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Research Article

OPTIMIZED RNA EXTRACTION FOR DETECTION OF BCR-ABL TRANSCRIPTS IN CHRONIC MYELOID LEUKEMIA PATIENTS DURING TREATMENT

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ABSTRACT

Objective: Monitoring of treatment response in chronic myeloid leukemia (CML) is difficult in leukopenia patients due to poor quality of blood cell RNA sample. Plasma RNA serves as an alternative for quantification of mutation gene but reproducibility depends on the quality and quantity of extracted RNA. The present study aimed to evaluate eight commercially available RNA extraction kits for detection of BCR-ABL transcripts in CML patients during treatment.

Methods: RNA extraction was carried out using eight types of commercially available kits on plasma samples of chronic myeloid leukemia patients on tyrosine kinase inhibitors therapy. Plasma samples of healthy individuals were used as controls.

Results: GeneAll® RiboEx™ with minor protocol modification demonstrated pure and sufficient amount of plasma RNA for quantification of ABL control gene and BCR-ABL mutation gene. This protocol produced a mean of 13.67±0.65 ng/µL plasma RNA whereas the purity indicator A260/280 ratio was 1.96±0.16 and A260/230 ratio was 2.07±0.12. As for gene expression analysis, 3561±392 copies of ABL control gene and 8.9±1.9 copies of BCR-ABL mutation gene were successfully amplified from 150 ng of plasma RNA.

Conclusion: GeneAll® RiboExTM with minor protocol modification produced purer plasma RNA extracts compared to other tested kits.

Keywords: Plasma RNA, Peripheral blood, Chronic myeloid leukemia, RNA extraction.

INTRODUCTION

There have been impressive advances in molecular testing to aid the diagnosis of cancers and treatment response monitoring. Free circulating nucleic acids in the peripheral blood cells and plasma are newly developed techniques in molecular field [1]. This technique is preferred over tissue biopsy because it is less invasive and convenient to obtain. Earlier studies have proven that plasma is rich in tumor-specific cancer genes [2], hence free circulating plasma nucleic acids is feasible for laboratory testing. For example, tumor suppressor gene TP53 and EGFR gene mutation were successfully identified in plasma DNA of ovarian cancer patients [3]; increment of EPAS1 and UBE2D3 genes was found in the plasma of colorectal cancer patients [4]; phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA), mediated complex subunit 1 (MED 1) and growth arrest specific 6 (GAS 6) and epidermal growth factor receptor (EGFR) mutation genes that cause therapy resistance were detected in the plasma DNA of relapsed breast, ovarian and lung cancer patients [5].

Nucleic acids in plasma sample offers ample opportunity for clinical translation. To date, plasma RNA sample has been used for prenatal diagnosis [6], detection of DNA-methylation changes [7], identification of viral infection using viral nucleic acids [8], and determination of tumor-induced microsatellite alteration [9]. However, the actual origin of free circulating RNA in plasma sample remains unclear. A few plasma RNA production mechanisms proposed earlier include active release of free circulating plasma RNA by tumor cells [10], produced from tumor cell necrosis [11] or apoptosis of proliferating cancer cells [12].

A majority of the RNA extraction kits available in the market is designed for blood cell specimens. Several commercial viral plasma RNA extraction kits may not be feasible for extracting plasma RNA for the analysis of mutation genes. This is either due to the lysis buffer used which produces low quantity of plasma RNA or the spin column's threshold is in efficient for capturing mutation genes. As a result, plasma RNA is hardly applied in molecular assays of mutation gene. Monitoring of treatment response in chronic myeloid leukemia (CML) is difficult in leukopenia patients. Poor quality of blood cell RNA sample remains as a common issue in diagnostic laboratories [13] which led to the suggestion of using plasma RNA as an alternative for quantification of mutation gene among CML patients [14]. Therefore, the present study aimed to evaluate eight commercially available RNA extraction kits and compare their quality and quantity for gene expression testing in plasma samples of CML patients during treatment.

MATERIALS AND METHODS

Study subjects

This study was approved by Medical Review & Ethics Committee (MREC), Ministry of Health, Malaysia and written informed consents were obtained from all subjects. Peripheral blood samples were collected from 80 CML patients and 20 healthy blood donors in ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged for 10 min at 1200 g to separate the plasma from blood cells. Pooled plasma specimens (n=10) were used for analysis using eight different types of RNA extraction kits.

Chemicals and kits

Eight RNA extraction kits available in the market were evaluated; namely TRIzol Reagent - Life Technologies, USA); E.Z.N.A.® Viral RNA Kit, Omega Bio-Tek, USA); PureLink® Total RNA Blood Kit (Life Technologies, USA); GeneAll® Hybrid-R™ blood RNA (GeneAll Biotechnology, Korea); NucliSENS® miniMag® (bioMerieux, France); ZR Whole-Blood RNA MiniPrep™ (Zymo Research, USA); QIAamp Viral RNA Mini Kit (Qiagen, USA); and GeneAll® RiboEx™ (GeneAll Biotechnology, Korea). The spin column for RNA extraction used was EconoSpin All-in-1 Mini Spin Columns (Epoch Lifescience, USA).

