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COMMENTARY

Stabilizing the Code—Methods to Preserve RNA Prove Their Worth

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Abstract: Commercially available platforms to stabilize messenger RNA (mRNA) and microRNA are critically designed to optimize and ensure the quality and integrity of those nucleic acids. This is not only essential for gene expression analyses, but would provide technical utility in providing concordant standard operating procedures in preserving the structural integrity of RNA species in multicenter clinical research programs and biobanking of cells or tissues for subsequent isolation of intact RNA. The major challenge is that the presence of degraded samples may adversely influence the interpretation of expression levels on isolated mRNA or microRNA samples and that in the absence of a concordant operating procedure between multiple collaborating research centers would confound data analysis and interpretation. However, in this issue of *Biomarker Insights,* Weber et al provide a detailed and critical analysis of two common RNA preservation systems, PAXgene and RNAlater. Such studies are lacking in the literature. However, the authors provide compelling evidence that not all conservation platforms are created equal and only one system proves its worth.

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Introduction—Stabilizing the Code

Since the discovery of nucleic acids by Friedrich Miescher in 1868, which he referred to as "nuclein",[1] there has been an unprecedented level of both discovery and functional appreciation in our understanding of RNA.[2–6] The pivotal role played by RNA in protein synthesis and the biochemical mechanisms of RNA synthesis has been already worked out in the late 1930's and 1950's.[2,3] Since then, RNA species have been sequenced.[4] and the process of gene sequences dampening other genes has been discovered—a process known as gene silencing.[5] Regulatory RNA sequences such as microRNAs (miRNA) are post-transcriptional gene regulators that function to silence messenger RNA (mRNA). What distinguishes miRNA from mRNA are that they are quite short RNA species, usually only 22 nucleotides in length, and as such are remarkably more susceptible to degradation than other RNA species such as mRNA.

It was not until the late 1990's and 2000's that miRNA were appreciated as a unique subgroup of conserved molecular regulators capable of repressing many hundreds of mRNAs.[6,7] The importance of miRNA in biological systems is pivotal since specific groups of miRNAs exert both negative and positive gene regulatory functions. It is no surprise then that mRNA species are thought to be tightly regulated by miRNA in a cell type and tissue/organ type specific manner and are likely to be involved in almost every biological or physiological process and in health as well as disease states.[8–10]

Isolation of highly pure intact mRNA and miRNA is critical for the successful quantification of the expression of those RNA species. In cancer research for example, cellular and tissue profiling requires precise quantification of mRNA and miRNA expression. Fortunately, the tools for such expression profiling are readily available and include reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time RT-PCR (qRT-PCR) as well as multiplex gene expression quantification arrays that permit simultaneous gene-expression analysis of disease- or molecular-pathway specific gene set matrices. However, despite such methodological platforms being widely available commercially or having been developed as a standard operating procedure (SOP) in the molecular biological research laboratory, the quality and integrity of the input RNA is a crucial variable that can not and should not be compromised.

The quality of gene expression data analyses obtained by the experimental platforms described above, are stringently dependent on the integrity and stability of the mRNA isolated and purified extracted from the tissue or population of adherent or suspension cell populations. This is of course also dependent proper optimal processing of the tissue or cellular on samples. The main concern in isolating highly pure and intact RNA is the exquisitely fragile nature of mRNA and miRNA and their susceptibility to degradation by RNAses during the isolation procedure. Though many other variables such as the source of RNA (cultured cells versus primary cells or tissue; hypoxic environment versus normoxic environment; good laboratory technique in handling RNA and so on) all play a role in retaining the intactness of RNA species during the isolation and purification procedure, there are commercially available tools to assist the investigator in preserving the intactness of RNA prior to subsequent extraction and purification.

Methods to Preserve RNA Prove Their Worth

The main challenge in the laboratory is the ubiquitous presence of RNAses—robust enzymes that can quickly and efficiently degrade purified RNA species. RNAses are present in both eukaryotes and prokaryotes, indeed almost every cell type in humans express RNAses, in part because of their microbicidal properties and their important role in nucleic acid metabolism. Standard practice in the research laboratory can minimize or avoid degradation of the purified RNA or miRNA species. This includes such basic considerations as good aseptic techniques during all downstream processes, ensuring materials and reagents that come into contact with RNA are RNAses-free, use of high-quality sterile plasticware that is guaranteed free of RNAses, frequent changes of nitrile gloves, and use of respirator-type dust masks and other personal protective equipment (PPE) can markedly dampen the risk of contamination from human skin, perspiration, tears, saliva and so on. Thus, methods to control RNAse contamination in the laboratory are

essential. Other useful laboratory precautions when handling mRNA and miRNA include pre-treatment of benchtops, pipetting devices, test tube racks and so on with a proprietary reagent called RNAseZap. In addition, water used for buffer preparations and solubilization of RNA should be RNAse-free or at least be treated with diethylpyrocarbonate (DEPC) though one needs to be particularly careful in matching buffer compatibility with the use of DEPC in the laboratory. For example, this would include buffers containing primary amine groups like Tris or those containing secondary and tertiary amines like HEPES. Such buffers can not be DEPC treated since they consume active DEPC quite quickly. However, alternative reagents can be used for inactivating RNAses in solution. One such reagent is RNAsecure resuspension solution which when introduced to a buffer or even purified RNA pellets can inactivate RNAses. By heating the buffer or other biological reagent solution to 60 °C for about 10 minutes, RNAsecure is "activated" and can thus be used to destroy RNAses introduced to a buffer or reagent solution "post-treatment".

