



Methodology

An alternate method for DNA and RNA extraction from clotted blood

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ABSTRACT. We developed an alternative method to extract DNA and RNA from clotted blood for genomic and molecular investigations. A combination of the TRIzol method and the QIAamp spin column were used to extract RNA from frozen clotted blood. Clotted blood was sonicated and then the QIAamp DNA Blood Mini Kit was used for DNA extraction. Extracted DNA and RNA were adequate for gene expression analysis and copy number variation (CNV) genotyping, respectively. The purity of the extracted RNA and DNA was in the range of 1.8-2.0, determined by absorbance ratios of $A_{260}:A_{280}$. Good DNA and RNA integrity were confirmed using gel electrophoresis and automated electrophoresis. The extracted DNA was suitable for qPCR and microarrays for CNV genotyping, while the extracted RNA was adequate for gene analysis using RT-qPCR.

Key words: Clotted blood; DNA extraction; RNA extraction; Sonication

INTRODUCTION

Medical genomic research has risen dramatically recently with the availability of various high-throughput technological advancements such as microarray and next-generation sequencing. However, obtaining DNA and RNA with good quality is challenging. Peripheral blood is one of the major sources for genomic DNA and RNA. New blood samples are usually obtained from consenting subjects for genomic studies, and providing these samples may be a nuisance to the subjects.

Methods have been developed to obtain DNA from human clotted blood, which is normally discarded (Kanai et al., 1994; Salazar et al., 1998; Basuni et al., 2000; Adkins et al., 2002; Se Fum Wong et al., 2007). These strategies could essentially minimize the volume of blood needed, especially when working with infants. Obtaining good-quality DNA from clotted blood for genomic studies is crucial for accurate analysis, as has been proven by Cukier et al. (2009).

The use of RNA for genetic studies has rapidly expanded in molecular research. Many advanced applications of gene expression in whole genomic studies require a certain amount of RNA for analysis. RNA is a much less stable molecule than DNA, and obtaining RNA with good quality and high yield from clotted blood is challenging. Precautions must be taken to avoid contamination with RNases, proteins, and genomic DNA, especially during RNA preparation and storage, while also obtaining maximum yield. Good-quality RNA is important in modern molecular biological methods such as reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and microarray analysis. In this study, we developed alternative method for extracting DNA and RNA from clotted blood and showed that both DNA and RNA is obtained with sufficient quality for use in copy number variation (CNV) genotyping and gene expression studies, respectively.

MATERIAL AND METHODS

Clinical material

Ethics approval was obtained for a study of the host genetics of dengue [Ministry of Health: NMRR-09-1128-4211; Universiti Teknologi MARA: 600-RMI (5/1/6), 600-RMI (5/1/6)]. Three milliliters of peripheral blood was collected from dengue patients at Sungai Buloh Hospital, Selangor, Malaysia, in plain tubes (BD, USA). Samples were spun, and the separated serum was used for serological tests. Clotted blood was kept at -80°C until use.

Genomic DNA extraction

Before extraction, clotted blood samples were thawed and sonicated at 100 Hz and 4°C for 3 h. Blood samples were then macerated with autoclaved disposable wooden applicator sticks. Approximately 200 µL was transferred to a sterile 1.5-mL microcentrifuge tube, and 400 µL 1X red blood cell (RBC) lysis buffer was added. The tubes were inverted for 30 s and quick-spun to ensure that none of the samples were stuck to the tube caps. The mixtures were incubated overnight at 37°C and then washed with RBC lysis buffer until a white pellet formed. Genomic DNA was extracted from the pellet using a QIAamp DNA Blood Mini Kit

(Qiagen, Germany) following manufacturer instructions. Briefly, 15 μL 100 mg/mL proteinase K and 200 μL buffer AL were added to the pellet, mixed thoroughly by inversion 10 times, and incubated at 56°C for 30 min. After incubation, the tube was briefly centrifuged, and 200 μL ethanol (96-100%) was added to the reaction mixture. The reaction mixture was then transferred to the QIAamp spin column and centrifuged at 8000 rpm for 1 min. The QIAamp spin column was then placed in a clean 2-mL tube and 500 μL buffer AW1 was added. The spin column was centrifuged at 800 rpm for 1 min and placed into a new collection tube. Five hundred-microliter buffer AW2 was added, and the column was centrifuged at 14,000 rpm for 3 min. The QIAamp spin column was then placed in a new 1.5-mL microcentrifuge tube, 75 μL buffer AE (elution buffer) was added, and the tube was incubated at room temperature for 20 min. Before the final centrifugation at 8000 rpm for 3 min, 50 μL double-distilled water was added into the spin column and incubated at room temperature for 15 min. The eluted DNA was stored at -20°C until further use.