RNA extraction procedure

Plasma RNA samples were extracted according to the manufacturers' protocols except for GeneAll® RiboEx™ in which minor modification of the protocol was done as described. Approximately 0.75 mL of Ribo-Ex reagent was added to each 0.25 mL of plasma. The homogenate was mixed well, incubated at room temperature for 10 minutes, and centrifuged at 12000 g for 10 minutes at 4°C. The supernatant was collected and 0.2 mL of chloroform was added. The tube was then shaken vigorously for 15 seconds, incubated for 10 minutes at room temperature followed by centrifugation at 12000 g for 15 minutes at 4°C.

RNA which remained exclusively in the aqueous phase was aliquoted and 0.25 mL of 1.2 M sodium chloride solution was added to precipitate the RNAs. The sample was centrifuged at 12000 g for 30 seconds at 4°C in spin column. Ethanol solution (75%) was added into the spin column, centrifuged at 12000 g for 30 seconds at 4°C and the filtrate was discarded to remove salts and phenol from the RNA sample. This RNA purification process was repeated for four times. RNase-free water was incubated in spin column membrane for five minutes at room temperature and finally centrifuged for two minutes at 12000 g, 4 °C to elute the RNA sample.

Determination of RNA sample quality and quantity

The quality and the concentration of RNA sample obtained were determined on the Nanadrop spectrophotometer by measuring the absorbance at 260 nm. The presence of protein and contamination in RNA samples were measured at A260/280 and A260/230. RNA samples were kept in -80°C until further analysis.

Quantification of BCR-ABL to ABL ratio using real time-polymerase chain reaction

Rotor-Gene 6000 instrument and Rotor-Gene Q series software were used for RT-qPCR. Three different levels of quality control which include high control, MMR control, negative control and the calibrators were provided in the MolecularMD's One-step qRT-PCR BCR-ABL kit. The forward and reverse primers used were specific to b2a2 and b3a2 BCR-ABL transcripts in which they spanned BCR exons b2 and b3 and ABL exon 2. The expected PCR amplification product is 210 base pairs.

A single reaction volume is 25 µL. The PCR amplification mixture consisted approximately 100 ng to 250 ng of genomic RNA (5 µL), 1 µL of primer/probe set , 0.5 µL Invitrogen SuperScript III/Platinum Taq Enzyme Mix, 12.5 µL 2X Reaction Mix (a buffer containing 0.4mM of each dNTP and 6mM MgSO4), 0.5 µL of RNaseOUT Recombinant Ribonuclease Inhibitor and 5.5 µL of PCR grade dH_2O. PCR was performed using the following parameters: 50°C for 15 mins (1 cycle); 95°C for 2 mins (1 cycle); 95°C for 15 seconds and 60°C for 60 seconds with data acquisition on green fluorescent signal (repeat for 42 cycles).

Statistical Analysis

The data are presented as mean±S.E.M. One way ANOVA with *post hoc* analysis was used to analyse any significant difference between the test groups. A *p* value of less than 0.05 was considered significant.

RESULTS

Comparison of the quality and quantity of plasma RNA extracted using eight different RNA extraction kits

The performance of eight types of RNA extraction kits was evaluated and the results are as shown in Table 1. TRIzol protocol produced the highest amount of RNA (23.58±1.42 ng/µL) followed by GeneAll® RiboEx™ with minor protocol modification (13.67±0.65 ng/µL). These two kits used guanidinium isothiocyanate reagent for RNA extraction. One way ANOVA accompanied with *Tukey* multiple comparison *post hoc* test [F(7,79)=72.657, p<0.01] revealed that GeneAll® RiboEx™ with minor protocol modification demonstrated significantly higher RNA yield compared to E.Z.N.A.® Viral RNA kit, PureLink® Total RNA Blood kit and NucliSENS® miniMAG® which used lysis buffer to extract RNA. The quantity of RNA yield was lower compared to the commercial kits which used guanidinium isothiocyanate reagent.

Table 1: Comparison of the quality and quantity of plasma RNA of CML patients extracted using eight types of RNA extraction kits.

RNA Extraction Kit	Total yield of RNA (ng/µL)	RNA	
		A260/280	A260/230
TRIzol reagent	23.58±1.42	1.69±0.02	0.32±0.02
E.Z.N.A.® Viral RNA kit	5.47±0.43	1.33±0.18	1.060±0.25
PureLink® Total RNA Blood kit	3.60±0.39	1.87±0.19	0.71±0.16
GeneAll® Hybrid-R™ blood RNA	11.72±0.69	2.17±0.14	1.54±0.11
NucliSENS® miniMAG®	1.63±0.21	1.71±0.15	0.28±0.05
ZR Whole-Blood RNA MiniPrep	11.10±1.08	1.61±0.09	0.49±0.11
QIAamp Viral RNA mini kit	12.90±0.92	1.78±0.49	0.37±0.34
GeneAll® RiboEx™ with minor protocol modification	13.67±0.65**	1.96±0.16	2.07±0.12##

