly. After incubation at room temperature for 15 min, the mixture was centrifuged at 12,000 x g at 4˚C for 15 min. The RNA was transferred to a fresh RNase-free centrifuge tube and mixed with 200 μl isopropanol at room temperature for 10 min. The total RNA was collected and centrifuged at 12,000 x g for 10 min at room temperature. The RNA precipitate was washed with 800 μl 70% ethanol, and re-precipitated and centrifuged at 8,000 x g for 5 min at 4˚C. After diluting the sample in 10 μl RNase-free water, the total RNA (~600 ng/μl) was stored at 80˚C until further experimentation.

Total RNA extraction using RNeasy Micro kit. Total RNA was extracted from cultured (HepG2 and Hep3B, 80% confluence in 6-well tissue culture plate) cells and PBMCs ($1x10^6$) using a RNeasy Micro kit (Qiagen GmbH) according to the manufacturer's protocol. Briefly, harvested cells were directly lysed with 350 μl RLT buffer containing 1% β-mercaptoethanol (Sigma-Aldrich; Merck KGaA) and homogenized using a pipette. To precipitate mRNA, 70% ethanol was added to the cell lysates, and mixed by pipetting. Then, the sample was transferred to a RNeasy MinElute spin column in a 2 ml collection tube and centrifuged immediately for 15 sec at 8,000 x g at 4˚C. After the flow-through was discarded, the collected mRNA was washed with 350 μl Buffer RW1 and centrifuged for 15 sec at 8,000 x g at 4˚C. The DNA in the sample was digested with 80 μl DNaseI solution for 15 min at room temperature. After washing with Buffer RW1 and centrifuging for 15 sec at 8,000 x g at 4˚C, 500 μl 80% ethanol was added to completely wash the sample. The RNeasy MinElute spin column was centrifuged in a new 2 ml collection tube at 13,000 x g for 5 min at 4˚C to dry the membrane. RNase-free water (14 μl) was added directly to the center of the spin column membrane to elute the total RNA, which was stored at -80˚C until further use.

cDNA synthesis. RT was performed using the SuperScript® III First-Strand Synthesis kit for RT-qPCR (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, each component was mixed and centrifuged at 2,000 x g for 15 sec at 4˚C before use. Random hexamer primers (5 ng/μl), dNTP mix (1 mM), total RNA (≤2.5 μg), and RNase-free water were added to a final volume of 5 μl. Samples were denatured at 65˚C for 5 min and subsequently cooled on ice at least for 2 min. The following reagents were added to a total volume of 10 μl: SuperScript® III (10 U), RNaseOUT (2 U), $MgCl_2$ (5 mM), DL-Dithiothreitol (10 mM) and RT buffer. The following temperature protocol were used: 25˚C for 10 min, 50˚C for 60 min, 85˚C for 5 min and 4˚C to infinity. cDNA was diluted 1, 5, 10 and 20 times in diethyl pyrocarbonate (DEPC)-treated water and stored at -20˚C, until further use.

Removal of PCR inhibitors and cDNA template enrichment. Saturated phenol was used to remove proteins in the diluted cDNA, and chloroform was used to remove the phenol dissolved in water. Briefly, 200 μl cDNA was added with an equal volume of the saturated phenol-chloroform mixture (ratio, 25:24), incubated on ice for 10 min and centrifuged at 12,000 x g for 10 mins at 4˚C to separate the cDNA and protein. The upper aqueous phase was transferred to a fresh 1.5 ml centrifuge tube. To precipitate the cDNA from the aqueous phase, 2 μg glycogen and 500 μl ethanol was added to the aqueous phase and the solution was stored at -80˚C for 8 h. The sample was centrifuged at 14,000 x g for 30 min at room temperature to separate the cDNA precipitate. After washing with 1 ml 70% ethanol, the cDNA was centrifuged for a final time at 10,000 x g for 5 min at room temperature. The enriched cDNA was then diluted in 10 μl DEPC-treated water and stored at -20˚C.

