Variation between the Purification Efficiency of microRNA with Different GC Content in Biological and Clinical Specimens

Bernard Lam[1], Taha Haj-Ahmad[2], Vanja Misic[2], Moemen Abdalla[1,3], Mohammed El-Mogy[1,4], Won-Sik Kim[1], Nezar Rghei[1] and Yousef Haj-Ahmad[1,2]

[1]Norgen Biotek Corp., Thorold, Ontario, Canada, [2]Department of Biology, Brock University, St. Catharines, Ontario, Canada

[3]Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt, [4]Molecular 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). The central role played by miRNAs in gene silencing mechanisms has been demonstrated unequivocally in all types of tissues and cell lines. Given such biological significance, the potential utilization of miRNAs as biomarkers in molecular diagnostics has been extensively studied. Expression profiling of miRNAs has been tested with various forms of clinical specimens including bodily fluids (urine, blood, serum, and plasma), tumor biopsies and formalin-fixed paraffin-embedded (FFPE) tissues. Among all the aforementioned samples and specimens, there is a wide spectrum of RNA content - from nanogram/sub-nanogram in bodily fluids to ten's of micrograms in various tissues. Many researchers have modified or adjusted standard protocols in order to maximize the recovery of total RNA from each of these samples. Interestingly, the majority of these methods are based on existing protocols for large mRNA purification which assume that all biomolecules purified are highly homogenous. However, in small molecules such as miRNAs, GC content could vary drastically, with published data suggesting a range of 20 - 80%. The diversity of GC base content could impact the effectiveness of miRNA purification using ion exchange resins (such as silica or silicon carbide) or phenol-based extractions. Hence, we studied the recovery of miRNAs of different GC contents from biological and clinical samples (cells, bodily fluids and tissues) of diverse RNA contents. Both column-based and phenol:chloroform-based RNA extraction methods were used. 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 efficiency of 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 certain miRNAs varied tremendously with the general abundance of RNA in a specific sample type. In particular, miRNAs with lower GC content tend to have worse recovery in most methods tested. Such bias is particularly prominent in samples of very low RNA abundance including bodily fluids such as plasma and urine. Moreover, it was evident that no single method (including the gold standard of phenol:chloroform extraction followed by alcohol precipitation) could work effectively as an "universal" protocol for all sample types. Hence, to ensure results obtained from miRNA expression studies can be biologically relevant, it is critical to select 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
   - Test with different levels of spike-in of microRNAs 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 leukocyte, human plasma and human urine. Total RNA was isolated (**Figure 1**) with different commercial products, including:

1) Phenol:chloroform extraction with alcohol precipitation
 - TRI Reagent (Sigma)
2) Phenol:chloroform extraction with silica column purification
 - miRNeasy Kit (Qiagen)
 - mirVana Kit (Ambion)
3) Non-phenol purification using silicon carbide columns
 - Total RNA Purification Kit (Norgen Biotek, Cat #17200)
 - Plasma/Serum Circulating RNA Purification Mini Kit (Slurry Format) (Norgen Biotek, Cat #51000)
 - Urine microRNA Purification Kit (Norgen Biotek, Cat #29000)

microRNA Spike-in

microRNAs of different GC content that was studied previously (**ref. 1**) were synthesized. The ID and sequence information of each microRNA used in this study is summarized in **Table 1**. The synthesized microRNAs were reconstituted in water and mixed in equal molar fashion. The equivalent of either 50 pmoles (High) or 50 fmoles (low) of microRNA mix was used in the RNA isolation by different methods.

