A sensitive method for low input amount of exosomes in mouse model liquid biopsy using two-stage PCR

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Abstract

Background Recently, many potential non-invasive biomarkers have been developed using exosome-based liquid biopsy for early detection, diagnosis, and treatment of cancer. However, exosome analyses from small liquid biopsy samples have been limited with regard to sensitivity and efficiency.

Methods We used a breast cancer model mouse with ERBB2 overexpression and collected liquid biopsy samples. We performed two-stage PCR for ERBB2, PPP1R1B, GRB7, and STARD3 genes. We compared conventional PCR methods with our two-stage PCR method. Both methods had same step of primer and cycle and amplification condition for RT-qPCR. PCR amplicons quality was validated by using the Sanger method.

Results Quantitative analysis of all samples by conventional PCR compared with two-stage PCR template dilution (1/200) showed similar Ct values in all sample groups (cell, supernatant, urine, and plasma). Sequencing of the two-stage PCR-derived products revealed no changes in product sequence compared to that of conventional PCR. The obtained sequences showed 98-99% match of target gene sequences in the databases according to BLAST searches.

Conclusion Two-stage PCR has better sensitivity and efficiency than conventional PCR methods for small-volume samples. Additionally, this method is useful for monitoring, as many genes can be assayed at once.

Background

Liquid biopsy is considered one of the most advanced, non-invasive diagnostic systems with which to obtain key molecular information relevant to clinical decisions and practice of precision medicine. Recently, the discovery that exosome-based liquid biopsy may have diagnostic and therapeutic applications has garnered considerable interest.

Exosomes are vesicles of 30–150 nm in length that were first identified in the 1980s and diameter membrane small vesicles of multi-vesicular endosomal origin. Similar to cells, exosomes are composed of a lipid bilayer and, at any given point, can contain all known molecular constituents of a cell, including nucleic acids (DNA, mRNA, miRNA, long and short noncoding RNA), proteins (cytoskeletal proteins, transmembrane proteins, heat shock proteins), and enzymes (GAPDH, ATPase, pgk1). Exosomes have been detected in many biological fluids, including cell culture media, urine, semen, saliva, amniotic fluid, cerebrospinal fluid, bile, ascites, tears, breast milk, and blood. [1–3] Cancer exosomes may serve as a liquid biopsy to aid in diagnosis of malignancies, including prostate, pancreatic, breast, and ovarian cancers.[4–6] Development of exosome-based liquid biopsy as a non-invasive method for management of breast cancer has gained much attention and used to protein and/or miRNA suggests the possibility novel biomarker.

At present, the methodologies of isolation are mainly based on physical (differential centrifugation, ultrafiltration, size-exclusion chromatography), chemical (polymeric-based precipitation [PBP]), or biological (immune-affinity) properties of exosomes. Among these methods, differentiated ultracentrifugation was considered the gold standard for separating exosomes from the RNA contained therein.[7,8] Recently, studies have found new isolation methods of exosomes from liquid biopsy samples (urine, serum, breast milk, and plasma of blood),[9,10] and several products have been developed to reduce time and cost, increase reproducibility between laboratories, and efficiently concentrate exosomes.[11–14]

However, because mouse models are associated with complications in collecting liquid biopsy samples, such as small sample volumes due to mouse size, there has not been much research on exosomes in this field. In particular, gene expression analysis using mRNA in exosomes has been difficult. Therefore, instead of focusing isolation or concentration of exosome specimen, our study suggests using a two-stage PCR method consisting of an initial multiplex PCR, which produces amplicon-enriched products. These products are subsequently used as templates in a singleplex PCR, with the same primer pairs and cycling parameters as in the multiplex PCR.[15]

This method is more effective than conventional PCR and can advance molecular research of exosomes by solving the problem of low molecular yields. Furthermore, we analyzed gene expression levels of exosomal mRNA using quantitative real-time PCR.