High-throughput qPCR. High-throughput qPCR was performed using the BioMark™ HD system and the 48.48 or 96.96 Dynamic Array™ integrated fluidic circuit (IFC) for gene expression according to the manufacturer's protocol (Fluidigm Corporation). Briefly, control line fluid was injected into each accumulator of the IFC. After the blue film was removed from the bottom of the IFC, the primer script was run in the instrument. For 10X assay preparation, 1.5 μl primer (10 μM; Shanghai Sangon Pharmaceutical Co., Ltd.), 1.5 μl probe (10 μM; Shanghai Sangon Pharmaceutical Co., Ltd.), and 2X assay loading reagent (Fluidigm, Corporation) were mixed together. All primers used are listed in Table SI. For the pre-mix preparation, 3 μl TaqMan Universal PCR master mix (2X; Thermo Fisher Scientific, Inc.), 0.3 μl 20X GE sample loading reagent (Fluidigm Corporation), and 2.7 μl enriched cDNA were mixed together. The primed IFC was removed from the instrument and 5 μl 2X assay and pre-mixed sample was pipetted into the assay and sample inlets, respectively. The following thermocycling conditions were used: 50˚ for 2 min, pre-denaturation at 95˚C for 1 min, denaturation at 95˚C for 15 sec, annealing at 56˚C for 30 sec, elongation at 72˚C for 50 sec, for 50 cycles, then the samples were held at 4˚C forever. Amplification data were analyzed using the Biomark Real-Time PCR analysis software version 1.3 (Fluidigm Corporation). The housekeeping genes, GAPDH and ACTB, served as internal controls. For quality control, in each test, a positive and a negative control was used, which was provided by the supplier (Fluidigm Corporation). If none of the

96 samples detected the result, then it was sufficient evidence that there was a problem with the detector. On the contrary, if none of the 96 genes detected the result, then there was have sufficient evidence that there was an issue with the sample.

Conventional qPCR. As performed in our previous study (17,18), the primer and probe [ABL proto-oncogene 1 (ABL1), cyclin dependent kinase inhibitor 1B (CDKN1B), CyclinA2, tissue inhibitor of matrix metalloproteinase (TIMP-1) and cyclin dependent kinase 7 (CDK7)] mixture solutions were prepared by adding 10 μM forward primer (2 μl), 10 μM reverse primer (2 μl), 10 μM probe (2 μl), and double-distilled (dd)H_2O (14 μl). The qPCR reaction solution was prepared with 2X TaqMan Universal PCR Master Mix (4 μl), cDNA diluted in DEPC-treated water (1 μl), ddH2O (1 μl), and primer and probe mixture solution (2 μl). The qPCR was run using a 384-well system using the aforementioned conditions. All primer and probes used are listed in Table SI.

Patients. A total of 21 residual whole blood samples (2 ml), collected from 4 patients infected with Hepatitis B virus, were obtained from the Clinical Laboratory of Beijing YouAn Hospital (Beijing, China). The Ethical Committee of Beijing YouAn Hospital, Capital Medical University, approved all studies (approval no. 2018011) and written informed consent was provided from all patients prior to the start of the study. The study methodologies conformed to the standards set by the Declaration of Helsinki. There were a total of four patients, three male and one female, which were between the ages of 43 and 67 years old. All samples were collected in May 2019.

PBMCs separation. Peripheral blood, 2 ml, was collected into heparin anticoagulation tubes and centrifuged at 500 x g for 10 min at room temperature. The cell pellet was diluted with an equal volume (~0.8 ml) of 1X PBS and mix gently with a disposable plastic dropper. A total of 4 ml lymphocyte isolate (Beijing Solarbio Science and Technology Co., Ltd.), was added to a fresh 15 ml centrifuge tube, following which the blood cells were added, gently down the side of the tube, on top of the lymphocyte separating fluid. After centrifugation at 500 x g for 10 min at 4˚C, PBMCs were removed into a fresh centrifuge tube and washed with 1X PBS, twice. The cell pellet was centrifuged in between each wash with PBS. The collected cell pellet was resuspended in 3 ml red blood cell lysis buffer to lyse red blood cells. PBMCs were washed with 1X PBS twice and centrifuged at 500 x g for 5 min at 4˚C to obtain the cell pellet.

Statistical analysis. Differences between groups were analyzed using Pearson's χ^2 test in SPSS v17.0 (SPSS, Inc.) for Windows. All experiments were repeated three times, the relative gene expression is presented as the Cq value and the positive rate is presented as a percentage. $P<0.05$ was considered to indicate a statistically significant difference. Heat maps were constructed using HemI v1.0 (19).