RT-qPCR

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

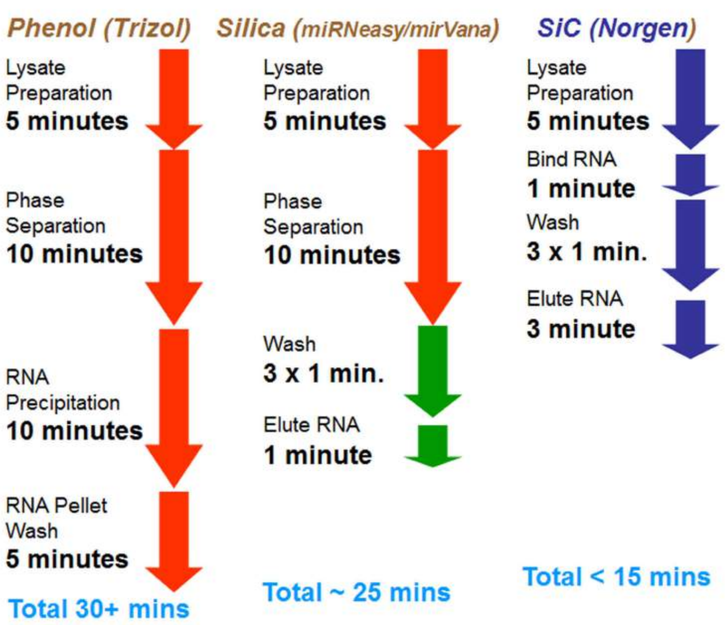

Figure 1. Generalized Illustration of Different RNA Isolation Methods used in this Study

Name	Accession	Sequence	No of Bases	GC No.	GC %	
hsa-miR-374a-5p	MIMAT0000727	UUAUAAUACAACCUGAUAAGUG	22	6	27	Low GC%
hsa-miR-450b-5p	MIMAT0004909	UUUUGCAAUAUGUUCCUGAAUA	22	6	27	Low GC%
hsa-miR-556-5p	MIMAT0003220	GAUGAGCUCAUUGUAAUAUGAG	22	8	36	Low GC%
hsa-miR-30b-5p	MIMAT0000420	UGUAAACAUCCUACACUCAGCU	22	9	41	Medium GC%
hsa-miR-125b-5p	MIMAT0000423	UCCCUGAGACCCUAACUUGUGA	22	11	50	Medium GC%
hsa-miR-132-3p	MIMAT0000426	UAACAGUCUACAGCCAUGGUCG	22	11	50	Medium GC%
hsa-miR-133a	MIMAT0000427	UUUGGUCCCCUUCAACCAGCUG	22	12	55	Medium GC%
hsa-miR-150-5p	MIMAT0000451	UCUCCCAACCCUUGUACCAGUG	22	12	55	High GC%
hsa-miR-937	MIMAT0004980	AUCCGCGCUCUGACUCUCUGCC	22	14	64	High GC%
hsa-miR-874	MIMAT0004911	CUGCCCUGGCCCGAGGGACCGA	22	17	77	High GC%
hsa-miR-615-5p	MIMAT0004804	GGGGGUCCCCGGGUGCUCGGAUC	22	17	77	High GC%

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

Results

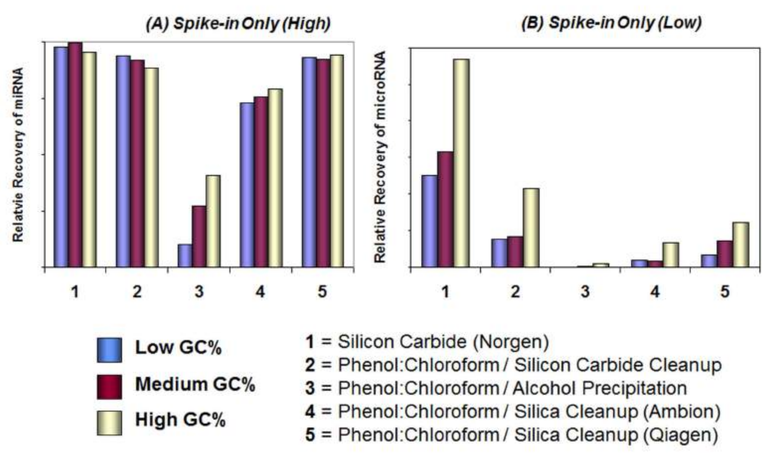

Figure 2. Difference in Recovery Efficiency of microRNA with Different GC Content in a Pure System

In order to study how each purification method recovers microRNA under the best optimized condition, **without the interference from components of the sample input** (such as contaminants and other biomolecules), we tested the miRNA 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.** Effectiveness of RNA precipitation by alcohol is concentration-dependent. With the low amount of spike-in used (< 50 pmoles), the RNA recovery by phenol:chloroform extraction coupled with alcohol precipitation is very low, particularly with miRNA with very low GC content.

