SHORT REPORT

A reliable method for total RNA extraction from frozen human bone marrow samples taken at diagnosis of acute leukaemia

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This report describes a newly developed method using Trizol LS® reagent that can reliably extract high quality total RNA from frozen human leukaemic bone marrow samples. Extraction of total RNA from 71 frozen leukaemic bone marrow samples obtained at the time of diagnosis produced a median yield of 145 µg/ml leukaemic bone marrow. Total RNA samples could be reverse transcribed into cDNA and used successfully in the reverse transcription polymerase chain reaction amplification of B2M transcripts in 68 of 71 cases. A multivariate linear regression analysis revealed that significant predictors of RNA yield were both sample volume (< 1 ml $v > 1$ ml; $p = 0.003$) and peripheral blood white cell count ($< 5 \times 10^9$ $v \geq 5 \times 10^9$ white blood cells/litre; $p = 0.011$). The percentage of blasts present, leukaemia subtype, and sample storage period at −80°C (up to 945 days) were not predictors of total RNA yield. This method of total RNA extraction should be of interest to diagnostic and research staff using frozen bone marrow samples for molecular analyses. Similarly, the lack of association between sample storage period at −80°C and total RNA yield should be of interest to the administrators of tumour banks housing frozen bone marrow samples.

Recent increased use of clinical specimens in cancer research has led to the widespread establishment of tissue and tumour banks,[1–3] including the tumour bank at the Children's Hospital at Westmead.[2] To focus our activities upon the provision of quality clinical specimens,[1] these are stored unprocessed at −80°C, after snap freezing in liquid nitrogen. The tumour bank collection includes a large number of frozen bone marrow (BM) samples from children with leukaemia.[2] However, there is little published information on methods for extracting total RNA from frozen BM samples or the anticipated RNA yields, and no information concerning the biological or sample storage determinants of these yields.

SAMPLES

The diagnostic BM samples analysed were collected between 28 May 1998 and 27 November 2000 from 71 children with acute leukaemia. Sixty samples were from patients with acute lymphoblastic leukaemia (ALL) and the remaining 11 were from patients with acute myeloid leukaemia (AML), a distribution that reflects the relative incidences of ALL and AML in the paediatric population.[4] Parameters recorded for each sample included leukaemia subtype, BM sample volume, lymphoblast/myeloblast percentage (according to the morphology report provided by the haematology department, the Children's Hospital at Westmead), and peripheral blood white cell count (WCC) at the time of leukaemia diagnosis. After collection in EDTA containing blood collection tubes, BM aliquots were transferred into Cryovials® (Greiner Labortechnik, Stonehouse, UK), snap frozen in liquid nitrogen, and stored without cryopreservatives at −80°C.

RNA EXTRACTION

Because tissue thawing in the absence of ribonuclease inhibitors leads to RNA degradation,[5] we developed a process where frozen BM pellets would remain frozen for as long as possible during the extraction process. Each Cryovial® tube containing a frozen BM pellet was held immobilised using a small desktop vice while a Stanley® knife (Stanley Tools, Sydney, Australia) was used to cut the tube open quickly. The frozen pellet was then homogenised using a high speed agitation Polytron® blender (Kinematica, Luzern, Switzerland) in the presence of Trizol LS® (Life Technologies, Paisley, UK), and 0.2M acetic acid to reduce genomic DNA contamination. The remaining RNA extraction procedure was carried out according to the manufacturer's instructions (Life Technologies). Total RNA was also extracted from SK-BR-3 breast carcinoma cells, which were cultured as described previously.[6] Total RNA samples (10 µg) were electrophoresed through 1% denaturing agarose gels, transferred to a nylon membrane (HybondN+; Amersham, Sydney, Australia), and stained with methylene blue to assess loading equivalence.[6]

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

For the reverse transcription polymerase chain reaction (RT-PCR) amplification of β_2 microglobulin (B2M) transcripts, 10 µg total RNA was reverse transcribed using Superscript® II reverse transcriptase according to the manufacturer's instructions (Life Technologies). RT-PCR amplification was then achieved in reactions including template cDNA (corresponding to 2.5 µg total RNA), 10mM Tris/HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200 µM each dNTP, 1 mM each of primers 5′-ATCTTCAAACCTCCATGATG-3′ and 5′-ACCCCCACTGAAAAAGATGA-3′, and 1 U Taq DNA polymerase (Roche, Sydney, Australia) in 40 µl reaction volumes. PCR cycle conditions involved 30 cycles of denaturation (94°C for one minute), annealing (60°C for one minute), and elongation (68°C for 45 seconds), and one final elongation cycle (68°C for seven minutes). Two negative controls were routinely performed where templates were omitted from the RT and/or PCR reaction steps, whereas cDNA from SK-BR-3 breast cancer cells was included in positive control RT-PCR reactions. All RT-PCR products were characterised by electrophoresis through 1.2% agarose gels.