Results

Limited cDNA template without pre-amplification. To prepare cDNA for high-throughput qPCR, the total RNA was extracted from HepG2 and Hep3B cells, followed by RT using SuperScript® III First-Strand Synthesis kit. The synthesized cDNA was diluted 20X DEPC-treated water. The standard temperature profile was performed using the BioMark™ HD system for the Cq value of target gene expression. However, only 56% of the 9,216 tests were detected using the Biomark™ HD (Fig. 1A). After the samples and detectors (primers and probes), which had failed completely were removed from the total number of samples, 70.18% of 7,329 tests were detectable (Fig. 1B). Notably, target genes failed to be detected in samples when the Cq values of housekeeping genes were >20 (Fig. 1A). Taken together, the low positive detection rate in the tested samples suggested that cDNA without pre-amplification was limited due to its limited template with high-throughput qPCR in the BioMark™ HD system. Thus, the aim of the present study was to identify an easy and reproducible strategy that enriches the cDNA template for high-throughput screening.

BioMark™ HD system fails to detect the target by directly reducing the dilution factor. To prepare a high concentration of cDNA template for high-throughput qPCR, the synthesized cDNA from HepG2 and Hep3B cells was diluted 1, 5, 10 and 20 times in DEPC-treated water for a serial gradient of cDNA. The standard temperature profile was performed as aforementioned for 48.48 Dynamic Array™ IFC. Consistently, in the 20-fold diluted cDNA, only 75.17% of 576 target samples were analyzed using the BioMark™ HD system (left, lines 35-46; Fig. 2A). By contrast, the 10-fold diluted cDNA exhibited a positive detection rate (74.83% of 576 tests), which did not change markedly (left, lines 23-34; Fig. 2A). However, in the 1- and 5-fold diluted cDNA samples, the positive detection rate decreased to 35.42% in 480 tests (left, lines 1-10; Fig. 2A) and 50.35% in 576 samples (left, lines 11-22; Fig. 2A), respectively. Taken together, it indicates that increasing the dilution factor increased the positive detection rate; however, PCR inhibitors (proteins and soluble salt ions) in the sample inhibited the subsequent PCR amplification. The PCR amplification requires a relatively high concentration of template but lower levels of PCR inhibitors (20).

Removal of PCR inhibitors using phenol-chloroform extraction. To remove proteins in the samples, an equal volume (~200 μl) of saturated phenol-chloroform mixture was mixed with the aforementioned 20x diluted cDNA. The high concentration of soluble salt ion was removed using centrifugation after being stored on ice for 10 min. The cDNA pellet was diluted in 10 μl DNase-free water. The standard temperature profile was performed as aforementioned using the 96.96 Dynamic Array™ IFC. Notably, 70.11% of 9,216 target samples were analyzed using the Biomark™ HD system (Fig. 3A). After samples and detectors which had failed were removed, the positive detection rate increased to 90.28% in 7,138 samples analyzed (Fig. 3B). Thus, following removal of PCR inhibitors (proteins and soluble ions), a higher number of cDNA samples were analyzed using high-throughput qPCR screening and the BioMark™ HD system.

High quality total RNA prepared using RNeasy Micro kit. The aforementioned results revealed that saturated phenol-chloroform extraction markedly improved the cDNA

