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ORIGINAL ARTICLE

Audit of Quality and Quantity of Nucleic Acid Yield from Pediatric Acute Leukemia Cases Following a Bio-banking Initiative

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Abstract Information which can be harvested from a biological sample has greatly improved with advancements in diagnostic technologies. However, in developing countries, the awareness about usefulness of bio-banking concept is lacking and centres which do offer it, depend mainly on − 20 or − 80 °C for sample storage due to lack of sophisticated infrastructure like vapour phase nitrogen storage preservation. Hence in these resource constraint settings, timely audit of quality of nucleic acids extractable from samples stored is of utmost importance. In this study, we explore the effect of − 20 °C storage over nucleic acids (DNA/RNA) isolated from blood samples of 180 patients with various leukaemia's following a bio-banking initiative. We observed that the integrity and quality of both DNA and RNA were maintained in 70 and 80% samples respectively over time as reflected by their concentration measurements and inherent uniform expression of housekeeping gene GAPDH. Only 3.7% of the RNA samples and 4.2% of the DNA samples yielded very low concentrations despite minimizing processing and technical loss. In nutshell, audit of our biobank sample yield highlights that storage of blood samples at − 20 °C does not compromise the fidelity of nucleic acids for future diagnostic and research work in a resource constraint setting.

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Electronic supplementary material The online version of this article (doi:https://doi.org/10.1007/s12288-018-0975-4) contains supplementary material, which is available to authorized users.

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Abbreviations
B-ALL   B cell acute lymphoblastic leukemia
T-ALL   T cell acute lymphoblastic leukemia
AML   Acute myeloid leukemia
FFPE   Formalin fixed paraffin embedded
PBMC   Peripheral blood mononuclear cells

Introduction

Advent of recent technologies like next generation sequencing, RNA sequencing etc. have revolutionized the investigations in the field of genome, transcriptome, epigenome, and proteome analysis. An increased world population has been predicted to increase the number of biomedical samples to a great extent with ∼ 600 million samples in 2010 in US alone [1, 2]. Freezing the tissue is a viable option for future studies as it allow the maintenance of DNA/RNA quality over the time as compared to FFPE (formalin fixed paraffin embedded) sample because paraffin embedding of specimens results in loss of RNA/DNA quality due to nucleic acid fragmentation and crosslinking [3]. To counteract the increasing demand for proper sample storage in pathology departments, bio banks or tissue banks are gaining popularity and becoming a "must to do" practice for detailed sample analysis to uncover the issues related to disease prognosis and treatment. Bio banks are mainly intended for long term storage of biological samples for future use. These are generally heterogeneous and patient sample storage capacity of a bio bank may range

from few thousands to million samples and ideally matched controls of particular diseases. The biological samples collected under this category include blood, saliva, urine, various tissue samples, hair etc. The quality control of different bio banks varies from initial collection, processing and final storage. In tertiary care hospital settings like PGIMER, Chandigarh, India the samples are mostly human tissues obtained after surgeries and autopsies. Post mortem interval and delay in the surgery time are one of the few factors which can result into nucleic acid degradation of samples obtained [4]. The typical strategy for sample preservation is procurement of sample in liquid ice and immediate storage in liquid nitrogen or at − 80 °C freezer to prevent further degradation. RNA is often considered as labile and very prone to degradation if not stored immediately in cold conditions. However, the studies on RNA integrity by RNA integrity number (RIN) analysis didn't show any degradation in RNA quality even after 4 h of incubation in cold and warm ischemic conditions [5, 6] and 24 h of room temperature storage prior to freezing in samples obtained from breast cancer patients [7]. However, in an interesting study, Viana et al. [8] have demonstrated the degradation in RNA quality after 30 min of cold ischemia. Few other studies reported a degradation in RNA quality after increased postmortem interval (PMI) [9], processing delay and fixation in FFPE samples [10] and other reports highlighted poor RNA quality after storage delay based on gene number and expression profile [11–13]. Recent study by Remany et al. [14] has shown deterioration in the yield and quality of both DNA and RNA even after storage at − 20 °C by using ethanol and RNA later based approaches.