However, in more complex situations such as in laser capture microdissection (LCM) analysis, maintaining or even guaranteeing high quality RNA in the microscopic isolation of cells for LCM analyses provides an additional challenge.[11–13] Analysis of gene expression by LCM requires the precise isolation of highly pure microscopic populations of cells from a quite heterogeneous tissue or organ source for subsequent purification of the RNA.[14] The problem with LCM is that RNA degradation occurs rapidly and frequently such that reliable gene expression analyses of the transcriptome of interest are severely compromised.[15,16] While frozen sections assist in the recovery of intact RNA,[17] use of fixatives and the chemical components contained within them as well as staining reagents can adversely affect RNA stability and integrity. For such techniques, often quite sophisticated, investigator-initiated protocols need to be applied.[18]

In this issue of *Biomarker Insights*, Weber et al, have critically assessed the stabilization of mRNA and miRNA species using human peripheral blood samples and two common platforms for RNA preservation: PAXgene and RNAlater.[19] The authors point out that a major goal in cancer research and related clinical trial programs is the identification and validation of certain tumor-associated biomarkers in biological samples that can be readily accessed such as peripheral venous blood. One of the key challenges of such biomarker discovery is the multi-center approach to such research. This provides the challenge of time and distance variables from the point at which samples are accessed, for example from participating study centers to the point where samples will bio- or tissue-banked for subsequent analyses. The main concern is that not all study centers participating in such a multi-center collaborative group have access to the expertise and necessary equipment that would permit extraction of intact, quality RNA species. Under such circumstances, it would be an appropriate additional step to focus the receipt of such samples to a common receiving laboratory that would have in place a concordant and reliable SOP for the extraction and purification of RNA species common to all participating study centers. This obvious step would remove one of the major variables that may otherwise compromise gene expression data analysis between collaborating laboratories.

In their study, Weber et al compared the utility of two common RNA preservation methods, namely PAXgene and RNAlater, both of which are commercially available.[19] Both platforms are capable of stabilizing RNA and providing the advantage that RNA species need not be isolated immediately on sampling a peripheral blood specimen from a study subject. While PAXgene has traditionally been employed for stabilization of RNA in human venous blood samples and particularly of mRNA,[20,21] it is less clear whether PAXgene stabilized venous blood similarly stabilizes the structural integrity of miRNA species. However, it has been reported that PAXgene fails to adequately stabilize specific gene transcripts, at least under particular circumstances.[22] For this reason, Weber et al explored the utility of the RNAlater system—a relatively common stabilizing system for studies that require intact RNA for gene expression analyses and biobanking. In a comparative analysis, Weber et al assessed the relative merits of both the PAXgene system and the RNAlater platform for RNA stabilization in human peripheral venous blood.

In a very well controlled and designed study, Weber et al tested both systems on freshly drawn anticoagulated blood held at ambient or room temperature, as well as blood samples that were anticoagulated and held on dry ice without storage at −80 °C, or anticoagulated and held on dry ice with storage at −80 °C for a period of 6 months prior to RNA isolation. All samples were sent to a central receiving laboratory by overnight courier service or regular mail and RNA isolated immediately or 6 months later for the samples held at −80 °C.

Weber et al found that the RNAlater system proved its worth in maintaining RNA integrity.[19] By assessment of RNA yields, RNAlater was found to be superior to PAXgene. Although this may have been caused by deviation from the manufacturer's protocol, the same variables were true for both RNA stabilization systems. One of the key quantitative measures that demonstrated the superior ability of RNAlater to preserve RNA integrity was by virtue of the RIN value, where a RIN value of >8 is considered optimal for downstream expression analyses. Peripheral venous blood samples stabilized in RNAlater consistently gave RIN values >8, while PAXgene was somewhat more variable. However, in terms of stabilizing mRNA, it was found that irrespective of shipping conditions (ambient versus frozen temperatures), both RNAlater and PAXgene performed equally well when assessing the expression of a single gene transcript, namely *ATM* (ataxia-telangiectasia mutated gene) by RT-PCR analysis.

Importantly, the authors point out that, similar to the challenges of mRNA integrity, the stabilization of miRNA species is variable, particularly with regard the stability of miRNA-26a and miRNA-26b.[19] Thus, not only is the choice of stabilization system important, but the choice of isolation and purification system for miRNA may differ somewhat from that optimized for mRNA isolation. However, despite some superior indices of yield and integrity of isolated mRNA and miRNA species using the RNAlater system, unlike the PAXgene system, there is currently no approved system of RNAlater containing vacutainer tubes used for collection of peripheral blood and stabilization of RNA. This additional step of introducing RNAlater to the collected blood sample runs the risk of cross-contamination of the sample as well as the research study personnel.

Conclusion

The RNAlater system provides a feasible alternative to the conventional use of PAXgene. However, much work is still required to properly validate and confirm the effectiveness of RNAlater in preserving a broad spectrum of mRNA and miRNA transcripts. Additionally, the broad clinical and research study utility of RNAlater for preserving RNA stability in human peripheral venous blood in multi-center clinical trials and collaborating groups requires thorough validation in double-blinded research studies.

Disclosure

This manuscript has been read and approved by the author. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The author and peer reviewers of this paper report no conflicts of interest. The author confirms that they have permission to reproduce any copyrighted material.

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