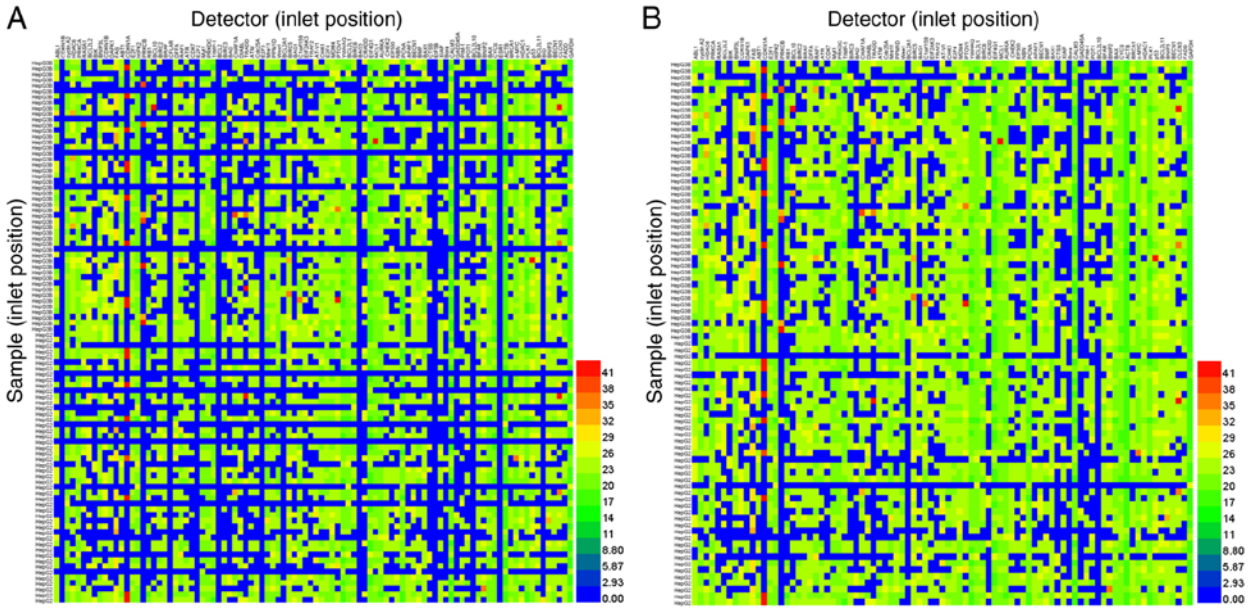

Figure 1. Results for high-throughput reverse transcription-quantitative PCR screening in BioMark™ HD system using the 96.96 Dynamic Array™ integrated fluidic circuit. (A) The top row shows the genes amplified in the assay, while the samples on the left-hand side are from Hep3B (1-48) and HepG2 (49-96). (B) The detectors that had failed were removed and the top row shows the genes that were successfully amplified, while the samples that had failed were also removed and those on the left-hand side were the successfully amplified Hep3B (1-43) and HepG2 (44-84) samples. Each square corresponds to an individual quantitative PCR. Cq values for each amplification reaction are indicated by color. Negative results are indicated using the blue color.

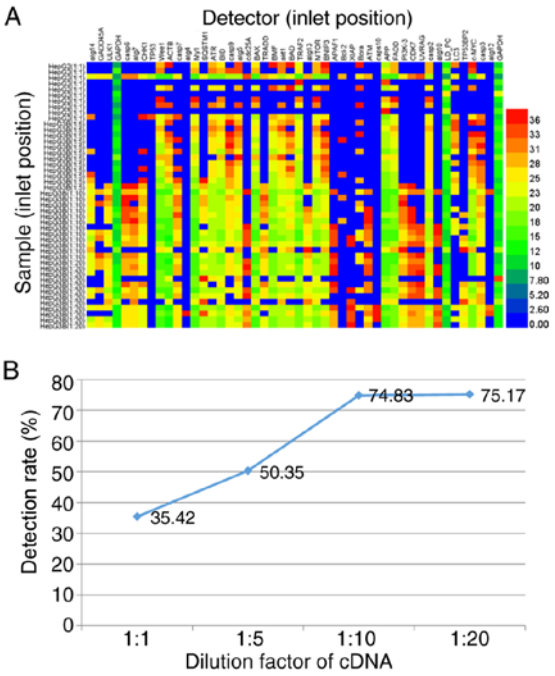

Figure 2. Results of dilution factors for high-throughput reverse transcription-quantitative PCR screening in BioMark™ HD system using the 48.48 Dynamic Array™ integrated fluidic circuit. (A) The top row indicates the genes amplified in the assay, while the samples on the left-hand side are from HepG2 cells, diluted 1:1 (1-10) and Hep3B cells diluted 5x (11-22), 10x (23-34) and 20x (35-46) with water. Each square corresponds to an individual quantitative PCR. Cq values for each reaction are indicated by color. Negative results are indicated using the blue color. (B) Detection rate increased as the dilution factor increased, the horizontal axis represents the dilution factor of cDNA and the vertical axis represents the positive dilution rate.