RNA extraction

The TRIzol method was combined with a Qiagen QIAamp spin column for the isolation of RNA from clotted blood. A total of 1 mL clotted blood was incubated at 4°C with 50 mL RBC lysis buffer for 30 min and spun down, and the supernatant was discarded. The step was repeated with 1 mL RBC lysis buffer followed by 1 mL phosphate-buffered saline until the resulting pellet was cleared. Eight hundred-microliter TRIzol reagent was added to the pellet and vortexed immediately for 15 s before incubation at room temperature for 5 min. Then, 200 μL chloroform was added to the tube, and the solution was centrifuged for 14,000 rpm for 15 min at 4°C. Approximately 200 μL aqueous phase was transferred to 700 μL Qiagen RLT buffer in a clean tube, precipitated with 500 μL 100% ethanol, and vortexed for 15 s. The samples were applied to the QIAamp spin column, centrifuged, and washed twice with 500 μL buffer RPE and 750 μL 80% ethanol. RNA was then eluted from the columns using 40 μL RNase-free water, quantitated with a NanoDrop ND-100 (Labtech International Ltd., UK), and stored at -80°C until further use.

Genomic DNA purity and integrity measurements

The concentration and purity of genomic DNA were determined by measuring the 260- to 280-nm absorbance ratios ($A_{260}:A_{280}$) using the NanoDrop ND-1000. Then, gel electrophoresis was used to determine the integrity of the DNA. Briefly, 3 μL DNA was loaded onto 1% agarose gel with 5 μL SYBR Safe (Invitrogen, USA) and electrophoresed for 30 min at 90 V followed by visualization under the gel imager (EasyDoc, Bio-Rad Laboratories, USA).

RNA purity and integrity measurement

RNA purity and concentration were determined by the absorbance ratios ($A_{260}:A_{280}$) using the NanoDrop ND-1000 with ideal values of 1.8-2.0. Analysis by Experion (Bio-Rad Laboratories), an automated capillary-electrophoresis system, provided a framework for the standardization of RNA quality control. RNA integrity number (RIN) was calculated.

Microarray analysis using genotyping

Genotyping was performed on DNA collected using the Genome-Wide Human single-nucleotide polymorphism (SNP) Nsp/Sty 6.0 assay (Affymetrix, USA), according to manufacturer instructions. Stringent filtering criteria were applied according to manufacturer recommendations, and the CNV of the sample was called using Genotyping Console (GTC 4.0, Affymetrix).

qPCR genotyping

CNV genotyping using qPCR was performed on the samples to countercheck the integrity of the DNA quality. The primers for genes FcGR3B F (5'-CAC CTT GAA TCT CAT CCC CAG GGT CTT G-3') and FcGR3B R (5'-CCA TCT CTG TCA CCT GCC AG-3') were used as targets. Primers for genes FOXP2 F (5'-TGA CAT GCC AGC TTA TCT GTT T-3') and FOXP2 R (5'-GAG AAA AGC AAT TTT CAC ACA GTC C-3') were used as references. Amplification was carried out for 3 min at 95°C, followed by 34 cycles of 20 s at 95°C, 61 cycles of 20 s at 60°C, 15 s at 95°C, and 30 s at 65°C, using an IQ4 Bio-Rad Thermocycler (Bio-Rad Laboratories).