Thus, presented data from this study will serve as a guide to optimizing low molecular yield biopsy exosome samples for desired applications in biomarker development, biological assays, and monitoring of health and disease.

Methods

- *Cell culture*

The human breast cancer cell line HCC1954 was purchased from KCLB (Korean Cell Line Bank) and maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and grown in 5% CO_2 at 37℃. Cells were seeded in 100-mm culture dishes at 2 x 10^6 cells/dish, maintained for 24 h in RPMI 1640 supplemented with 10% FBS, and washed with PBS and RPMI supplemented with serum-free medium. After 72 h, the medium was collected from the dishes and centrifuged at 300 x g for 10 minutes to remove cells. The supernatant was transferred to a new tube and centrifuged at 16,000 x g for 30 minutes to remove any remaining cell debris.

- *Mouse xenograft model*

HCC1954 cells (6 x10^6 per mouse) were injected into the mammary fat pads of female BALB/c mice. Tumor volume was calculated using the following formula: volume = (largest diameter x smallest diameter)^2 / 2. Mice were sacrificed when the tumor volume reached 1000 mm or was ulcerated.
We used mouse urine from collect up to 800 ul and plasma collect up to 200 ul for mouse blood. The

urine and plasma were centrifuged at 300 x g for 10 minutes to remove cells, and the supernatant was transferred to a new tube and centrifuged at 16,000 x g for 30 minutes to remove any remaining cell debris.

- *Exosome isolation*

We isolated exosomes using the Exo-spin™ Exosome Purification system (Cell Guidance Systems, St. Louis, MO) according to the manufacturer's instructions. The supernatant was collected from cell culture media to remove cell debris. Also, the mouse urine was filtered (0.2 um syringe filter) to remove cell debris. Plasma was separated from the mouse blood using centrifugation.

- *Extraction of total RNA and analysis*

Total exosomal RNA was isolated and purified using the MasterPureTM RNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's protocol. The RNA concentration was determined using absorbance measurements at wavelengths of 260 nm and 280 nm on a Nanodrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA). RNA quality was determined by capillary electrophoresis (Agilent 4200 Tapestation system, Agilent Technologies, Foster City, CA, USA). Exosomal total RNA was analyzed with Agilent Tapestation analysis software 3.1.

- *cDNA synthesis*

cDNA was synthesized from 200 ng total exosomal RNA using a High-Capacity RNA-to-cDNA kit (Applied Biosystems™, Foster City, CA, USA) according to the manufacturer's protocol.

- *The two-stage PCR protocol*

First stage: Each sample was amplified using multiplex PCR with the primer sequences shown in Table 1. All the primers had the same annealing temperature of about 60℃. The cycling parameters included 3 minutes of initial denaturation at 95℃; 13 cycles consisting of 30 seconds of denaturation at 95℃, 30 seconds of annealing at 60℃, and 30 seconds of extension at 72℃; followed by 5 minutes of final extension at 72℃. PCR was carried out using 20-ul volumes of pFU DNA Polymerase, 0.1 uM primers, and 40 ng cDNA template.

Second stage: The first-stage PCR products were diluted 1:200, and 2 ul of diluted PCR product was used with the same primers as in the singleplex PCR. The PCR cycles at 35 and using same condition from first stage PCR.

- *The conventional PCR protocol*

Each sample was amplified using singleplex PCR with the same primers (table1) and conditions as described for the second stage of PCR. We compared these results with those of the two-stage PCR protocol described above.

- *Quantitative real-time reverse transcription PCR*

Quantitative reverse transcription PCR was performed using a SensiFAST™ SYBR® Lo-ROX kit (Bioline, Luckenwalde, Germany), and PCR reactions were executed using a ViiA 7 Real-Time PCR System. PCR reactions were performed in triplicate, and samples were normalized to GAPDH expression.

- *Sequencing*
The PCR products were sequenced using the Sanger method. The sequences of the products from the conventional PCR and two-stage PCR methods were compared using chromatogram analysis and interpreted using CLC Main workbench 8.1.2 version software.