templates for qPCR. However, as shown in Fig. 3A, the sample concentration (left, lines 24, 25, 49, 58-60, 70, 72, 82, and 84; Fig. 3A) was too small to be detected using the BioMark™ HD system. Factors from total RNA extraction using TRIzol® (i.e., protein pollution in RNA separation and precipitation of soluble salts in RNA centrifugation) limited the subsequent qPCR reaction. A commercially available RNA extraction kit (RNeasy Micro kit; Qiagen GmbH) was used for an easy and reproducible RNA extraction. After dilution in 10 μl DEPC-treated water, the standard temperature profile was performed aforementioned for 96.96 Dynamic Array™ IFC. Notably, 86.09% of 5,148 tests were analyzed using the BioMark™ HD system (Fig. 4A). After detectors which had failed were removed (top, line numbers 12, 17, 22, 24, 36, 48, 57, 60, 71, 72, and 78; Fig. 4A), the positive detection rate increased to 97.04% in 4,590 samples analyzed (Fig. 4B). To compare the results of BioMark™ qPCR and conventional RT-qPCR, the same samples and 5 detectors (ABL1, CDKN1B, CyclinA2, TIMP-1 and CDK7) were added to a 384-well plate for conventional qPCR. As shown in Fig. 4C, the positive detection rate increased to 96.6% in 270 tests. Thus, there was no difference between BioMark™ HD system and conventional qPCR (97.04 vs. 96.6%); however, the gene expression detected by the new method was higher compared with that in conventional PCR. Taken together, the results showed that using a combination of the commercially available RNA extraction kit from Qiagen GmbH and saturated phenol-chloroform extraction, cDNA sample preparation was easy and reproducible for high-throughput qPCR screening using the BioMark™ HD system.

Preparation of cDNA template from PBMCs. As aforementioned, the cDNA template preparation was easy and reproducible. Furthermore, the assay was performed in cultured cell lines, therefore the same method was used with cDNA prepared from a limited PBMC sample to determine

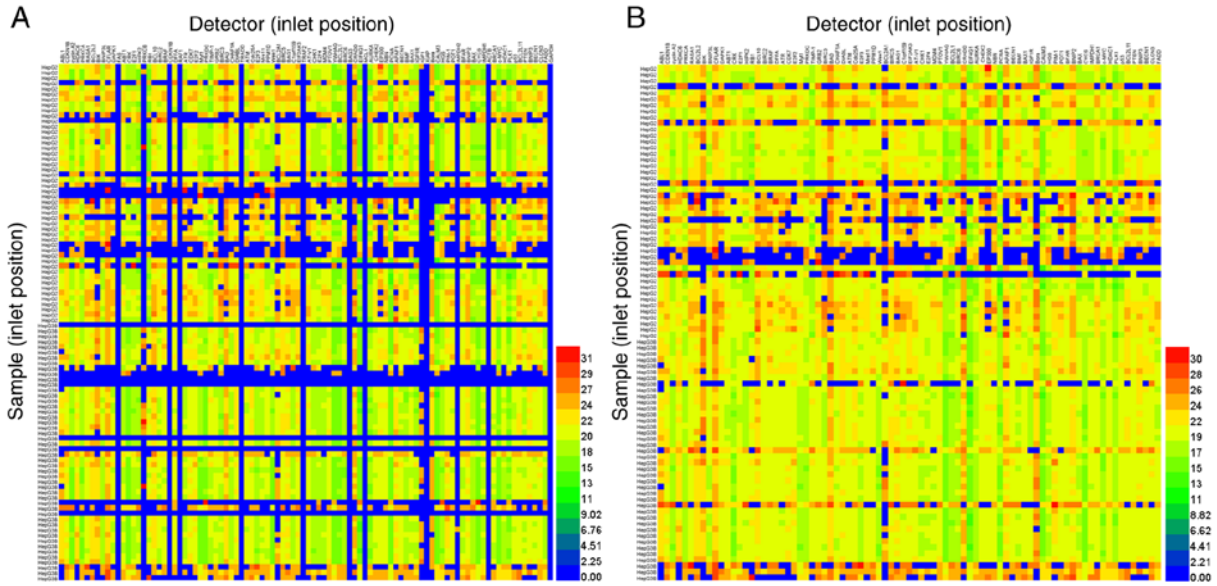

Figure 3. Results of total RNA extraction using TRIzol® for high-throughput reverse transcription-quantitative PCR screening in BioMark™ HD system using the 96.96 Dynamic Array™ integrated fluidic circuit. (A) The top row indicates the genes amplified in the assay, while the samples on the left-hand side are from HepG2 (1-48) and Hep3B (49-96) cells. (B) The detectors that had failed were removed and the top row indicates the genes that were successfully amplified, while the samples that had also failed were removed and those on the left-hand side were the successfully amplified HepG2 (1-45) and Hep3B (46-85) samples. Each square corresponds to an individual quantitative PCR. Cq values for each reaction are indicated by color. Negative results are indicated using the blue color.

