Effect of microRNA Content on RNA Purification Efficiency from Biological and Clinical Specimens using Various Isolation Procedures

Bernard Lam1, Taha Haj-Ahmad2, Vanja Misic3, Moemen Abdalla3, Mohammed El-Mogy4,5, Won-Sik Kim6, Nezar Rgehi7 and Yousef Haj-Ahmad1,2

1Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt; 2Molecular Biology Department, National Research Centre, Dokki, Cairo, Egypt

Abstract
MicroRNAs (miRNAs) are small RNAs that play important roles in gene regulatory networks by binding to and repressing the activity of specific target messenger RNAs (mRNAs). A large volume of basic research has been done to characterize miRNA biology in various tissues and cell lines of model organisms. Moreover, the potential utility of these RNAs as biomarkers in molecular diagnostics has been extensively studied. Expression profiling of miRNAs has been tested with various forms of clinical specimens including urine, blood, serum, plasma, tumor biopsies and formalin-fixed paraffin-embedded (FFPE) tissues. A number of procedures have been developed to purify total RNA for profiling these miRNA datasets. Most of these protocols are generally divided into two main categories: 1) traditional, phenol-chloroform extraction followed by acid precipitation, and 2) column-based purification with affinity resin including silica and silica columns. The majority of these methods are modifications or derivatives of existing protocols for large mRNA purification which assume that all nucleic acids are purified simultaneously. However, in small molecules such as miRNAs, parameters such as GC content could vary drastically, with published GC contents ranging from 25 to 80%. Hence, we studied the relationship between miRNA GC content and the purification efficiency of different methods using various biological or clinical specimens. Total RNA isolated using RNA methods was isolated from different inputs (saliva, bodily fluids and tissues). In addition, synthetic miRNAs of different GC contents (from 20-70%) were spiked in at the point of isolation. RT-qPCR expression profiling was performed on the isolated RNA to determine the recovery of different miRNAs (both endogenous and spiked-in). Interestingly, the purification efficiency was not uniform for miRNAs of different GC contents or for different isolation methods. Percentage recovery of miRNAs varied tremendously with the percent abundance of RNA in a specific sample and type. For example, the gold standard of phenol-chloroform extraction followed by acid precipitation performed poorly in low abundance input such as plasma and serum. In addition, different miRNA GC contents were recovered with different efficiency, with low GC miRNA generally recovered less than high GC miRNA. Hence, to ensure results obtained from miRNA expression studies can be biologically relevant, it is critical to develop a proper purification procedure that provides the least bias to miRNA composition and abundance.

Purpose of the Study
1. To test if GC content of microRNA affects its recovery during RNA isolation process across different levels of spike-in of microRNA with different GC content
2. To test if different RNA isolation methods affect the recovery of microRNA of different GC content
3. To test if the recovery of microRNA of different GC content differs in different input sample types

Materials and Methods
RNA Isolation
RNA was isolated from various input samples including HeLa cells, hamster liver, human lung, and human gastric cancer cells. Total RNA was isolated (Figure 1) with different commercial products, including: 1) phenol/chloroform with alcohol precipitation; 2) TRI Reagent (Sigma); 3) phenol/chloroform extraction with silica column purification — miNeasy Kit (Qiagen); miRNeasy bead kit (Qiagen); miRNeasy Extraction Kit (ThermoFischer); 4) non-polar purification using silica column cartridges — Total RNA Purification Kit (Romeo Biotech, CA, USA); Plasma/Serum Circulating RNA Purification Mini Kit (Stanford (Norgen) Biotek); Cell culture microRNA purification kit (Norgen Biotek, Cat. #20000)

microRNA Spike-in
miRNAs of different GC content that was studied previously (ref 1) were synthesized. The 3D and sequence information of each miRNA used in this study is summarized in Table 1. The synthesized miRNAs were reconstituted in water and mixed in equal molar fashion. The equivalent of either 50 picomols (high) or 50 femtols (low) of microRNA mix was used in the RNA isolation by different methods.