Values are in mean±S.E.M. **p<0.01 *vs*. all kits except TRIzol reagent; ##p<0.01 *vs*. all kits except GeneAll® Hybrid-R™ blood RNA

PureLink® Total RNA Blood kit, GeneAll® Hybrid-R blood RNA and GeneAll® RiboEx™ with minor protocol modification produced pure RNA with A260/280 ratio of more than 1.8 (Table 1). One way ANOVA accompanied with *Tukey* multiple comparison analysis revealed no significant difference in RNA purity (A260/280) produced by all the kits. However, GeneAll® RiboEx™ with minor protocol modification demonstrated the lowest level of contaminant in the extracted RNA samples with A260/230 ratio of 2.07±0.12. One way ANOVA followed by *post hoc Tukey* multiple comparison analysis showed that the purity (A260/230 ratio) of RNA extracted by GeneAll® RiboEx™ with minor protocol modification and GeneAll® Hybrid-R™ blood RNAkit were higher ($p < 0.01$) compared to other RNA extraction kits [$F(7,79) = 25.213$, $p < 0.01$].

In addition, the quantity of RNA samples extracted using GeneAll® RiboEx™ with minor protocol modification from healthy individuals was 4.20±1.21 ng/µL which produced good purity with A260/280 ratio of 1.90±0.05 and A260/230 ratio of 2.10±0.05.

Quantification of ABL gene and BCR-ABL gene in plasma RNA

Plasma RNA extracted using GeneAll® RiboEx™ with minor protocol modification was reverse-transcribed by specific ABL control gene and BCR-ABL mutation gene primers. ABL control gene was successfully amplified in plasma RNA of CML patients with ABL gene copies of 3561±392 (Table 2).

Table 2: Quantification of ABL gene and BCR-ABL gene in plasma RNA extracted using the selected GeneAll® RiboEx™ protocol with minor modification.

Source of plasma RNA	No. of BCR-ABL gene copies	No. of ABL gene copies
CML patients	9±2	3561±392
Healthy individuals	0	1543±154

Values are in mean±S.E.M.

BCR-ABL mutation gene detected was 8.9±1.9 copies. Similarly, 1543±154 ABL gene copies were amplified in plasma RNA of healthy individuals but no BCR-ABL mutation gene was detected.

DISCUSSION

In the present study, it was demonstrated that GeneAll® RiboEx™ with minor modification to the protocol produced high purity of RNA and sufficient quantity for further gene expression analysis. Approximately 3561±392 copies of ABL control gene were amplified from 150 ng of plasma samples although low number of BCR-ABL gene was detected. The results are in agreement with the work done by Narita and colleagues showing that control gene could be detected in plasma RNA samples of CML patients but the amount of mutation gene was very low probably due to TKI therapy which act by blocking the production of BCR-ABL mutation gene [15].

In this study, GeneAll® RiboEx™ protocol was modified by using spin column in the washing protocol. This device works like a filter to capture RNA and enables washing solution to pass through while eliminating contaminants in the RNA. In addition, RNA washing procedure was repeated for four times to ensure all impurities were removed without affecting the RNA yield. The RNA sample purity is an important element in molecular diagnostic test because phenol and sodium chloride used in the extraction could inhibit polymerase chain reaction [16] which may lead to false low amount or false negative results. Reagents used in GeneAll® RiboEx™ kit were developed based on the guanidinium-thiocyanate-phenol-chloroform extraction technique [17]. Deng and colleagues reported that although guanidinium isothiocyanate-phenol protocol produced high quantity of RNA, the purity of extracted RNA was unsatisfactory with purity indicator A260/230 ranging from 1.48 to 1.67 [18]; indicating the presence of contaminants in RNA sample. Jakovljevic and colleagues recommended that the RNA extraction methodology has to be improved especially on RNA sample purity [19]. Various efforts have been taken to find the best method to produce pure and high quantity of RNA extracts. It has been shown that RNA sample extracted by spin column technique could also be used for further gene amplification assay [20].

Plasma RNA levels in patients with cancer such as hematological malignancies, hepatic, pancreatic, colonic, and sarcoma have been reported to be higher than healthy individuals [21]. Similar findings have been reported in renal cell cancer [22], other malignancies [23], and trauma patients [24]. The research in this field has also progressed to the extent that quantification of nucleic acids in plasma could be used as an indicator to predict survival of patients with prostate cancer [25]. Therefore, the development of a very efficient method to extract RNA sample is deemed necessary. Our results showed that plasma RNA samples extracted using GeneAll® RiboEx™ with minor protocol modification were high in amount, pure and free from contaminants (phenol and sodium chloride salt). This protocol is efficient and less labor-intensive than conventional guanidinium-phenol-chloroform RNA extraction technique. Therefore, this optimized plasma RNA extraction technique should be tested in other cancer types or diagnostics to determine its feasibility and efficiency.

CONCLUSION

GeneAll® RiboEx™ with minor protocol modification offered high purity but moderate quantities of amplifiable plasma RNA compared to other kits tested. Further studies using this kit on quantification of plasma and blood cells RNA in other advanced stage cancers are warranted.

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