RT-qPCR for gene expression

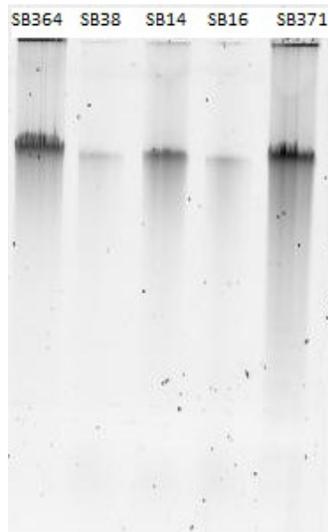
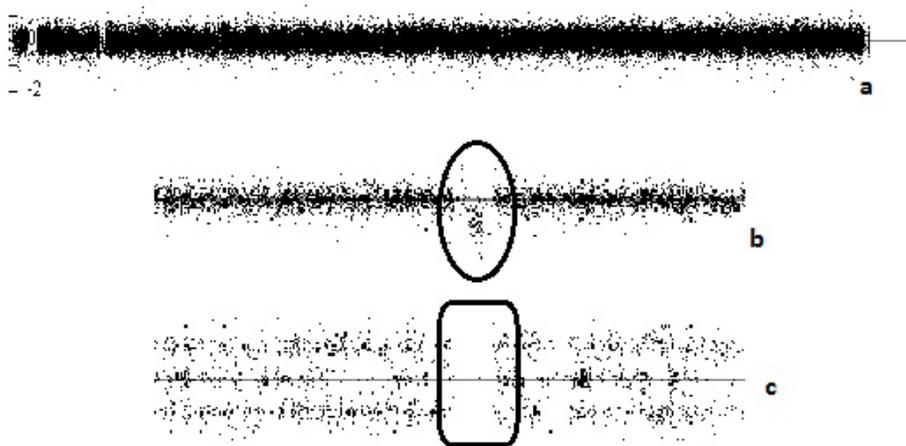
Total RNA was reverse transcribed to complementary DNA using 5xi Script Reverse Transcriptase Supermix (Bio-Rad Laboratories). The primers for interleukin 6 (IL-6) were designed based on sequences published by Abe et al. (2005). Primer sequences were as follows: IL-6 forward primer (5'-CCA GTA CCC CCA GGA GAA GAT-3') and IL-6 reverse primer (5'-CGT TCT GAA GAG GTG AGT GGC-3') were used as targets, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer (5'-TGC ACC ACC AAC TGC TTA GC-3') and GAPDH reverse primer (5'-GGC ATG GAC TGT GGT CAT GAG-3') were used as references. PCR was conducted using an IQ4 Bio-Rad Thermocycler (Bio-Rad Laboratories) in a 25- μ L reaction mixture. Amplification was performed for 3 min at 95°C and 45 s at 60°C, followed by 49 cycles of 30 s at 72°C, 1 min at 95°C, and 1 min at 55°C.

RESULTS

The quality of purified DNA from clotted blood was compared, and its integrity was evaluated with 3 assays. First, yields and purity of genomic DNA were determined through spectrophotometric analysis (Table 1). Second, the quality of DNA was observed using gel electrophoresis (Figure 1). Finally, microarray and qPCR analyses were performed to prove the good integrity and purity of the DNA (Figures 2 and 3). The purity (260/280) of the sample was expected to be within the range of 1.8-2.0, which is crucial for microarray and qPCR analysis. The success of the assay was an indicator of DNA quality.

Table 1. Genomic DNA yield and purity.

Sample ID	Concentration (ng/ μ L)	A_{260}/A_{280}	Volume (μ L)	Amount (ng)
SB364	175.0	1.86	15	2625.0
SB38	29.4	1.69	125	3675.0
SB14	37.5	1.70	125	4687.5
SB16	8.4	1.60	125	1050.0
SB371	92.0	1.88	15	1380.0

**Figure 1.** DNA banding patterns electrophoresed on 1% gel electrophoresis.**Figure 2.** **a.** Log_2 ratio graph of a sample revealed from GTC 4.0 of Affymetrix indicates no copy number variation observed on chromosome 1. **b.** Log_2 ratio graph on chromosome 3 observed with a copy number loss. **c.** B allele frequency graph showing a loss at the same position.

