Levels of 5′ RNA tags in plasma and buffy coat from EDTA blood increase with time

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Background For biological sample banking it is important to precisely document sample treatment prior to extraction and storage. A major variable is the interval between blood sampling and subsequent processing and storage. We have determined the relationship between this time interval and frequency of 5′ transcript tags. This study was designed to establish guidelines for collecting RNA from blood in prospective studies and ensure maximum availability of RNA analytes.

Methods Venous blood was collected from 40 healthy volunteers. Samples were processed immediately, 12, 24 and 36 h post collection and buffy coat and/or plasma removed. Total RNA was extracted and reverse transcribed, assays were optimized and levels of 5′ RNA tags quantified by qPCR.

Results Stably expressed reference genes were selected to examine 5′ tags in plasma and buffy coat blood fractions. Whole blood was processed at various time points post collection to determine the affect on the presence and stability of 5′ RNA tags. A significant increase ($P < 0.05$ to $P < 0.001$) in 5′ RNA tags was observed at 12 h and up to 36 h in plasma and buffy coat samples isolated from EDTA blood which was maintained at 4°C prior to processing when compared with plasma and buffy coat isolated from EDTA blood processed immediately.

Conclusions Over time 5′ RNA tags increase in both plasma and buffy coat samples. It has been previously shown that removing cells from their normal environment produces cellular activation and up-regulation of pathways resulting in increased transcript expression. Positive correlation was observed between the time interval from sample collection to storage and amount of 5′ transcript tags present. This increase could be due to white blood cells undergoing necrosis and lysis, or from RNA protected within apoptotic bodies. As 5′ RNA tags were targeted using random primers for reverse transcription, even RNA partly degraded by RNases would have been detected.

Keywords Plasma, buffy coat, RNA, gene expression, real-time PCR

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Introduction

Recent debate has propounded whether whole blood collected from patients and subsequently stored at 4°C for up to 36 h is suited to certain downstream applications such as analyses including gene expression studies. The general dogma is that RNA is very unstable both in vivo and in many in vitro situations, and therefore the appropriateness of samples for use in studies after storage might be dubious and highly variable.1 Prospective cohort studies and banking facilities are storing progressively larger numbers of samples from both healthy and unwell individuals. Increasing importance is being placed on understanding how sample treatment and storage conditions affect variability and downstream applications. Advances in this area will assist in preserving sample analytes and to permit their accurate investigation in future studies. RNA has a relatively low half life and is subject to degradation by endo- and exonucleases.1 The potential exists to study transcripts under such conditions and this is normally overlooked. Wong et al. have shown that mRNA fragments are present in maternal plasma with a preponderance of 5’ mRNA fragments.2 Using quantitative polymerase chain reaction (qPCR) as a method of detection we were able to design assays which targeted sequence-specific regions towards the 5’ end of the gene. Using this approach we were able to measure the presence and level of 5’ RNA tags and determine their relationship with time interval between sample collection and processing.

The majority of expression studies utilize reference genes in order to normalize data for meaningful comparisons across samples to be made. For a transcript to be considered as an endogenous reference control it must be stably expressed with respect to the target pathogenesis ‘system’ of interest.3 To maximize the chance of successfully detecting 5’ RNA tags, a panel of transcripts were selected which have been widely used in leukocyte studies as reference genes/endogenous control,4,5 many of which have also been reported to occur in high abundance.6,7

Methods

Venous blood samples were collected into EDTA tubes from 40 healthy volunteers.8 Samples were processed immediately according to the protocol or maintained at 4°C for 12, 24 and 36 h post collection, and buffy coat and/or plasma removed. Aliquots of plasma and buffy coat were stored at −80°C. Total RNA was extracted from 1 ml plasma and 100 μl buffy coat using Trizol LS™ (Invitrogen) followed by column purification. Trizol LS™ was added to the samples in a 3:1 ratio and samples were incubated at room temperature for 5 min. Chloroform was added to the samples which were mixed and then centrifuged for 15 min. The aqueous phase was removed and transferred into an Eppendorf tube and an equal volume of 70% ethanol added. The sample was mixed and applied to an RNeasy mini column (QIAGen). The column was washed and finally the RNA eluted in 30 μl RNase free water.