Blood is an intricate component and a preferred sample for storage and future analysis in clinical research. Various fractions of the blood can be stored depending on the study design. Different storage strategies for blood storage are in use which includes the use of − 20 °C freezer at some places and adoption of ultra-low temperature storage of − 80 to − 150 °C at others. Vapor phase nitrogen storage (− 150 °C) is a preferred method over liquid phase (− 196 °C) because of the risk of sample contamination due to errant floaters present in liquid nitrogen. But the main complications involved with storage in nitrogen involve health hazards, spillage, nitrogen burns, explosion of nitrogen tanks and suffocation caused due to nitrogen leaks in the enclosed spaces. So many centers in our subcontinent either use − 80 or − 20 °C ultra-low temperature freezers for sample storage but there is little data available about the effect of storage at this temperature on nucleic acids extracted in cases with Leukemia. Measurements by spectrophotometers and nanoquant instrument have been accepted as a great indicator of RNA/DNA quality simultaneously reporting any protein contaminants

[15]. Measurements of optical density values and polymerase chain reaction with expression of housekeeping genes are effective approaches to analyze the effect of long term storage on quality yield of nucleic acids [16, 17]. So this study was conducted following a start-up for pediatric leukemia sample bio-banking initiative to assess the sample nucleic acid quality and quantity extractable after variable period of storage at − 20 °C. As per best of our information, this is the first study auditing the delay in nucleic acid extraction after blood sample storage at − 20 °C in pediatric leukemia patients from our subcontinent.

Methodology

Patient recruitment and sample collection

A total of 180 blood samples were collected for bio-banking from acute leukemia cases (B-ALL/T-ALL and AML, 1–12 years of age) diagnosed at hematology oncology unit, department of pediatrics, PGIMER, Chandigarh, India. Bio-banking initiative was started in our unit in year 2016 and audit of all acute leukemia cases bio-banked till January 2018 was carried out in terms of nucleic acid quality and quantity. The EDTA blood samples after routine blood tests were used for PBMC separation by Ficoll density gradient centrifugation. Briefly, 1 − 2 ml of blood was layered carefully upon 2 ml of Ficoll (Ficoll-paque™ plus, GE Healthcare) in a 15 ml falcon tube after diluting blood volume with PBS (phosphate buffered saline) to maintain blood: Ficoll ratio of 3:1. The samples were then centrifuged at 3000 rpm for 20–25 min at room temperature in a centrifuge with brakes off. Buffy layer (white ring at interface of plasma and RBCs) thus obtained was separated in a separate tube and added with equal volume of phosphate buffered saline (PBS) and centrifuged again. One part of the PBMCs presented in pellet was stored in RNA later for RNA isolation and one part was stored in PBS for DNA isolation. The pellets were stored at − 20 °C for nucleic acid isolation in batch (time taken from sample storage to nucleic acid isolation varied from 1 week to a maximum of 1 month).

DNA and RNA isolation

DNA and RNA were isolated from the stored pellets by using QIAamp DNA blood mini kit (Qiagen) and QIAamp RNA blood mini kit (Qiagen) respectively as per manufacturer's instructions. The nucleic acids extracted were then subjected to quantitative and qualitative measurements.

Assessment of DNA and RNA quantity and quality

Isolated DNA was measured in terms of yield and optical density by using Spectrophotometer cum ELISA reader provided with a nanoquant plate (Tecan). Blanking was performed by AE buffer and 2 μl of DNA sample was loaded onto the well of nanoquant plant and absorbance was taken at 260:280. The concentration (ng/μl) and quality of DNA was measured by optical density values obtained and used further for quality assessment of DNA. The samples were also run on agarose based gel system using 1.5% agarose gel in TBE buffer. Bands were stained with EtBr (ethidium bromide) stain and visualized under ultraviolet light for determination of DNA quality.

