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

D Barbaric, L Dalla-Pozza, J A Byrne

This report describes a newly developed method using Tri-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 1.45 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 β2M transcripts in 68 of 71 cases. A multivariate linear regression analysis revealed that significant predictors of RNA yield were both sample volume (< 1 ml vs > 1 ml; p = 0.003) and peripheral blood white cell count (< 5 x 10^9 vs > 5 x 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, including the tumour bank at the Children's Hospital at Westmead. To focus our activities upon the provision of quality clinical specimens, 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. 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. 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 Laborteknik, 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, 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 Polytorn® blender (Kinetematica, 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. 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.

Reverse Transcription Polymerase Chain Reaction
For the reverse transcription polymerase chain reaction (RT-PCR) amplification of β₂ 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 MgCl₂, 200 µM each dNTP, 1 mM each of primers 5’-ATCTCTCAAACCTCCATGATG-3’ and 5’-ACCCCCCTGAAAAAGATGA-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, β₂ microglobulin; BM, bone marrow; RT-PCR, reverse transcription polymerase chain reaction; WCC, white blood cell count
commercially available single step reagents, namely Trizol LS, BM using Trizol LS. Their study compared the abilities of three high quality total RNA. Trizol LS most consistently produced satisfactory quantities of reagents did not differ significantly, it was concluded that Trizol LS is suitable for extracting high quality total RNA suitable for RT-PCR analyses from frozen human BM sample (cross) contamination, which is essential if the result-LS is suitable for extracting 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.

Our present study confirms that 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.

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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.

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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 colleagues1 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.2 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.13 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.

**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 A260/A280 ratios ranged from 1.0 to 2.0, and were distributed unimodally with negative skewing. The median A260/A280 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) and peripheral blood white cell count (WCC) (fig 1A, and in 68 of 71 cases). 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.

**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. Using BM from healthy volunteers and patients with breast cancer, Chadderton and colleagues1 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.2 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.13 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.

**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.
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