2. **Low amount of miRNA extracted by phenol:chloroform extraction could be recovered efficiently using either silicon carbide or silica columns.** Minimal bias by GC% was observed with the higher spike-in amount (50 pmoles, **Panel A**). However, there was some biased towards the high GC miRNAs with the lower spike-in amount (50 fmoles, **Panel B**)

3. **Non-phenol purification of miRNA spike-in using silicon carbide columns resulted in the best recovery with minimal bias by GC content.** Silicon carbide columns were previously shown to have better affinity to microRNAs than silica columns. The study here showed that it could recover the miRNA spike-in as effectively, without use of phenol.

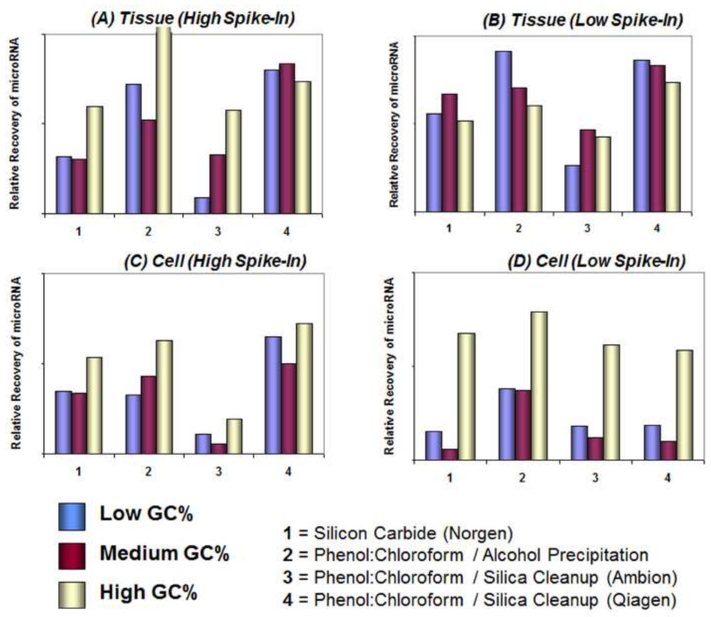

Figure 3. Difference in Recovery Efficiency of microRNA with Different GC Content using biological inputs (Mammalian Cells and Tissues) with high intrinsic RNA content

We then tested the recovery of the various microRNA spike-in from hamster liver (**Panel A** for high spike-in and **Panel B** for low spike-in) and HeLa Cells (**Panel C** for high spike-in and **Panel D** for low spike-in):

1. **All methods recovered similar amounts of the microRNA spike-in.** With the abundance of intrinsic total RNA in these tissues (in the range of >5 mg), all RNA methods (including phenol:chloroform extraction with alcohol precipitation) showed similar recovery of the microRNA spike-in. Note that all of the microRNA spike-in studied here had relatively low intrinsic level (data not shown)

2. **Minimal, non-significant GC bias for recovery was observed with high level (50 pmoles) of spike-in.** With high amount of total RNA from the input, there was not any significant difference in recovery of microRNA spike-in with different GC content (**Panel A and C**)

3. **However, microRNA with higher GC contents showed significantly better recovery when spiked in a lower level.** Both cell and tissue isolation by all methods tested showed lower recovery of microRNA with medium to low GC contents, when spiked at a lower level (**Panel B and D**)