RT-qPCR
RNA was reverse-transcribed using iScript's SuperScript III system. As a control, equivalent amounts of microRNA spike-in without isolation was also reverse-transcribed in parallel. The qPCR generated was used as template in qPCR using iCycler iQ SYBR Green Mastermix on a Bio-Rad Cycler real-time PCR system. Relative loss of microRNA was calculated as the difference in Ct values between isolated RNA and control.

Results
By measuring the levels of control microRNA (miR-483) across different isolation methods and GC contents, we observed that miRNAs of different GC contents could be isolated with different efficiencies. For example, miR-483 (GC content of 25%) was more efficiently isolated by miNeasy kit (57%) compared to phenol/chloroform extraction with silica column purification (41%).

Table 1. microRNA Spike-in and the Respective GC Content. The miRNAs are divided into 3 groups (High, Medium and Low GC content) according to ref 1.

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<th>miRNA</th>
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<th>Recovery (%)</th>
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<td>miR-483</td>
<td>25</td>
<td>57</td>
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<tr>
<td>miR-130</td>
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<td>miR-153</td>
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Discussion
In order to study how each purification method recovers microRNA under the best optimized condition, without the interference of components of the sample input (such as contaminants and other nucleic acids), we tested the microRNA spike-in recovery using each isolation method without any biological input. The following observations were made:
1. Recovery of low amounts of miRNA, particularly with low GC, is very poor using phenol/chloroform extraction coupled with alcohol precipitation. Effective DNA precipitation by alcohol is concentration dependent. With the low amount of spike-in used (< 50 picomols), the RNA recovery by phenol/chloroform extraction coupled with alcohol precipitation was very low, particularly with miRNAs with very low GC content.
2. Low amount of miRNA extracted by phenol/chloroform extraction could be recovered efficiently using either silica column or silica columns. Minimum loss by GC was observed with the highest spike-in amount (30 femtols, Panel A). However, there was some bias targeted to the high GC miRNAs with the lower spike-in amount (50 femtols, Panel B).
3. Non-polar purification of miRNA spike-in using silica column cartridges resulted in the best recovery with minimal bias by GC content. Silica column cartridges were previously shown to have better affinity to miRNAs than silica columns. The study here showed that it could recover the miRNA spike-in efficiently, without use of alcohol.

Figure 1. Generalized illustration of different RNA isolation Methods used in this study

Figure 2. Difference in Recovery Efficiency of microRNA with Different GC Content in a Pure System

Figure 3. Difference in Recovery Efficiency of microRNA with Different GC Content using biological inputs (Mammalian Cells and Tissues) with high intrinsic RNA content

Figure 4. Difference in Recovery Efficiency of microRNA with Different GC Content using biological fluids (blood, plasma and urine) with medium to low intrinsic RNA content

Summary
1. General microRNA recovery is similar among different isolation methods when RNA is abundant. However, microRNA recovery from samples with low RNA abundance is significantly poorer when alcohol precipitation is used.
2. The difference in isolation efficiency affected by RNA abundance is important in considering the choice of RNA isolation methods particularly when using biological fluids such as blood, plasma, serum and urine. Use of simple, non-polar methods such as utilizing silica column cartridge is highly recommended.
3. GC content bias in microRNA recovery was not significant when the microRNA of interest is of high abundance.
4. However, there could be significant loss of microRNA with low GC contents, when such microRNAs is of low abundance. This is particularly significantly critical for studies using biological fluids. Methods that utilize silica column cartridge were shown to have least GC content bias when microRNA is present in low amount.

References
1. Davis et al. 2008. Computational Biology and Chemistry. 32:222-226

Contact Information:
Norgen Biotek
3430 Schönir Parkway
Toronto, ON M3J 2Z5
Phone: (905) 227-8848 Fax: (905) 227-1061
nezar.rgehi@norgenbiotek.com

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