- *Transmission electron microscopy*
The isolated exosomes (20 ul) were fixed with 1% (v/v) glutaraldehyde in phosphate buffer solution (PBS) and dried at room temperature. After staining, samples were imaged by JEM-F200 TEM (Jeol Ltd., Tokyo, Japan).

Results

- *Exosome isolation and total RNA analyses*

Exosome isolation was performed using commercial kits for cell culture media and mouse model urine and blood. The size and shape of exosomes were confirmed by transmission electron microscopy (TEM). The TEM images of exosomes showed them to be contained in extracellular vesicles with a spherical shape of approximately 100-200 nm in size (Fig. 1 A). Qualitative RNA analysis showed the exosomal total RNA of supernatant (Blue), urine (Green), and plasma (Red) (Fig. 1 B).

- *Quality of the two-stage PCR method*
We tested the two-stage PCR template quality using the Sanger method. The two-stage PCR template sequences matched those of the conventional PCR amplicon sequences (Fig. 2 A). Also, when the obtained sequences were queried on BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), they showed a 98-99% match to the target gene sequences in the databases (Fig. 2 B).

- *Amplification efficiency between the conventional PCR and the two-stage PCR protocols*
We compared the amplification cycle threshold (Ct) values of four target genes (PPP1R1B, GRB7, ERBB2/HER2, and STARD3) and the reference gene GAPDH of conventional PCR and two-stage PCR template serial dilution (Table 2). We used 40 ng cDNA from the conventional PCR and 2 ul of the two-stage serial dilution templates of the 20 ng of cDNA from the first stage. The results showed that conventional PCR Ct values ranged from 24.3±0.0 to 26.1±0.1, with similar Ct values for the two-stage PCR template dilution (1/200).

- *Specificity of the two-stage PCR method using melting curve analysis*
Meting curve analysis showed a single melting peak without demonstrable amounts of primer dimers or nonspecific fragments, verifying primer sensitivity and sample quality. It was detected that some samples of the conventional PCR templates introduced error during the amplification (Fig. 3 A). The two-stage PCR protocol showed stable melting curves (Fig. 3 B), with similar results to serial dilution templates.

- *Quantitative RNA expression*
Quantitative real-time reverse transcription PCR (RT-qPCR) analysis of all samples compared

conventional PCR with two-stage PCR. The conventional PCR protocol used 20 ng cDNA, and the two-stage PCR protocol used 2 ul templates (1/200 dilution products of first-stage PCR). All samples (cell, supernatant, urine, and plasma) showed similar Ct values for conventional and two-stage PCR (Fig. 4 A). Using the stable reference gene GAPDH for RT-qPCR, relative gene expression levels were calculated. We showed that the target genes (PPP1R1B, GRB7, ERBB2/HER2, and STARD3) were the same across cell and supernatant groups but differed across the urine and plasma groups (Fig. 4 B). While the expression of target genes seemed to be lower with conventional PCR, the expression appeared to increase in the two-stage PCR, especially in the urine and plasma groups.

Discussion

This study sought to overcome the difficulties associated with using small amounts of exosomal mRNA in breast cancer research.

According to the WHO, cancer is a leading cause of death worldwide, with breast cancer being the fifth most common cancer and one of the most common malignancies in women.[16] Breast cancer is a heterogeneous disease with several sub diseases with differences in tumor biology, histopathology, and prognosis. About 20% of all breast cancers are characterized by amplification of 17q12, leading to overexpression of epidermal growth factor receptor 2 (ERBB2/HER2/neu).[17]

ERBB2 is a transmembrane tyrosine kinase with oncogenic potential and belongs to the epidermal growth factor (EGF) receptor family. Since identification of ERBB2 amplification in breast cancer, multiple genes have been reported to be co-amplified with ERBB2 and a 2 Mb region at the 17q12–21 and their relation to trastuzumab (Herceptin) resistance.[18] The 17q12-q21 region has been heavily implicated in breast cancer and was identified as a common region of amplification and overexpression in breast tumors.[19–21] In this study, we observed the expression levels of ERBB2, along with three genes (PPP1R1B, GRB7, and STARD3) that are commonly co-amplified with ERBB2, in breast cancers.

