RNA Preservation and Stabilization

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Abstract

RNA quality and integrity is a prominent issue in gene expression analysis which emerged as a critical tool in life science researches, drug discovery and optimization of bioproduction. Handling and preservation methods including formalin fixation, flash freezing and chemical preservatives (sulfate solution and TRIZol) and commercial compounds (RNAlater, Allprotect and PAXgene) are widely applied to keep high quality RNA within fresh tissue samples. In this article, we tried to give a general idea on basic aspects of the above methods.

Keywords: RNA; RNAlater; TRIPol; RNase; Allprotect; PAXgene

Introduction

Gene expression analysis provides information at which a particular gene pattern may be expressed by cellular responses. Gene expression is evaluated by means of reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time RT-PCR (qRT-PCR) as well as multiplex gene expression quantification arrays and, data analysis depends on the RNA integrity and stability isolated from cells [1]. RNA quality is influenced by warm and cold ischemic durations, cellular stress responses, tissue processing protocols and storage conditions [2,3]. Isolation of highly pure intact RNA is vital for successful quantification of gene expression [1,4] so that RNA preservation is an essential subject during handling processes [5]. Since RNA degradation takes place by cytoplasmic RNase [4] thus, RNase inhibition is a main approach in RNA extraction and subsequently gene expression analysis [1,4].

Current methods

Formalin fixation

RNA degradation correlates with the length of delay in sample fixation or preservation. Most pathology departments still rely on tissue preservation with formalin fixation followed by paraffin embedding step [3]. Although it is suitable for morphologic assessment, it is unable to keep intact RNA [5].

In fact, formalin fixative keeps tissue structure via cross-linking of cellular proteins. In addition, there also happen cross-links between proteins and nucleic acids. Mono-methylol groups (-CH2OH) added to all four bases of RNA create methylene bridges between two amino groups. Although it makes formalin an ideal fixative hardening agent for histopathological analysis, cross-linking of proteins and nucleic acids causes intact mRNA extraction from formalin-fixed tissues to be difficult [3,4,6,7]. RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues is chemically modified and dramatically degraded. It is often present in fragments less than 300 bases in length [4,5,8] and may produce reliable data if selected genomic sections be heavily amplified using specific primers and short amplicons (<300 bps) [4,10]. In addition, RNA chemical modifications in FFPE tissues can be reversed with proteinase K digestion and preheating in guanidinium or TE (10 mM Tris-HCl, pH 7.0, 1 mM EDTA) or citrate (pH 4.0) buffers making it more amenable to RT-PCR yet, the major drawback of formalin fixation (i.e. fragmentation) remains uncorrected [4,9,10]. On the other hand, labile and low mRNA transcripts of specific genes may also remain unamplifiable [4]. Therefore, biopsy tissues have to be preserved with alternative methods providing higher-quality RNA for gene expression analysis. It should be noted that small RNA molecules (such as mature mi RNA) are less affected by formalin and can be recovered more easily and efficiently in the extraction process [8].

This highlights the FFPE archived tissues are valuable resources for miRNA expression analysis [8,11].

Formalin can be substituted with other fixatives such as ethanol, methanol, Carnoy fixative (60% ethanol, 30% chloroform and 10% glacial acetic acid) and methacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid). These fixatives would avoid hydroxymethylene cross-linking between proteins and nucleic acids and, keep tissue morphology and RNA integrity [5,12,13].

Flash freezing

Flash freezing (also called snap freezing) is a better method for providing high quality RNA for high throughput expression analysis as compared to formalin fixation [4]. RNA stability in intact fresh frozen tissue is due to both preserved cellular structure [14] and RNase inactivation [4]. In this method, small fragments of fresh tissue (approximately 0.1 cm3) are immediately frozen (after half an hour of surgical resection) using immersion within liquid nitrogen (~196°C) or on dry ice (~78°C) and finally stored at -80°C or below and, never allowed to thaw [4,14-17].

Although optimal RNA quality is preserved using flash freezing, this method also has a few limitations. It may not be practical if there is not access for freezing facilities and sample collection is decentralized (i.e. samples are gathered from medical services in different locations) [4]. To reach the place where freezing facilities are available, transportation of fresh tissues on ice would be a practical solution, as it does not compromise RNA integrity and gene expression profiles in tissue [4,18]. Even brief thawing of flash frozen sample before homogenization in a guanidinium-based lysis solution (containing 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl and 0.1 M 2-ME) [19] can lead to RNA degradation [5,15] so that, it has to be grounded at cryogenic temperatures (below ~150°C) [15].

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Received January 02, 2014; Accepted February 04, 2014; Published February 10, 2014


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general, flash freezing disadvantages including availability, expense of cold shipping and storage as well as risk of RNA degradation prior to homogenization [3,5,6,12,18] impose great needs for applying other preservative methods.

**Chemical preservatives**

Aqueous sulfate solutions (containing 25 mM sodium citrate, 10 mM EDTA and 70 g ammonium sulfate/100 ml solution, pH 5.2) precipitate degenerative RNases and other solubilized proteins resulting in preserving tissue with intact RNA. In this way, tissue can be stored at room temperature for weeks and at -60°C for long-term periods [18,20]. Other chemical reagent called TRIzol, consisting of phenol and guanidine isothiocyanate can preserve high RNA quality and quantity [21]. However, it destroys tissue structure, making it unamenable for histomorphological studies [22].