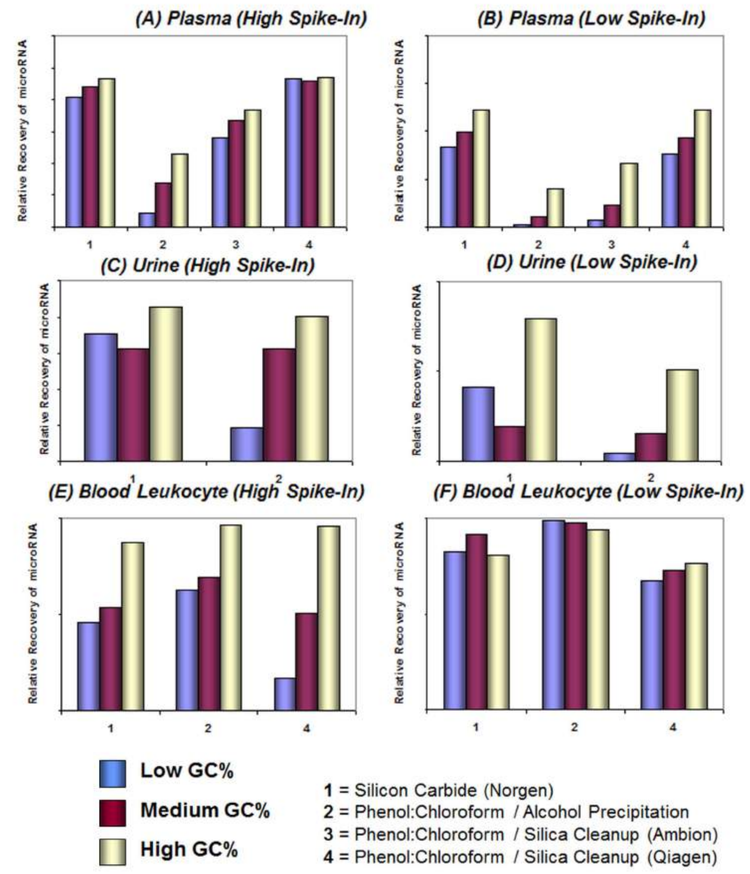

Figure 4. Difference in Recovery Efficiency of microRNA with Different GC Content using biological fluids (blood, plasma and urine) with medium to low intrinsic RNA content

There are great interests in using microRNAs as a biomarker in various biological fluids. Tremendous amount of research has been done in blood and plasma microRNA profiling. Urine, while not as frequently studied, could be a good non-invasive alternative source. The challenge in these biological fluids is that the RNA content is usually very low (< 1 to 100 ng per 100 µL). Here, we tested the recovery of the various microRNA spike-in from human plasma (**Panel A** for high spike-in and **Panel B** for low spike-in), human urine (**Panel C** for high spike-in and **Panel D** for low spike-in) and leukocyte of human blood (**Panel E** for high spike-in and **Panel F** for low spike-in):

1. **High variation in microRNA spike-in recovery by different RNA isolation methods for biological fluids.** Very poor isolation with phenol:chloroform extraction coupled with alcohol precipitation, particularly in plasma and urine (**Panels A-D**). In contrast, best recovery of RNA from plasma and urine using a non-phenol method using silicon carbide column.

2. **There were observable loss in microRNA with lower GC content.** All methods tested for plasma and urine showed relatively lower recovery of microRNA with lower GC content (**Panels A-D**). However, the non-phenol method using silicon carbide column showed slightly better low GC microRNA recovery, mainly due to the overall better recovery.

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-phenol method such as that utilizing silicon carbide column 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 microRNA is of low abundance. This is particularly significantly critical for studies using biological fluids. Methods that utilize silicon carbide column were shown to have least GC content bias when microRNA is present in low amount.

References

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Contact Information:
Norgen Biotek
3430 Schmon Parkway
Thorold, ON CANADA L2V 4Y6
Phone: (905) 227-8848 Fax: (905) 227-1061
nezar.rghei@norgenbiotek.com

Brock University

NORGEN BIOTEK CORPORATION