Samples were quantified using a NanoDrop ND-1000 spectrophotometer. RNA (100 ng) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and 250 ng random primers (Invitrogen), the manufacturers guidelines for reverse transcription with random primers was followed. Briefly RNA, random primers and dNTP were heated to 65°C for 5 min and quickly cooled on ice. Buffer, DTT and RNaseOUT were added and the samples incubated at 25°C for 2 min. Superscript II reverse transcriptase was added and the samples incubated at 25°C for 10 min followed by 42°C for 50 min. The reaction was inactivated by heating to 70°C for 15 min. cDNA was diluted 1:10 for use in qPCR assays.

The Universal ProbeLibrary Assay Design centre (Roche Diagnostics) was used to design PCR amplicons directed to RNA sequences towards the 5’ end of the transcripts (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; beta actin, ACTB; beta-2-microglobulin, B2M; hypoxanthine phosphoribosyltransferase 1, HPRT1; phosphoglycerate kinase 1, PGK1 and ribosomal protein L32, RPL32). The assays comprised sequence specific primers (Metabion) which were used in conjunction with a fluorescently labelled LNA probe (Universal ProbeLibrary, Roche Diagnostics). The GeNorm software was used to calculate which of the transcripts showed the least variability across the plasma and buffy coat samples.3 Briefly, samples were screened for the six reference genes, the algorithm works out the stability of each transcript, and sequentially removes the least stable transcript until the two most stable transcripts remain. These were; GAPDH (glyceraldehyde 3 phosphate dehydrogenase) and B2M (Beta-2-microglobulin).

GAPDH forward primer 5’-AGCCACATCGCTACAGACAC-3’, GAPDH reverse primer 5’-GCCCAATACGACCAAA-TCC-3’, GAPDH Universal Probe #60.

B2M forward primer 5’-TTCTGGCCTGGAGGCTATC-3’, B2M reverse primer 5’-TCAGGAAATTTGACCTTCCATTC-3’, B2M Universal Probe #42.

To quantitate the levels of transcript present standard curves were developed utilizing a synthetic template oligonucleotide (TO) representative of one strand of full length amplicons.9 GAPDH TO 5’AGCCACAUCCGCTAGACACACCAUUGGGAAAGCTGAGCGAGTCAACAGGATTGGTGGTGAGC-TGGC-3’

B2M TO 5’-TGGGCTCUGAAGGCTATCAGCGCTACCUCCAAAGATTCCAGTATGTCACUAUCCAGGACAGAATGGGAGUCAAATTTGCAGGA-3’
Deoxyuracil bases were substituted at strategic sites of the TOs allowing for decontamination via incubation with uracil-N-glycosylase (UNG) in subsequent reactions to remove any potential contamination caused from the TOs.

Standard curves were constructed by performing 10-fold serial dilutions ranging from 1 nM down to 10 aM and the exact number of molecules calculated and run 10 times to check for reproducibility (data not shown). Assays were performed on a Roche LightCycler 480, real time PCR machine using a commercially available 2× Probe Mastermix without UNG (Roche). All samples and controls were assayed in a final volume of 10 μl in triplicate using a thermocycling profile of 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. TO quantities were calculated via the application of Avogadro’s number. The highest concentration on the standard curve was 1 nM. To determine the number of molecules in 1 μl of the 1 nM stock solution the following calculation was applied:

$$1 \text{ mole} = 6.022 \times 10^{23} \text{ molecules}$$

$$1 \text{ nM} = (1 \times 10^{-9}) \times (6.022 \times 10^{23})$$

$$= 6.022 \times 10^{14} \text{ molecules/1 \mu l}$$

Therefore, there are $6.022 \times 10^8$ molecules per microlitre of 1 nM solution.

For the assays 4 μl of the standard TO were aliquoted into the reaction well, hence a total of $4 \times 6.022 \times 10^8$ molecules $= 2.4088 \times 10^9$ molecules was added per well for the highest value of the standard curve with a 10-fold decrease with each point of the dilution series.