RNA concentration and quality was also measured with the help of Tecan ELISA cum spectrophotometer by employing nano-quant plate as explained above. In addition, RNA quality was assessed by converting it to cDNA by conventional Polymerase chain reaction using thermo revert aid cDNA synthesis kit (thermo scientific). A total of 1 μg RNA was used per sample for cDNA synthesis. The reaction conditions for cDNA synthesis were initial denaturation at 94 °C for 3 min (1 cycle), and denaturation at 94 °C for 30 s (35 cycles). It was then followed by annealing at 58 °C for 30 s and final extension at 72 °C for 30 s (35 cycles each). The synthesized cDNA was then tested for expression of housekeeping gene GAPDH by using conventional PCR. PCR product obtained after GAPDH PCR was run on 1% agarose gel and bands were visualized under UV light to gain insights into quality and integrity of cDNA formed ultimately reflecting the RNA quality.

Results

A total of 180 patient samples had been biobanked from different pediatric leukemia cases before the start of audit, out of which 104 samples were from B-ALL patients, 18
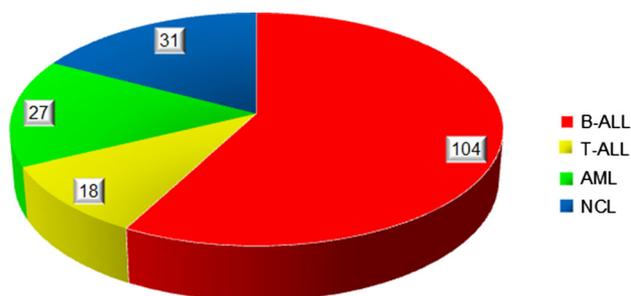

Fig. 1 Distribution of total 180 samples collected into different leukemia categories viz. B-ALL, T-ALL, AML and NCL (non classified Leukemia)

samples from T-ALL patients and 27 samples from AML patients (Fig. 1). 31 samples were non classified leukemia samples which comprised of blood samples obtained from cases that had refused treatment at our unit and hence were not processed for immunophenotyping and the group also included a subgroup of leukemia relapse as well as bi-phenotypic leukemia cases. The range for 260/280 ratio for DNA was taken as ∼ 1.8–2.0 while for RNA it was taken as > 1.8–2.1 as per standard literature.

Assessment of RNA integrity and yield from leukemia samples

Out of the 180 leukemia samples bio-banked, RNA was isolated in a total of 166 (92%) samples from B-ALL, T-ALL and AML patients and out of these 120 (72%) samples were satisfactorily processed for cDNA synthesis and ultimately housekeeping GAPDH PCR. Of the 104 B-ALL cases where RNA was extracted, 6 (6%) yielded very poor quantities and 3 (3%) gave instrument overflow reading error. The statistics for the same are shown in Fig. 2. Of 18 T-ALL, 2 (11%) yielded poor RNA quantity while rest yielded good amount of RNA as confirmed by the spectrophotometric measurements. RNA was satisfactorily extracted in 26/27 (96%) cases of AML, while one case was not processed due to presence of small clot in sample. Out of total 31 unclassified leukemia samples RNA was successfully isolated from 23 (74%) samples while one sample showed overflow error. The optical density measurements (260/280 ratio) as a measure of DNA/RNA quality for different leukemia cases are shown in Fig. 3. To further test the quality of the RNA isolated from all samples, cDNA was synthesized and GAPDH was run as housekeeping gene to assess the quality of synthesized
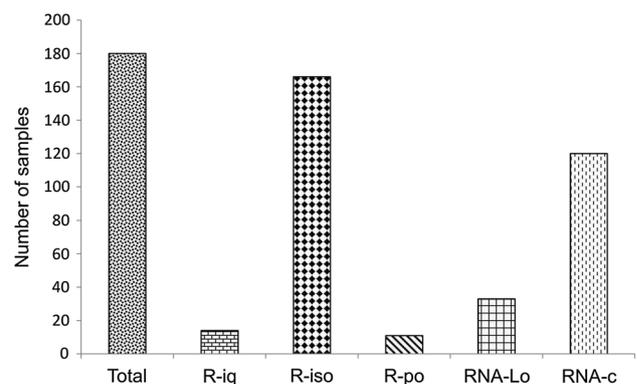