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; B2M, β_2 microglobulin; BM, bone marrow; RT-PCR, reverse transcription polymerase chain reaction; WCC, white blood cell count

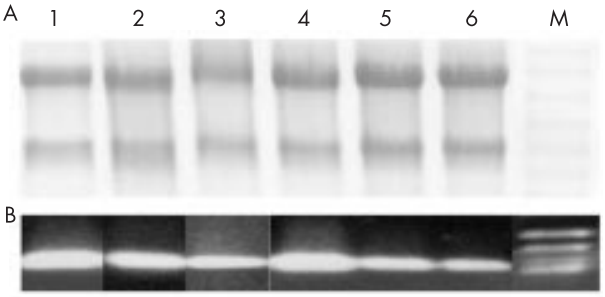

Figure 1 (A) Total RNA extracted from SK-BR-3 cells (lane 1) and five frozen leukaemic bone marrow (BM) samples (lanes 2–6), after northern blot transfer and methylene blue staining. Lane M shows the positions of RNA markers of 6.6 kb, 5.0 kb, 3.6 kb, 2.6 kb, 1.9 kb, and 1.4 kb (from top to bottom). (B) Successful amplification of the 120 bp B2M reverse transcription polymerase chain reaction (RT-PCR) product from cDNA derived from SK-BR-3 cells (lane 1) and the same BM samples shown in (A) (lanes 2–6). Lane M shows the positions of DNA markers of 190 bp, 140 bp, 124 bp, and 110 bp (from top to bottom). Negative control RT-PCR reactions in which templates were omitted from the RT and/or PCR reaction steps produced no RT-PCR products in all cases (data not shown).

RESULTS

Total RNA yields ranged from 1.3 to 840 µg/sample. The distribution of these yields was unimodal and positively skewed, with a median yield of 99 µg, and an interquartile range of 32–213 µg (n = 71). When total RNA yields were expressed as yield/ml BM sample, these ranged from 1.3 to 560 µg/ml. The distribution of these yields was again unimodal and positively skewed, with a median yield of 145 µg/ml, and an interquartile range of 32 to 286 (n = 71). The A_{260}/A_{280} ratios ranged from 1.0 to 2.0, and were distributed unimodally with negative skewing. The median A_{260}/A_{280} ratio was 1.6, with an interquartile range of 1.6 to 1.7 (n = 71). Purified RNA was generally of high quality, as demonstrated by its appearance following electrophoresis under denaturing conditions (fig 1A), and in 68 of 71 cases, total RNA samples were used successfully in subsequent RT-PCR amplification of B2M transcripts (fig 1B and data not shown). To identify factors influencing total RNA yield, a multivariate linear regression analysis was performed between yield and the following variables: BM volume, WCC at diagnosis, percentage of BM blasts, leukaemia subtype, and sample storage period at –80°C. Significant predictors of RNA yield were identified to be both BM sample volume (< 1 ml *v* > 1 ml; $p = 0.003$) and WCC (WCC $< 50 \times 10^9$/litre *v* WCC $\geq 50 \times 10^9$/litre; $p = 0.011$).

DISCUSSION

The ability to extract high quality total RNA from frozen leukaemic BM is of practical value in both clinical and research settings, where patient samples are often collected at times when laboratory staff are unavailable to carry out immediate analyses. This also facilitates the retrospective molecular analysis of stored patient material in tumour banks for both clinical and research purposes. The lack of sample manipulation required by single step total RNA extraction techniques using reagents such as Trizol LS also minimises the chances of sample (cross) contamination, which is essential if the resulting total RNA is to be analysed using RT-PCR based techniques.[7] Chadderton and colleagues[8] previously reported obtaining a mean total RNA yield of 28 µg/ml frozen human BM using Trizol LS. Their study compared the abilities of three commercially available single step reagents, namely Trizol LS, RNA-STAT 50 LS®, and Ultraspec-3®, to extract total RNA from fresh and frozen clinical samples. Although the mean total RNA yields and A_{260}/A_{280} ratios obtained with these three reagents did not differ significantly, it was concluded that Trizol LS most consistently produced satisfactory quantities of high quality total RNA.[8] Our present study confirms that Trizol LS is suitable for extracting high quality total RNA suitable for RT-PCR analyses from frozen human BM (fig 1). Moreover, we found that total RNA yields of 145 µg/ml can be reasonably expected from frozen leukaemic BM samples collected at diagnosis. This indicates that using our extraction method, adequate amounts of total RNA for molecular analyses (20 µg) could be expected to be obtained from approximately 140 µl frozen leukaemic BM.