We designed a mouse model using breast cancer cell line HCC1954, in which the ERBB2 gene is overexpressed, to determine the degree of expression of ERBB2 co-amplified genes (Table 1).[22–26] One of the several recently developed systems to enrich exosomes efficiently from culture supernatant and liquid biopsy was used in our study.[27]

Electron microscopy confirmed that the structures studied were exosomes (Fig. 1 A). We determined the quality of the RNA collected from supernatant, urine, and plasma using the Agilent 4200 Tapestation system. All samples yielded RNA of equivalent size distribution and abundance (Fig. 1 B), indicating good-quality RNA including 18S and 28S rRNA peaks. In contrast, exosomal RNA differed in profile due to absence of two peaks. Since the algorithm is based on ribosomal RNA, little of which is contained in exosomes, RNA integrity number (RIN) values are only valid for cellular RNA quality assessments.[28–30] For this reason, RNA purity was evaluated spectrophotometrically at absorbances of 230, 260, and 280 nm (Table 2). Obtaining high-quality RNA is a critical step in many molecular techniques, such as RT-

qPCR and transcriptome analysis by next-generation sequencing or hybridization to microarrays. The quality and reproducibility of RNA sample preparation is crucial for diagnostic development, especially for clinical samples.[31]

Exosome isolation has limitations including difficult extraction of exosomal RNA from urine and plasma in mouse models. To overcome these shortcomings, we used a two-stage PCR method. To reduce PCR errors caused by primer dimers and/or nonspecific fragments, which may occur in multiplex-PCR amplification, we used Pfu-DNA polymerase with lower error rate than taq polymerase and limited the pre-amplification step of the first PCR to 13 cycles.[32, 33]

We compared the conventional PCR protocol with a two-stage PCR protocol using the same primers and cycle and amplification conditions. The quality values of the amplicons produced by the two-stage and conventional PCR were compared using the Sanger method. Sequencing of the two-stage PCR-derived products revealed no changes in product sequence compared to that of the conventional method, and the obtained sequences were queried using BLAST, which revealed a 98–99% match to the target gene sequences in the database (Fig. 2 B).

We compared the amplification cycle threshold (Ct) values of four target genes from the conventional PCR and the two-stage PCR templates serial dilution, and found similar Ct volumes of two-stage PCR template dilution (1/200).

Conventional PCR detected some sample errors introduced during amplification, detected from meting curve analysis. This suggests the presence of primer dimers or nonspecific fragments.[34] On the contrary, the two-stage PCR templates showed stable melting curves, is same results at serial dilution templates (Fig. 3).

In quantitative RNA expression analysis of all samples by conventional PCR compared to those of two-stage PCR template dilution (1/200), similar Ct volumes were observed for all sample groups (cell, supernatant, urine, and plasma) and same result of cell and supernatant groups. But it showed different aspect of urine and plasma groups in relative quantitation (Fig. 4). While the expression of target gene seems to have decreased in the conventional PCR, the expression of target gene appears to be increasing in the two-stage PCR, especially in the plasma group.

Conclusions

In conclusion, the two-stage PCR protocol was more sensitive than conventional PCR and was superior in amplifying exosome samples that often show insufficient yields. The presented data from this study will serve as a guide to choose and further optimize low molecular-yield biopsy exosome samples for applications in biomarker development and/or biological assays and monitoring of health and disease.