Fig. 2 Metrics of the RNA with respect to total blood samples collected, samples obtained in inadequate amount (R-iq) which was not sufficient for RNA isolation, samples from which RNA was isolated (R-iso), RNA obtained in poor quality (R-po), low quantity (RNA-Lo) RNA and the number of RNA samples from which successful cDNA was synthesized and corresponding GAPDH was run (RNA-c)
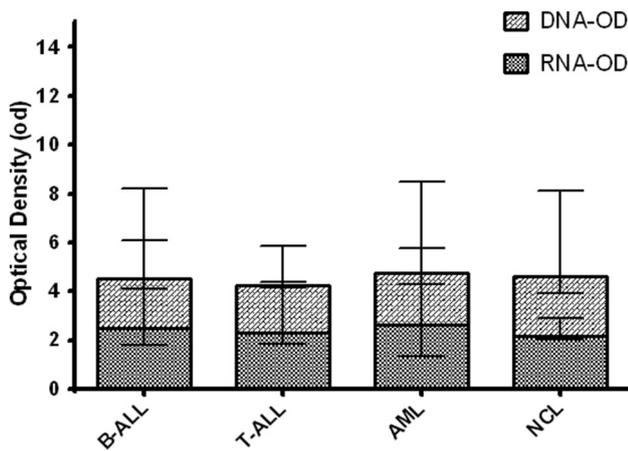
Fig. 3 Bar chart shows summary of optical density for DNA and RNA in different patient cohorts. Respective bars for DNA and RNA are stacked for comparison. Height of the bar indicates mean and error bars shows range of the data
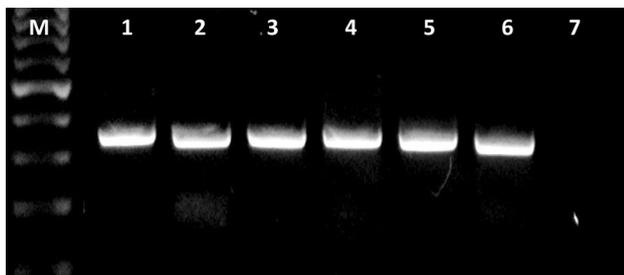

Fig. 4 Agarose gel electrophoresis (1.5% TBE) based profile for GAPDH of cDNA synthesized from RNA of B-ALL, T-ALL and CML samples-100 bp marker, 1—B.ALL-1, 2—B.ALL-2, 3—AML-1, 4—B.ALL-4, 5—T.ALL-1, 6—B.ALL-5, 7—negative control

cDNA. The integrity and quality of cDNA was found to be optimally good as confirmed by the presence of sharp and uniform bands of GAPDH (Fig. 4).