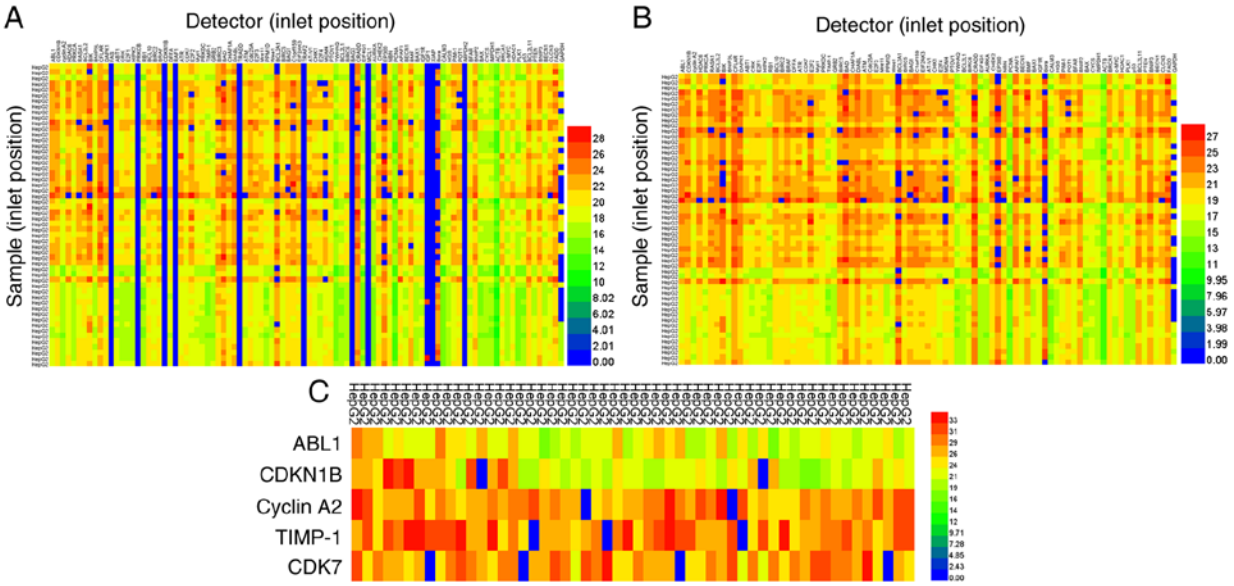

Figure 4. Results of total RNA extraction using RNeasy Micro kit for high-throughput reverse transcription-quantitative PCR screening in BioMark™ HD system using the 96.96 Dynamic Array™ integrated fluidic circuit and conventional qPCR. (A) The top row indicates the genes amplified in the assay, while the samples on the left-hand side were from HepG2 cells. (B) The detectors which had failed were removed and the top row indicates the successfully amplified genes in the assay, while the samples on the left-hand side were from HepG2 cells. (C) The genes, ABL1, CDKN1B, CyclinA2, TIMP-1 and CDK7 were amplified using conventional qPCR assay and the QuantStudio™ Real-Time PCR System in HepG2 cells. Each square corresponds to an individual qPCR. Cq values for each reaction are indicated by color. Negative results are indicated using the blue color. q, quantitative.

its suitability with high-throughput qPCR screening using the BioMark™ HD system. The residual blood samples were obtained from patients with Hepatitis B virus, recruited at the clinical laboratory of Beijing YouAn Hospital (Beijing, China). Following extraction from the blood cells, cDNA templates from PBMCs were prepared as aforementioned. After dilution in 10 μl DEPC-treated water, the standard temperature profile was performed as aforementioned for 96.96 Dynamic Array™ IFC. A total of 70.4% of 2,016 tests were analyzed using the BioMark™ HD system (Fig. 5A). After the detectors, which had failed detectors (top, line numbers 34, 45, 62, 71, 87, and 94) and samples (left, line numbers 13, 14, and 21) were removed, the positive detection rate increased to 81.55% of 1,729 samples (Fig. 5B). Taken together, in addition to cultured cells, PBMCs were suitable for cDNA sample preparation for high-throughput qPCR screening using the BioMark™ HD