Take home messages

- We have developed a method using Trizol LS® reagent that can reliably extract high quality total RNA from frozen human leukaemic bone marrow samples—a median yield of 145 µg/ml leukaemic bone marrow was achieved
- Total RNA samples could be reverse transcribed into cDNA and used successfully in the reverse transcription polymerase chain reaction amplification of B2M transcripts in 68 of 71 cases
- Multivariate linear regression analysis found both sample volume and peripheral blood white cell count to be significant predictors of RNA yield
- The percentage of blasts present, leukaemia subtype, and sample storage period at –80°C (up to 945 days) were not predictors of total RNA yield
- This method of total RNA extraction should be of interest to diagnostic and research staff using frozen bone marrow samples for molecular analyses and the administrators of tumour banks housing such material

> "The lack of sample manipulation required by single step total RNA extraction techniques using reagents such as Trizol LS also minimises the chances of sample (cross) contamination"

Our study investigated the biological determinants of RNA yields from frozen leukaemic BM, and whether these yields were influenced by sample storage periods at –80°C. Increased total RNA yields were predicted by both increased sample volume and WCC, indicating the reliable nature of our extraction procedure. A positive association between WCC and total RNA yields is also supported by comparing our median total RNA yield with the lower yields reported by other studies using Trizol LS with non-leukaemic BM and peripheral blood samples. Using BM from healthy volunteers and patients with breast cancer, Chadderton and colleagues[8] reported mean total RNA yields of 42 µg/ml and 28 µg/ml from fresh and frozen samples, respectively, and a mean total RNA yield of 32 µg/ml from fresh peripheral blood. An independent study reported an average total RNA yield of 20 µg/ml from fresh peripheral blood from healthy volunteers.[9] We did not find an association between total RNA yields and either the proportion of blasts or the leukaemia subtype present. Importantly, we also found no relation between sample storage period at –80°C and total RNA yields for storage periods up to 945 days. Therefore, the storage of human BM at –80°C does not greatly compromise total RNA yields within this time frame, although further studies will be necessary to determine whether total RNA yields are similarly unaffected by storage at –80°C for longer periods. However, the abundance of low abundance collagen α1(IV) transcripts (as measured by RT-PCR) did not differ significantly in renal biopsies that had been stored for 1–10 years at –70°C before total RNA extraction.[10]

In summary, this method of total RNA extraction should be of interest to both diagnostic and research staff accessing frozen BM samples for molecular analyses, and the administrators of tumour banks housing such material.

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REFERENCES

1 **Grizzle WE**, Aamodt R, Clausen K, *et al*. Providing human tissues for research: how to establish a program. *Arch Pathol Lab Med* 1998;**122**:1065–76.
2 **Coupland LA**, Cooke-Yarborough CM, Dalla-Pozza L. The New Children's Hospital tumour bank. *Med J Aust* 1999;**170**:284–5.
3 **Balleine RL**, Humphrey KE, Clarke CL. Tumour banks: providing human tissue for cancer research. *Med J Aust* 2001;**175**:293–4.
4 **McWhirter WR**, Dobson C, Ring I. Childhood cancer incidence in Australia, 1982–1991. *Int J Cancer* 1996;**65**:34–8.
5 **Sambrook J**, Fritsch EF, Maniatis T. Extraction, purification and analysis of messenger RNA from eukaryotic cells. In: *Molecular cloning: a laboratory manual*, Vol. 1, 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989:7.2–7.6.
6 **Balleine RL**, Schoenberg Fejzo M, Sathasivam P, *et al*. The D52 (TPD52) gene is a candidate target gene for events resulting in increased 8q21 copy number in human breast carcinoma. *Genes Chromosomes Cancer* 2000;**29**:48–57.
7 **Mannhalter C**, Koizar D, Mitterbauer G. Evaluation of RNA isolation methods and reference genes for RT-PCR analyses of rare target RNA. *Clin Chem Lab Med* 2000;**38**:171–7.
8 **Chadderton T**, Wilson C, Bewick M, *et al*. Evaluation of three rapid RNA extraction reagents: relevance for use in RT-PCRs and measurement of low level gene expression in clinical samples. *Cell Mol Biol (Noisy-le-Grand)* 1997;**43**:1227–34.
9 **Gandini O**, Celi FS, Magnanti M, *et al*. A rapid, simple, and inexpensive step facilitates RNA extraction from whole blood cells. *Lab Invest* 1999;**79**:1731–2.
10 **Eikmans M**, Baelde HJ, De Heer E, *et al*. Processing renal biopsies for diagnostic mRNA quantification: improvement of RNA extraction and storage conditions. *J Am Soc Nephrol* 2000;**11**:868–73.