Monitoring quality and quantity of gDNA from leukemia samples

Out of total 180 samples, gDNA isolation was carried out from 104 B-ALL samples and the concentration and integrity of gDNA from 100 (96%) samples was found to be sufficient for carrying out various molecular biology related experiments (Fig. 5). Of the 18 T-ALL and 27 AML cases, 17/18 (94%) and 26/27 (96%) yielded good gDNA respectively. In non-classified leukemia samples, 26/31 (84%) samples were processed and yielded satisfactory gDNA except for one case that gave instrument overflow error. The detailed breakdown of samples with successful RNA and DNA isolation in all leukemia cases is shown in a comprehensive table (Table 1).
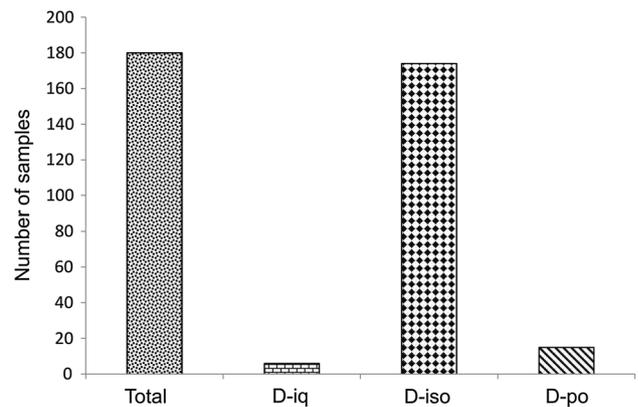

Fig. 5 Bar diagram showing statistics of DNA with respect to total blood samples collected, samples obtained in inadequate amount (D-iq), DNA isolated (D-iso), DNA obtained in poor quality (D-po)

Discussion

Bio-banks hold a great importance in the era of personalized medicine because the proper storage of human samples provides for valuable source of material for future research to understand disease biology and develop targeted therapeutic approaches. Storage of biological samples also helps to understand the prognosis as well as the cause of disease. One famous example of sample bio-banking includes the AIDS samples which were stored in bio-banks of ASB (UCSF AIDS specimen bank) much before anybody had any clue about the causative agent. Later on HIV was identified from the samples which were provided by ASB for identification of agents causing AIDS and Kaposi's sarcoma [18]. Standard literature also suggests that pilot studies before initiating a bio-bank are helpful in establishment of standard operating protocols which can be ultimately useful in proper processing and storage of pediatric samples [19].

There is not even a single method which can be considered perfect for storage and preservation of divergent samples. So it is always advisable to test the method of choice on a low number of samples by a pilot study to get details into its integrity and stability before opting for storage of large number of samples. The 260/280 ratio method is a reliable method for nucleic acid quantity and quality assessment [20]. In our study, integrity of the RNA/ DNA obtained from the samples in case of B-ALL/T-ALL/ AML indicated that − 20 °C freezing was sufficient to maintain the integrity and yield of nucleic acids in these samples even after long time of storage (∼ 1.5 years). In our study, optimal quantity RNA and DNA could be isolated in 74–96 and 84–96% of different groups of leukemia cases respectively. The overall satisfactory RNA quality yield as observed by cDNA assessment was noted in 72% cases. In cases where RNA and DNA isolation from

Table 1 Percentage distribution of DNA/RNA samples from different types of Leukemia samples according to 260/280 ratio

	B-ALL (total-104) (%)	T-ALL (total-18) (%)	AML (total-27) (%)	NCL (total-31) (%)
RNA				
+	21 (20.1)	2 (11.1)	7 (25.9)	2 (6.4)
OK	74 (71.1)	14 (77.7)	18 (66.6)	21 (67.7)
−	0	0	1 (3.7)	0
NA	9 (8.6)	2 (11.1)	1 (3.7)	8 (25.8)
DNA				
+	16 (15.3)	0	2 (7.4)	8 (25.8)
OK	78 (75)	17 (94.4)	24 (88.8)	18 (58)
−	5 (4.8)	0	1 (3.7)	0
NA	5 (4.8)	1 (5.5)	0	6 (19.3)