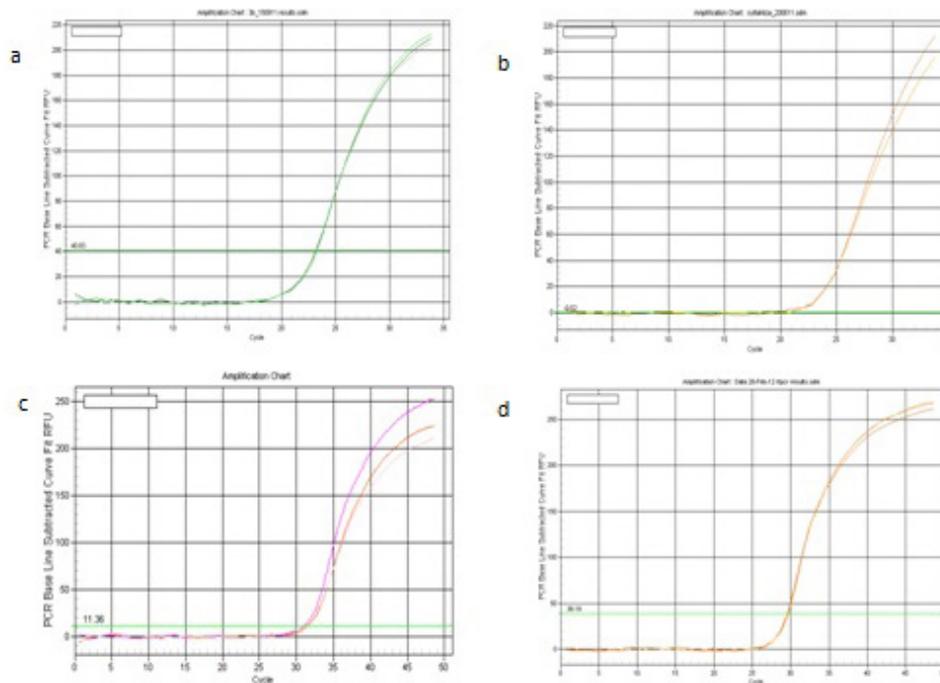


Figure 3. Triplicate amplification curves (C_t curves) of qPCR for DNA extracted from: **a.** whole blood and **b.** clotted blood. **c.** and **d.** are C_t curves for the expression of the IL-6 gene from clotted blood-extracted RNA.

Microarray analysis (Affymetrix SNP 6.0) showed that the genomic DNA used in this platform had a contrast quality control (QC) of more than 1.4 (Table 2). Contrast QC is a recommended QC metric for Genome-Wide SNP 6.0 assays, and the value of ≥ 0.4 indicated that all steps of the assay were working as expected (GTC 4.0 user manual). QC call rate is another QC for this assay, and the manufacturer recommends $>86\%$ as the cut-off value for good data (Nishida et al., 2008; Vogler et al., 2010). However, a Korean research group has used samples that have a genotyping rate of $>93\%$ to eliminate false-positive results from low-quality genotyping data (Kang et al., 2008). Our research showed that the samples displayed good-quality genotyping with an average QC call rate of 93%. The \log_2 ratio graph (see Figure 2) of the processed samples indicated a tentatively high-quality signal-to-noise ratio, proving the reliability of the DNA sample quality.

Table 2. Contrast quality control (QC) and QC call rate of genomic DNA run on Affymetrix SNP 6.0 assay.

Sample ID	Contrast QC	QC call rate
SB106	1.82	97.09
SB27	2.47	97.29
SB41	1.41	91.29
SB263	1.00	90.77
SB364	1.40	92.36

For qPCR amplification, DNA quality was observed via triplicate amplification curves (C_t curves), as shown in Figure 3a and b. The amplification time for the triplicates of each samples occurred at approximately the same C_t value indicating good-quality DNA.

Optical density measurements via NanoDrop and Experion (Bio-Rad Laboratories) were used in RNA quality assessment (Table 3; Figure 4). Both measurements showed similar readings, indicating the reliability of the assays. Additional measurement - namely, RIN - was calculated to assess RNA integrity by visualizing the 18S and 28S RNA bands. An RIN of <5 indicates total good-quality RNA, and >8 indicates perfect total RNA for genomic applications (Fleige and Pfaffl, 2006). We obtained an RIN of 7 from clotted blood samples. In addition, a decreased 28S:18S ratio or elevated threshold baseline indicates degradation of samples (Mueller et al., 2004). Gene expression profiles of the samples extracted were investigated using RT-qPCR, as shown in the C_t curves in Figure 3c and d. Analysis of gene expression patterns in 4 samples (Figure 5) are presented as units of IL-6 as a target gene after normalization with GAPDH as the reference gene. The impact of RNA quality and purity also influenced the significance of differential gene expression. The data in Table 4 further show that the gene expression results we obtained appear to be influenced most by the purity of the individual samples themselves (see Table 3).