List Of Abbreviations

miRNA: micro RNA

mRNA: messenger RNA

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

ATPase: adenylpyrophosphatase

Pgk1: phosphoglycerate kinase 1

TEM: transmission electron microscopy

CT: cycle threshold

PPP1R1B: protein phosphatase 1 regulatory subunit 1B

GRB7: growth factor receptor-bound protein 7

ERBB2: receptor tyrosine-protein kinase erbB–2

HER2: human epidermal growth factor receptor 2

STARD3: StAR-related lipid transfer domain containing 3

cDNA: complementary DNA

RT-qPCR: quantitative real-time reverse transcription PCR

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Declarations

•Ethics approval and consent to participate

- All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee, Yonsei University Health System.

- All experiments involving the use of live animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees (IACUC) and the protocols 2016-0331 and 2019-0072 were approved by IACUC at the Yonsei University Health System.

•Consent for publication

- Not applicable

•Availability of data and materials

- Not applicable

•Competing interests

- The authors declare that they have no competing interests

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Consent for publication

Not applicable

Competing interests

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Figures

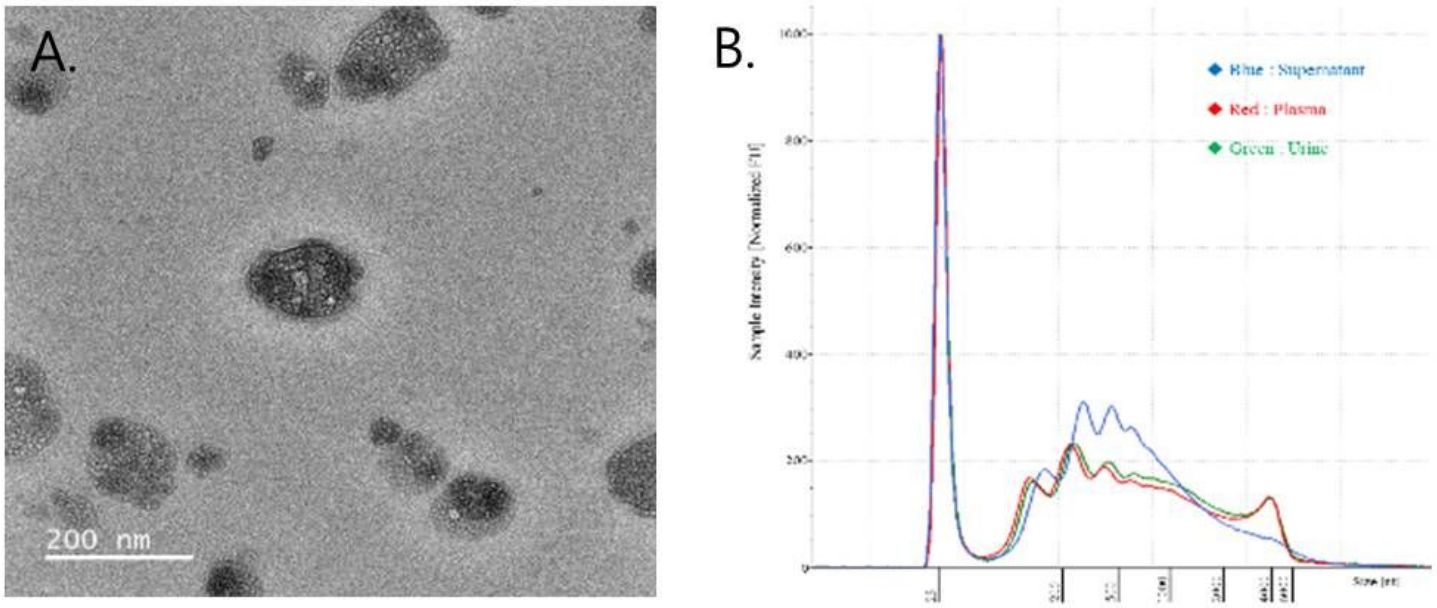

Figure 1) Exosome isolation and total RNA analysis.

A) Transmission electron microscopy of native exosomes isolated from HCC1954 cell media supernatant.