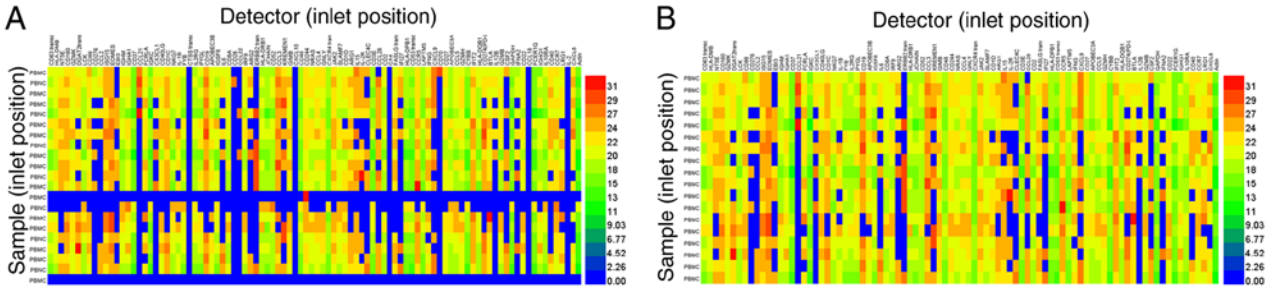

Figure 5. Results of PBMC for high-throughput reverse transcription-quantitative PCR screening in BioMark™ HD system using the 96.96 Dynamic Array™ integrated fluidic circuit. (A) The top row indicates the genes amplified in the assay, while the samples in the left-hand side are from PBMCs extracted from patients infected with Hepatitis B virus. (B) The failed detectors were removed and the top row indicates the genes that were successfully amplified in the assay, while the failed samples were also removed and those on the left-hand side were the successfully amplified PBMCs. Each square corresponds to an individual quantitative PCR reaction. Cq values for each reaction are indicated by color. Negative results are indicated using the blue color. PBMC, peripheral blood mononuclear cell.

system, using a combination of a commercially available RNA extraction kit (Qiagen GmbH) and saturated phenol-chloroform extraction.

Discussion

Limited sample amounts are increasingly used in laboratory research and in clinical laboratories. At present, various analytes, such as protein, RNA and DNA, can be accurately analyzed and quantified, even from an individual single cell (21-23). Next-generation sequencing and qPCR are emerging as the two most commonly used techniques to analyze mRNA sequence and expression levels, respectively (24). However, pre-amplification is typically required to increase the template of limited samples (25). The pre-amplification step is not necessary when few genes (≤10), intermediately or highly expressed, are to be accurately analyzed (26). When analyzing only one gene, pre-amplification should be avoided as the conventional qPCR method is sufficient (27). In the present study, in the cultured cell lines, HepG2 and Hep3B, individual gene expression failed to be detected using high-throughput q-PCR in Biomark™ HD. Target gene expression of a sample with a Cq value >20 for the housekeeping genes had poor detectability, which may be due to the limited amount of the template. Therefore, template concentrations were increased by reducing the dilution factor. The cDNA prepared using the novel method showed no difference in the positive detection rate compared with that using the Biomark™ HD system; however, the Cq value was higher. The possible reason may be that some cDNA templates were lost. In addition, when the PCR inhibitor was removed, success of PCR depends on the concentration of the cDNA template.

However, the presence of PCR inhibitors (e.g., blood, aqueous and vitreous humors, heparin, ethylenediaminetetraacetic acid, urine, polyamines, and plant polysaccharides) are common limiting products in PCR-based methods and can lead to failed amplification (28-31). By reducing the dilution factor, it was found that the cDNA template concentration increases. However, the PCR amplification reaction was still inhibited due to the presence of PCR inhibitors.

There are 4 common methods for removing PCR inhibitors in samples, including the Power Clean® DNA clean-up kit (MO BIO Laboratories, Inc.; Qiagen, Inc.), DNAIQ™ System (Promega Corporation), Chelex 1-100 method (Sigma-Aldrich; Merck KGaA), and phenol-chloroform extraction (Tiangen Biotech Co., Ltd.) (32-35). To remove PCR inhibitors and increase the concentration of the cDNA template, it was found that secondary extraction using saturated phenol-chloroform for the library preparation of cDNA could be used to analyze mRNA quantification for high-throughput qPCR screening using the BioMark™ HD system. Although saturated phenol chloroform extraction was added here, pre-amplification was avoided in high-throughput qPCR, which makes the widespread use of high-throughput qPCR screening using the BioMark™ HD system possible. Importantly, the positive detection rate of individual target gene expression was increased to 90.28% (Fig. 3B).