Table 3. Total RNA yield and purity.

Sample ID	Concentration (ng/ μ L)		A_{260}/A_{280}	Volume (μ L)	Amount (ng)
	NanoDrop	Experion			
SB72	18.75	11.14	1.61	50	937.5
SB166	18.24	10.63	1.63	40	729.6
SB362	29.64	15.89	1.73	40	1185.6
SB89	42.48	15.74	1.60	40	1699.2
SB69	22.28	43.88	1.99	50	1114.0

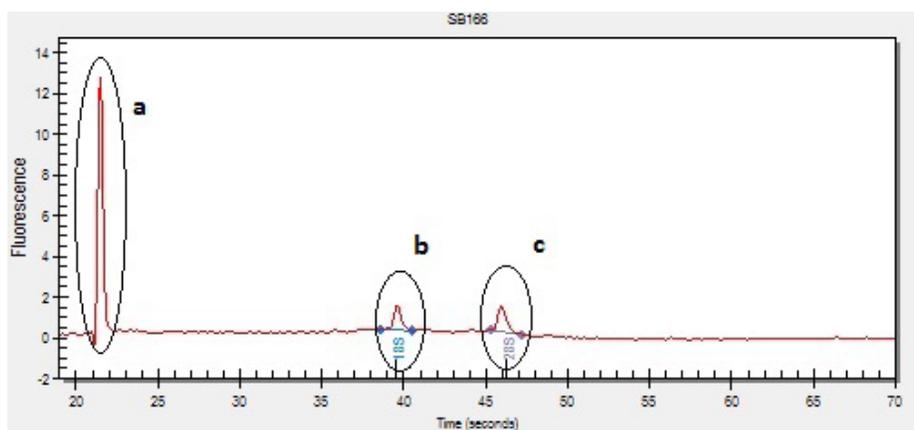


Figure 4. Chromatogram of micro-capillary electrophoresis [Experion (Bio-Rad, USA)] from RNA samples showing a small 28/18S rRNA peak ratio with RIN = 7.0 was indicated by a shift in the electropherogram to shorter fragment sizes. **a.** marker. **b.** 18S. **c.** 28S.

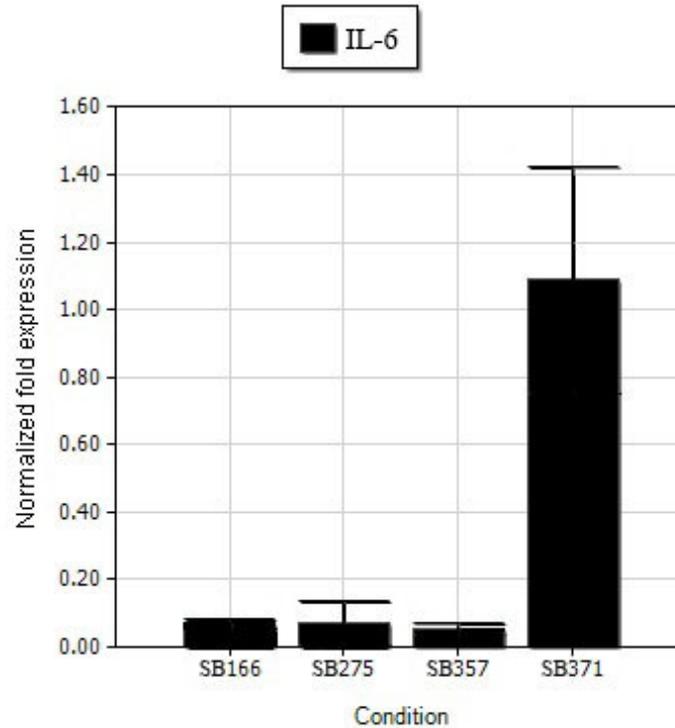


Figure 5. Higher expression of the IL-6 gene in the SB371 sample compared with others. Results are reported as relative units of IL-6 as a target gene compared with GAPDH as the reference gene.