B) Quality of exosomal total RNA analysis using the Agilent 4200 Tapestation system. Representative electropherograms show the size distribution of exosomal RNA from supernatant (blue), plasma (red), and urine (green).

Figure 1

Exosome isolation and total RNA analysis

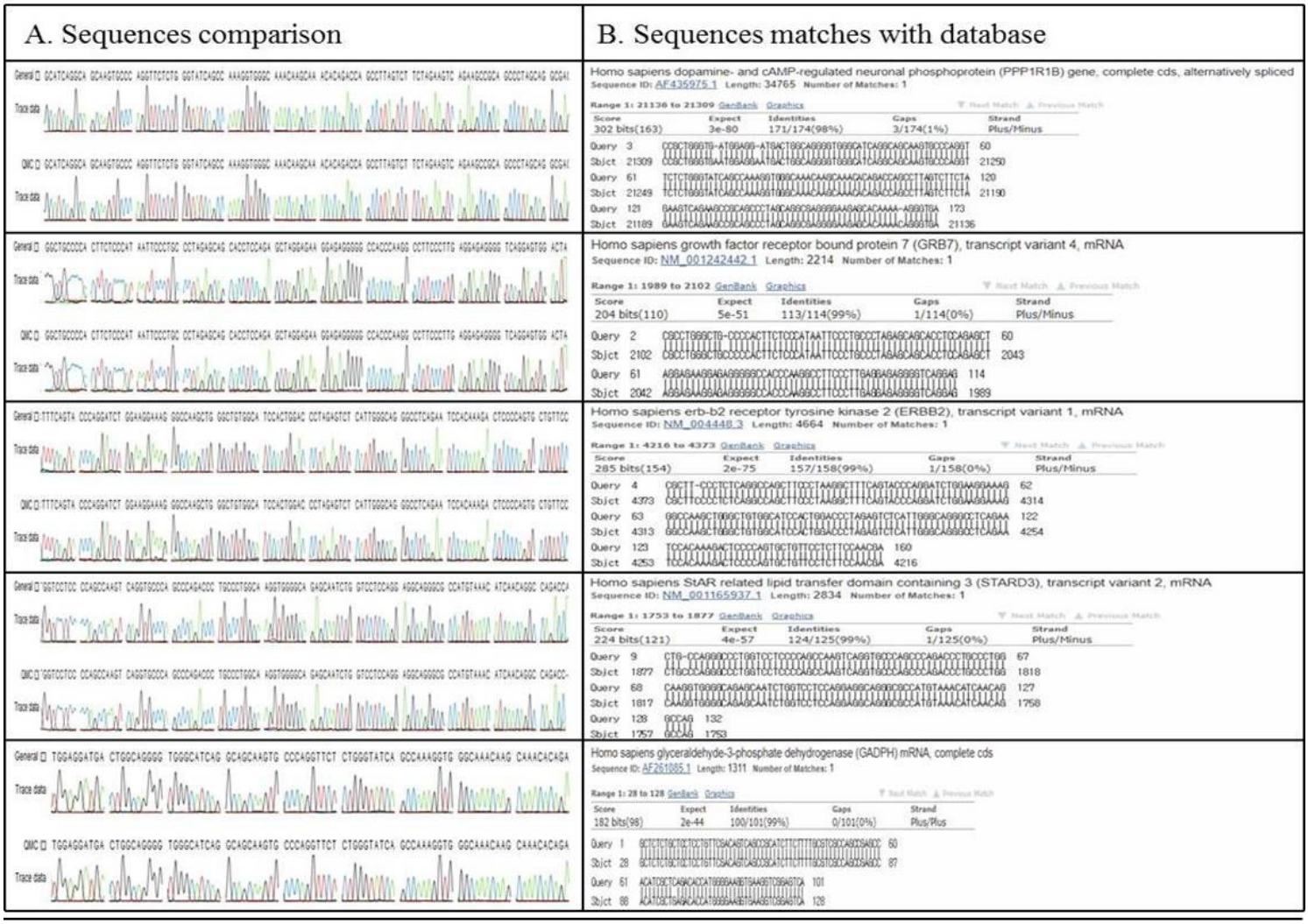

Figure 2) Sanger sequencing of conventional and two-stage PCR.