**Commercial compounds**

RNAlater (Ambion, Austin, TX, USA) is an available preservative in which small tissue pieces (less than 0.5 cm thickness) are immediately submerged to stabilize cellular RNA without the need to freeze samples. This solution preserves intact RNA by precipitating out RNases into an aqueous sulfate salt solution [4,18,20]. With RNAlater, tissue can be stored at room temperature for up to 1 week, at 4°C for up to 1 month and at -80°C for a long time. Based on the passive diffusion of reagent, thin tissue pieces must be prepared to penetrate rapidly into the tissue before RNA destruction by RNases. Moreover, aggregation of fragments may prevent fine diffusion that can be obviated either by gentle agitation or placement in a large container [18].

Allprotect tissue reagent (Qiagen) is another commercial stabilizer designed to preserve DNA, RNA and proteins in tissue samples concurrently [23-25]. DNA, RNA and proteins can be stabilized in freshly harvested tissues if the tissues are immediately submerged in Allprotect at room temperature, eliminating the need for liquid nitrogen and dry ice. It is claimed that stabilized tissues can be transported at 15-25°C for up to 7 days, or stored at 2–8°C for up to 12 months. Long term storage of tissues is also possible at -20°C or –80°C [25].

PAXgene (Paxgene Tissue System, PreAnalytix GmbH, Hombrechtikon) is other commercial product used to improve tissue molecular quality and morphological analysis. PAXgene fixation reagent is a non-carcinogenic, non-cross-linking mixture of different alcohols and other chemical compounds that can rapidly preserve tissue morphology and bio-molecules up to 26 months at –20°C [26]. It has been reported that fixed, paraffin embedded (PFPE) tissues with PAXgene are morphologically similar to those preserved in neutral buffered formalin and can be used for immunohistochemical staining. In addition, RNA quantity and quality extracted from PFPE tissues are almost similar to that from flash frozen tissues and, is significantly better than what obtained from their FFPE counterparts [3,27,28].

PAXgene Blood RNA System (PreAnalytiX GmbH, Hombrechtikon, Switzerland) is a well-described product for RNA stabilization in human blood [29]. PAXgene Blood RNA tubes are prefilled with a RNA stabilization reagent that stabilizes intracellular RNA for 3 days at 18-25°C and, 5 days at 2-8°C. Additionally, it can be kept at -20 to -70°C for long terms (Table 1).

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sample</th>
<th>Period</th>
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<td></td>
<td>Blood</td>
<td>6 months</td>
<td>Weber et al. [29] PAXgene vs. RNAlater</td>
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**Table 1:** Studies on current methods for RNA preservation.
RNA later versus other preservations

Some studies suggested that RNA later (Ambion Inc, Austin, TX) improves RNA integrity and yield in comparison to flash freezing method [2]. Florell et al. showed that RNA later (Ambion Inc, Austin, TX) not only preserves RNA integrity but also keeps histologic structure [22]. On the contrary, other studies reported that results of using RNA later and flash freezing in short and long term periods are comparable [17,30,31]. Another study even showed large variability in gene expression level and RNA integrity in immersed samples within RNA later (Ambion, Austin, USA) [14]. However, ease of use and handling of decentralized samples are considered with RNA later. Moreover, it is a safe, non-toxic solution that can be stored at ambient temperature precluding the need for specialized equipment such as liquid nitrogen and thawing RNA preserved tissues [18,22,30].

RNA later is also compared with other commercially available RNA preservatives. In a study assessing stabilization of mRNA and miRNA, RNA later (Ambion, Austin, TX, USA) and PAXgene (Pre Analytix, Hombecthickon, Switzerland) were shown to be suitable for RNA stabilization in blood and, isolation of good quantity and quality RNA which was well suited for qRT-PCR [29]. Another study showed no significant differences in RNA, DNA and protein qualities and yields between RNA later (Qiagen, Cat. No. 76104) and Allprotect (Qiagen, Cat No. 76405) reagents. They also noted a modest trend towards better yields of RNA when Allprotect was used. This was in contrast with the results of another study demonstrating higher yields of RNA in samples immersed in RNA later [23]. In addition, it is noteworthy that using RNA later would be more desirable since Allprotect is more expensive than RNA later and more difficult to work due to its viscosity [23,32].

Conclusion

In general, decision on the use of RNA preservatives is based on availability of required equipment, expenses, ease of work, handling and preservation periods. If freezing facilities are available and sample collection is centralized, flash freezing as a suitable method for tissue RNA stabilization is preferred. Otherwise, the use of chemical preservatives such as sulfate solution or TRIzol may be advisable. In this circumstance, if preserved tissue is intended for both molecular and histopathological studies, the commercial compounds such as RNA later, Allprotect and PAXgene would be recommended.

However, apart from preservation methods, other parameters such as timing of tissue collection and preservation, use of different fixatives, RNA extraction procedures, tissue quantity and checking methods for RNA quantity and quality would also directly or indirectly influence RNA integrity and gene expression.

References

15. Top Ten Ways To Improve Your RNA Isolation.


30. Storage of tissue over extended periods prior to RNA extraction can degrade RNA resulting in altered gene expression patterns.