Table 4. IL-6 gene expression data of RNA extracted from clotted blood samples.

Sample ID	Expression	Expression SD	Corrected expression SD	Mean C_t	C_t SD
SB166	0.06920	0.01587	0.01587	32.74	0.25020
SB275	0.07292	0.06183	0.06183	35.70	1.21015
SB357	0.05620	0.01674	0.01674	35.87	0.00000
SB371	1.09037	0.33224	0.33224	28.89	0.07868

SD = standard deviation.

DISCUSSION

The increasing volume of research that requires genomic DNA and RNA for either basic experimental procedures or high-throughput technology has led to a need to collect larger amounts of biomaterials - notably, peripheral blood - with excellent quality. Various types of blood collection tubes are often required to perform different biological investigations, including biochemical tests, serological tests, and also genomic studies. Volunteers or patients are inconvenienced when asked to provide high volumes of blood to meet the needs of researchers or diagnostic tests. Therefore, minimizing the blood volume taken from subjects is essential.

Several reports have suggested that DNA and RNA can be extracted from clotted

blood with hazardous organic solvents such as phenol-chloroform (Basuni et al., 2000) and ethanol precipitation (Santella, 2006). Others have reported the use of a combination of 100% isopropanol and 70% ethanol to extract DNA from clotted blood (Adkins et al., 2002). Clements et al. (2008) have compared 3 methods for DNA extraction - namely, a phenol-chloroform method, a silica gel membrane column method, and a modified salt precipitation method - and revealed that the silica column method retrieves DNA from both clotted blood and ethylenediaminetetraacetic acid-preserved blood with equally good results.

We developed an alternative method for extracting DNA from clotted blood; this method involved the use of sonication to enhance the rate of cell lysis, followed by manual grinding of clotted blood to maximize the volume of blood in liquid form. We believe that sonication breaks down the cell wall of clotted blood, easing the lysis process. Ultrasonication is a known method of mechanical cell disruption and is based on the high shear created by high-frequency ultrasound. Schoppee Bortz and Wamhoff (2011) used sonication for full-cellular disruption and maximum DNA recovery from fixed rat aortic smooth muscle cells. They suggested that sonication should be performed over a certain period. We performed sonication with hourly time intervals and found that 3 h was the most effective sonication time for clotted blood (data not shown) that would be later macerated. This mechanical cell disruption may also effectively break the walls of some fungi (Klimek-Ochab et al., 2011). Although we did not standardize the volume of the clotted blood samples during sonication, we made sure that they were nearly identical. Schoppee Bortz and Wamhoff (2011) have shown that cell density has no effect on outcome when using the same sonication treatment.

The lysed samples we obtained were sufficient for further DNA extraction using commercially available kits. The extracted DNA was genotyped using an SNP 6.0 Affymetrix platform with an average QC call rate of 93% and contrast QC of 1.62. These values showed that the quality of the extracted DNA was sufficient for its use in microarray analysis. Similarly, the use of the extracted DNA for qPCR analysis was also successful.

On a separate note, conventional methods are nonspecific for single-stranded RNA and often exposed to interference from contaminants present in the sample (Imbeaud et al., 2005). De Paula et al. (2001) evaluated 3 well-established methods of RNA extraction from blood samples. The TRIzol and Chomczynski-Sacchi techniques showed an absence of amplification, and the authors believed it to be due to a loss of genetic material during the precipitation and washing procedures of these methods. The RNA yield obtained in this current study ranged from 730 to 5260 ng, with an average purity of 1.8. Although obtaining RNA of good quality from clotted blood is challenging, our alternative method proved its reliability, as evidenced by the RT-qPCR results. Gene expression profiles obtained from partially degraded RNA samples with still visible ribosomal bands exhibit a high degree of similarity (Schoor et al., 2003).

Therefore, our results are promising and proved that the sample integrity was reliable. Ultimately, our aim is to use collected biological materials fully while minimizing donor and patient discomfort. In summary, we successfully developed an alternative method to extract DNA and RNA from clotted blood for use in various genomic and molecular investigations.

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