A) Comparison of conventional PCR and two-stage PCR product Sanger sequences.

B) BLAST analysis of the Sanger sequences shows matches with the database sequences (PPP1R1B, GRB7, ERBB2, STARD3, and GAPDH).

Figure 2

Sanger sequencing of conventional and two-stage PCR

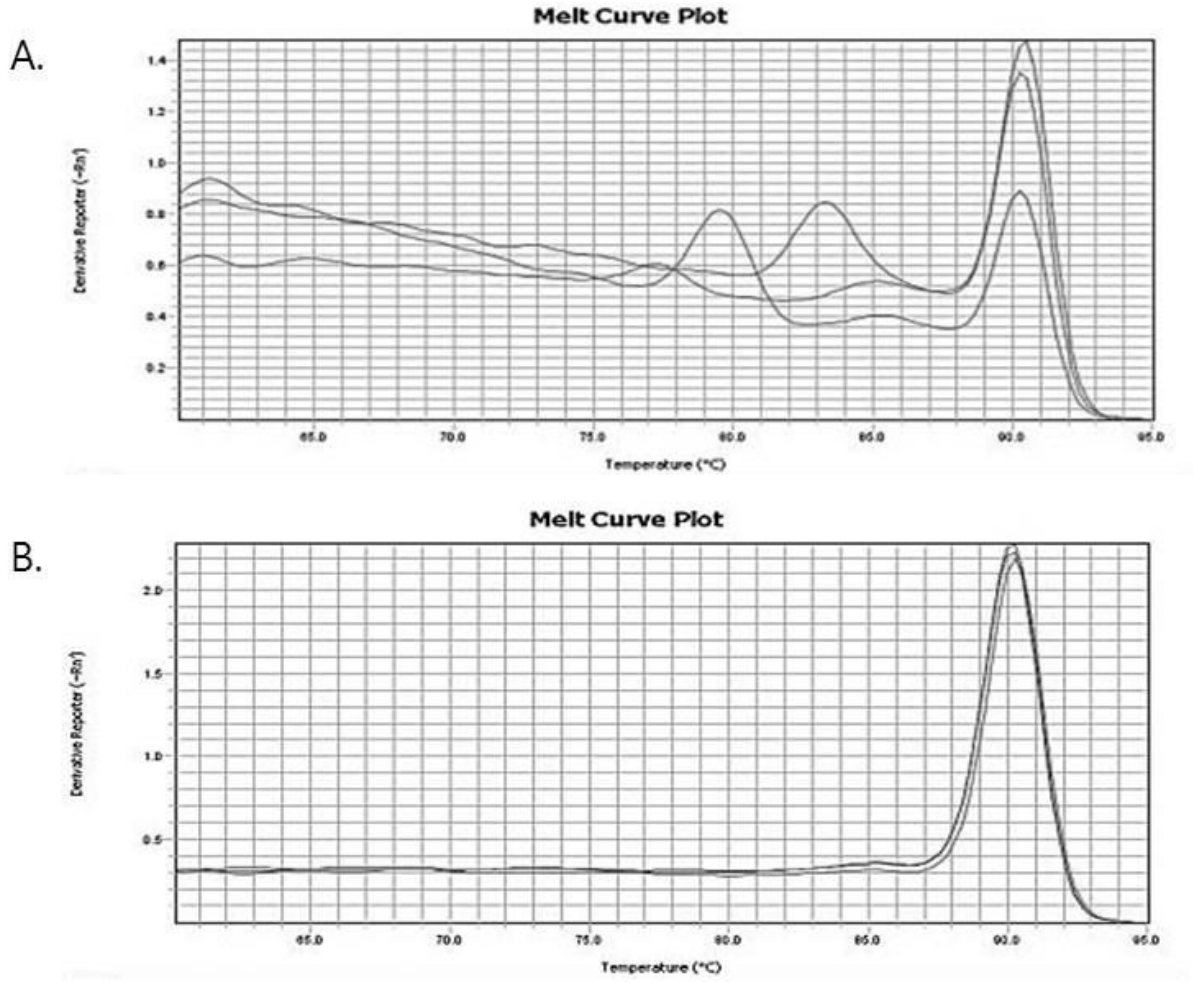

Figure 3) Melting curve analysis.

A) Melting curve analysis for the conventional PCR protocol has amplified un-sensitivity templates temperature, but B) the two-stage PCR protocol has amplified equal- templates temperature.

Figure 3

Melting curve analysis

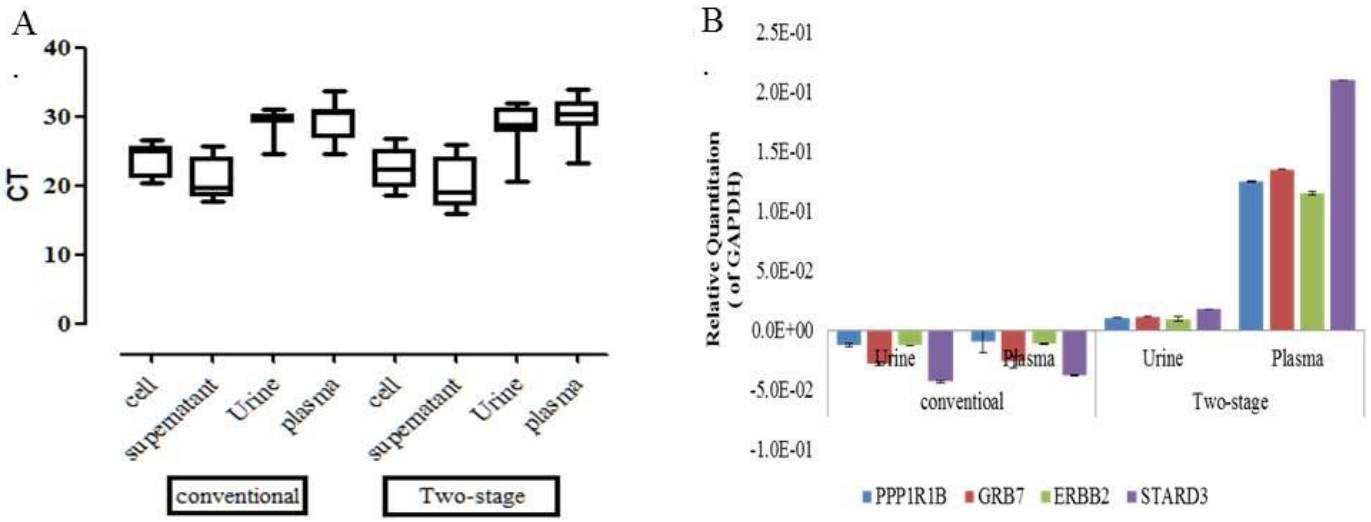

Figure 4) Quantitative testing of the quality of conventional PCR and two-stage PCR protocols regarding ERBB2 gene expression.

A) Expression of exosomal RNA of cell, supernatant, urine, and plasma for the conventional and diluted (1/200) two-stage PCR by RT-qPCR. Ct values are each transcripts, and data are presented as mean ± s.d ; n=12, p-values <0.3796, and ****<0.0001

B) The expression of target gene seems to have decreased in the conventional PCR, the expression of target gene appears to be increasing in the two-stage PCR, especially in the plasma group.

Figure 4

Quantitative testing of the quality of conventional PCR and two-stage PCR protocols regarding ERBB2 gene expression

Supplementary Files

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