+, 260/280 ratio > standard range

OK, 260/280 ratio ∼ standard range

−, 260/280 ratio < standard range

NA, sample not available

samples did not yield optimal quantities (6% B-ALL, 11% T-ALL and 22.5% non classified cases for RNA isolation) and (3% B-ALL, 6% T-ALL and 16% non classified leukemia cases for DNA isolation), the most common reason was availability of a very low initial sample quantity (0.5–1 ml) for bio-banking processing. Since, we avoided separate pricks from children for keeping bio-banking samples (difficult withdrawing large sample volumes in pediatric children) and utilized their routine hemogram samples for processing and nucleic acid isolation, hence sample volume was a limiting factor especially in cases with relatively low TLC count or less number of blasts in peripheral blood. Although the quality of RNA/DNA checked through 260/280 and gel electrophoresis methods were suitable for our downstream processes such as, PCR applications, MLPA etc. however, it is to be noted that more sensitive techniques like using qubit fluorometer and or bioanalyzer, especially for RNA integrity assessment, should be included in the quality control procedure if the downstream applications required are next generation sequencing and or digital PCR.

Typical 260/280 ratio for RNA and DNA has been defined to be 2.0 and 1.8 respectively. In case of DNA a ratio of < 1.8 indicates a protein contamination (samples should be re-precipitated in ethanol and further stored into Tris EDTA buffer) while ratio > 1.8 is an indicator of RNA contamination [20]. The overall 260:280 ratio fluctuated from optimal values in 30% RNA and 20% DNA samples in our study but the same did not affect the downstream applications as indicated by uniformity of DNA bands obtained in gel run. This variation could be due to improper technical expertise and or inter-personal sample handling errors as different research staff are involved in sample processing and isolation on a rotational basis. In addition as per studies, although the 260/280 ratio and

profile of the spectra obtained are an important criteria for assessment of sample quality, the best parameter for RNA/DNA quality is their suitability and functionality for downstream applications as per requirement [20]. Higher 260/280 ratio are generally not an indicator of any issue. It may be worth mentioning that, occasionally there are problems encountered with samples even if the purity ratios are within the defined limits. Shabihkhani et al. [1] have reported the degradation of RNA after long time of storage at − 80 °C while DNA was reported to maintain its integrity even after 5 years. Since all our RNA extraction pellets were stored in RNA later before extraction, the study highlights the utility of RNA later in maintenance of quality of RNA for long time storage which can be especially useful in settings where less sophisticated infrastructure is available. Moreover post cDNA synthesis, the remaining RNA can be stored in ethanol for even longer period of time. As in our pilot study we have checked the integrity of nucleic acids up to 1.5 years at − 20 °C, quality checks in the following years will be required to further assess the longer period storage of nucleic acids at − 20 °C and amendments in the storage procedure may be required if the quality/quality falls down in subsequent years. It may be worth mentioning that T-ALL samples in our study yielded very good quantity and quality of RNA and DNA which may be due to the fact that most T-ALL cases have high TLC (> 100×10^9/L) at presentation and hence even low sample volumes resulted in a good cell pellet for nucleic acid extraction.

In nutshell, our study highlights the fact that DNA/RNA from pediatric leukemia samples are quiet stable at − 20 °C and can be successfully bio-banked for a long period of time especially where resource constraints are paramount. However, for RNA extraction, the pellets need to be stored in RNA later to maintain the long term yield.

Further, the limited facilities should resort to timely audit of their bio-banked samples so that corrective measures can be taken to improve quality and be able to preserve valuable genetic information from their samples.

Conclusion

This study is a preliminary step towards establishment of a bio-bank in a tertiary care hospital. It is imperative to say that sample collection, processing, handling, preservation, freeze–thaw cycles can affect the quality of sample to a great extent. This study sheds light on the effect of conventional − 20 °C on the integrity and quantity of nucleic acids obtained from blood samples. It's a pilot study which can be helpful in standardizing and establishment of standard operation procedures (SOPs) for a robust functional bio-bank for proper storage of large number of pediatric leukemia samples. However further deep investigations involving use of rigorous parameters for assessment of integrity and quantity of nucleic acids are needed to be carried out regularly to ensure the same.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was taken as a standard practice before biobanking of samples. The article is not funded by any support.

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