Results obtained from the standard curves for the samples were in number of molecules per well. As cDNA samples had been diluted 1:10 for use in the assays with 4 μl used per well, this number was converted into the number of molecules per microlitre of stock cDNA and finally into the number of molecules in the entire reverse transcription reaction which was originally from 100 ng RNA. All values expressed in the results are numbers of molecules per 100 ng RNA. Data was analysed using the mean difference for each time point. Paired t-tests were used to determine statistical differences.

### Results

To determine the effect of time on the level of RNA transcripts, firstly stably expressed transcript tags in both plasma and buffy coat were identified. To do this the software package GeNorm was used which determined the most stable reference transcripts from a set of tested candidate reference transcripts in a given sample panel. Six commonly used endogenous control transcripts (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; beta actin, ACTB; beta-2-microglobulin, B2M; hypoxanthine phosphoribosyltransferase 1, HPRT1; phosphoglycerate kinase 1, PGK1 and ribosomal protein L32, RPL32) were used to screen the plasma and buffy coat samples. Assays were performed in triplicate and expression levels analysed using GeNorm software. The two most stably expressed transcripts across plasma and buffy coat were GAPDH and B2M. All samples were then further assayed in triplicate for these two transcripts and quantified against a standard curve. 5′ RNA tags were readily detected for both plasma and buffy coat. In addition, there appeared to be an increase in 5′ RNA tags over time for both GAPDH and B2M in comparison to the levels observed if the samples had been processed immediately. In plasma samples, a 3–4-fold increase in 5′ RNA tags was observed in those processed after 12, 24 and 36 h in comparison to those processed immediately. For GAPDH 1554 ($T = 0$), 5218 ($T = 12$ h), 4844 ($T = 24$ h), 5157 ($T = 36$ h) molecules per 100 ng RNA; for B2M 251 240 ($T = 0$), 782 968 ($T = 12$ h), 798 297 ($T = 24$ h), 935 695 ($T = 36$ h) molecules per 100 ng RNA. This increase in 5′ RNA tags was more pronounced in the buffy coat samples where the amount of 5′ RNA tags detected continually increased across the timeline studied. For GAPDH 3544 ($T = 0$), 20 042 ($T = 12$), 40 131 ($T = 36$) molecules per 100 ng RNA; for B2M 373 809 ($T = 0$), 2 223 769 ($T = 12$ h), 4 459 066 ($T = 36$). This equates to a 6-fold increase after 12 h and an 11–12-fold increase after 36 h. Figure 1 shows the mean difference of B2M and GAPDH 5′ tags for plasma and buffy coat. There is a significant increase in 5′ tags when EDTA tubes are maintained at 4°C rather than being processed immediately.

### Conclusions

As RNA degradation is a likely event in samples left for up to 36 h at 4°C prior to sample processing, the experimental design was specifically tailored to look for 5′ mRNA tags. By utilizing random primers in the reverse transcription phase as opposed to oligo d(T) and specifically designing assays to target the 5′ end of stably expressed, high abundant genes, the assays were able to quantify the presence of specific transcripts. Using this approach all degraded RNA can be detected.

We have shown that it is possible to detect 5′ RNA tags even after an interval of 36 h between blood sample collection and RNA extraction. However, the results do clearly indicate that for the transcripts studied (GAPDH and B2M) abundance levels were increased with prolonged processing lag times.

With increasing time RNA transcript tags increased in both plasma and buffy coat samples. This supports previous studies which have shown that once cells have been removed from their native environment numerous factors are involved in cellular activation and associate with an increase in expression of many transcripts. This study has shown there is a
Figure 1  The mean difference of 5’ tags per 100 ng RNA for B2M and GAPDH for samples processed immediately (T = 0) or maintained at 4°C for 12, 24 and 36 h (T= 12 h, T = 24 h and T = 36 h). The open squares represent plasma and the closed squares represent buffy coat. The error bars show the 95% CIs for the mean difference. There is a significant increase in the amount of 5’ tags detected in plasma during the first 12 h incubation at 4°C, there was no further increase in 5’ tags after 24 or 36 h. There is a significant increase in the amount of 5’ tags detected in buffy coat after the initial 12 h incubation at 4°C for both B2M and GAPDH which further increases at 36 h for B2M. Significant P values from a paired t-test are represented by *P < 0.05, **P < 0.005, ***P < 0.001

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Conflict of interest: None declared.

References