Total mRNA extraction is an important process that influences the RT-qPCR reaction. In addition to the amount of mRNA in cells, the efficiency of RNA extraction may also have a significant impact on the PCR template. Some common methods to extract mRNA from samples include phenol (Tiangen Biotech Co., Ltd.), anionic detergent, LiCl-urea (LiCI, 3 M; urea, 6 M; NaOAc, 10 mM), modified Gomez, bismuth isothiocyanate (Amresco, LLC), cetyl trimethylammonium bromide (Amresco, LLC), modified or conventional hot boric acid (Chemical Book), and TRIzol® reagent rapid extraction (36-40). The results in the present study revealed (Figs. 1 and 3) that although TRIzol® reagent rapid extraction is currently widely used in laboratories, in order to avoid contamination of phenol and protein this method requires an experienced experimenter. Thus, the reproducibility of the results are unpredictable, which is why in the present study commercially available kits were used for RNA extraction. RNase in cells and the mRNA extraction process degrade mRNA, and therefore protein, DNA, and soluble salts can have a notable negative impact on the subsequent qPCR reaction (33,41). Finally, Trizol® prepares poor quality cDNA. Using the commercially available RNeasy Micro kit (Qiagen GmbH) good quality cDNA was prepared. As a result, almost none samples were below the detection limit (Cq values of housekeeping genes were >20), and the positive detection rate increased to 97.04%. Notably, in addition to the cultured cell lines, high-quality and high-throughput PCR cDNA samples were prepared using the novel strategy, and the positive detection rate of samples from PBMCs extracted from patients with

Hepatitis B virus infection was notably increased using this protocol (Fig. 5).

The novel method described in the present study produced an easy and reproducible method for template preparation and high-throughput qPCR, however, the quality and quantity of the sample were essential factors that could influence the final result. RNase is commonly found in the environment (42). Thus, once the permeability of the cell membrane changes, RNase in the environment can enter the cell and degrade mRNA rapidly (43). Therefore, fresh or preserved samples at -80˚C are required. Unlike traditional pre-amplification, the concentration of the template was increased by reducing the dilution factor. Thus, the right number of samples, 80% confluent HepG2 and Hep3B cells in 6-well tissue culture plate or $1x10^6$ PBMCs, was required.

Normally, 2 days are required to perform all the experimental procedures. When cDNA was precipitated overnight at -80˚C, a total of 3 days was required. The traditional techniques could be completed in <2 days; however, the design of the pre-amplification primers can be a time-consuming and complicated process (44). Notably, the novel method may overcome the pre-amplification bias in cDNA template preparation, which was the primary reason for the development of a cDNA enrichment method. However, automatic procedures of for this method were not developed. At present, litter cDNA in the sample was not sufficient to recycle using the commercial kit, therefore glycogen was added to promote the precipitation of cDNA. However, using the principles in the present study, commercial kits could be developed in the future to achieve automatic cDNA preparation.

In summary, high-quality total RNA was repeatedly extracted using a commercially available RNeasy Micro kit (Qiagen GmbH). PCR inhibitors in samples were removed using saturated phenol-chloroform extraction. By decreasing the dilution factor, the positive detection rate for high-throughput qPCR screening in the BioMark™ HD system was increased to 97.04%. Notably, the easy and reproducible novel method is suitable for both cultured cell lines and PBMCs separated from blood cells. Therefore, large sample preparation would be possible for high-throughput qPCR screening using the BioMark™ HD system.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DC and YZ designed the study and wrote the manuscript. TY drafted the manuscript and prepared the cDNA. YO helped to operate the BioMark™ HD system and analyzed data. YG performed cell cultures. DL helped collect blood and performed PBMC separation. All authors read and approved the final manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of Beijing YouAn Hospital, Capital Medical University, (Beijing, China) approved all studies involving patients and informed consent was provided from all the patients prior to the start of the study (approval no. 2018011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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