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

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## 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 miRNAs biology in various tissues and cell lines of model organisms. Moreover, the potential utilization of miRNAs as a biomarker 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 including miRNAs from these aforementioned samples. These protocols are generally divided into two main categories; 1) traditional, phenol:chloroform extraction followed by alcohol precipitation; and 2) column-based purification (with affinity resin including silica and silicon carbide). The majority of these methods are modifications or derivatives of existing protocols for large mRNA purification which assume that all biomolecules purified are highly homogenous. However, in small molecules such as miRNAs, parameters such as GC content could vary drastically, with published data suggesting a range of 20 - 80%. Here, we studied the relationship between miRNA GC content and the purification efficiency of different methods using various biological or clinical specimens. Total RNA including miRNA was isolated from different inputs (cells, 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 general abundance of RNA in a specific sample type. For example, the gold standard of phenol:chloroform extraction followed by alcohol precipitation performed poorly in low abundance input such as plasma and serum. In addition, miRNAs of different GC contents were recovered with different efficiency, with low GC miRNAs generally recovered less than high GC miRNAs. 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.

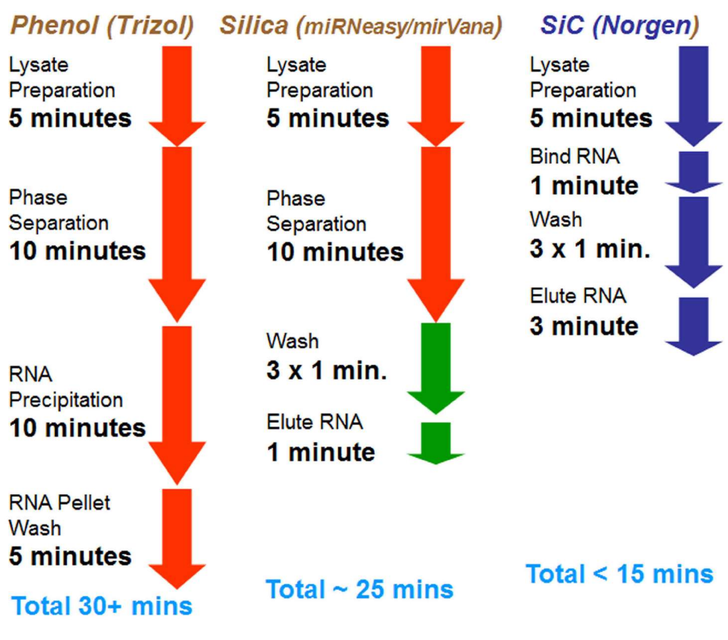


**Figure 1. Generalized Illustration of Different RNA Isolation Methods used in this Study**

| 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 | |
| hsa-miR-556-5p | MIMAT0003220 | GAUGAGCUCAUUGUAAUAUGAG | 22 | 8 | 36 | |
| hsa-miR-30b-5p | MIMAT0000420 | UGUAAACAUCCUACACUCAGCU | 22 | 9 | 41 | |
| hsa-miR-125b-5p | MIMAT0000423 | UCCCUGAGACCCUAACUUGUGA | 22 | 11 | 50 | Medium GC% |
| hsa-miR-132-3p | MIMAT0000426 | UAACAGUCUACAGCCAUGGUCG | 22 | 11 | 50 | |
| hsa-miR-133a | MIMAT0000427 | UUUGGUCCCCUUCAACCAGCUG | 22 | 12 | 55 | |
| hsa-miR-150-5p | MIMAT0000451 | UCUCCCAACCCUUGUACCAGUG | 22 | 12 | 55 | |
| hsa-miR-937 | MIMAT0004980 | AUCCGCGCUCUGACUCUCUGCC | 22 | 14 | 64 | High GC% |
| hsa-miR-874 | MIMAT0004911 | CUGCCCUGGCCCGAGGGACCGA | 22 | 17 | 77 | |
| hsa-miR-615-5p | MIMAT0004804 | GGGGUCCCCGGUGCUCGGAUC | 22 | 17 | 77 | |

**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

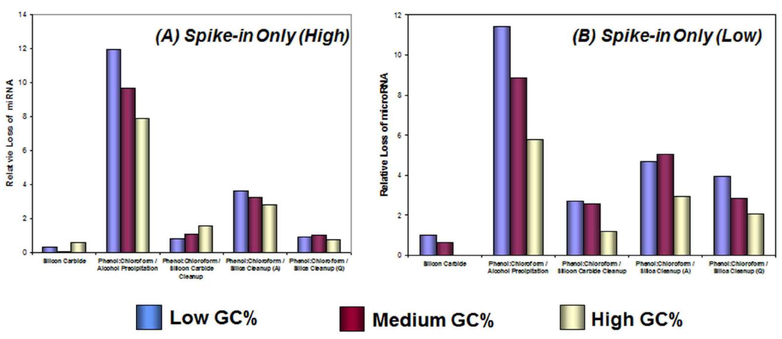


**Figure 2. Difference in Recovery Efficiency of microRNA with Different GC Content in a Pure System**

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.

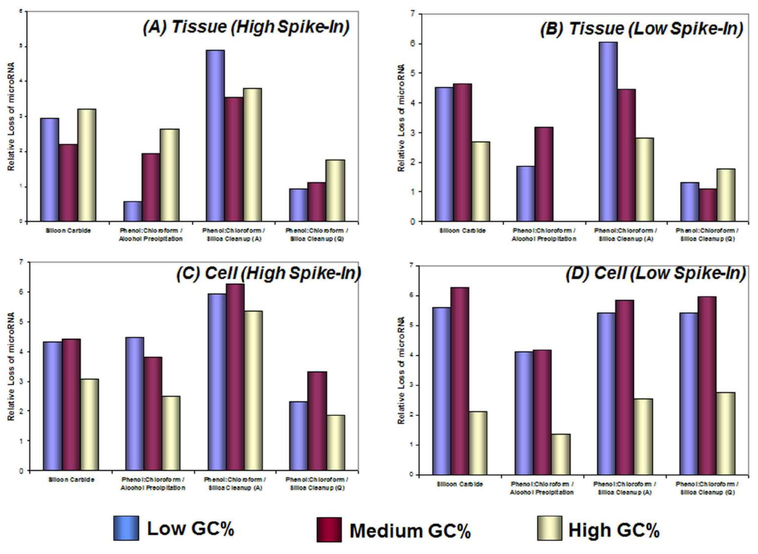


**Figure 3. Difference in Recovery Efficiency of microRNA with Different GC Content using biological inputs (Mammalian Cells and Tissues) with high intrinsic RNA content**

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**)

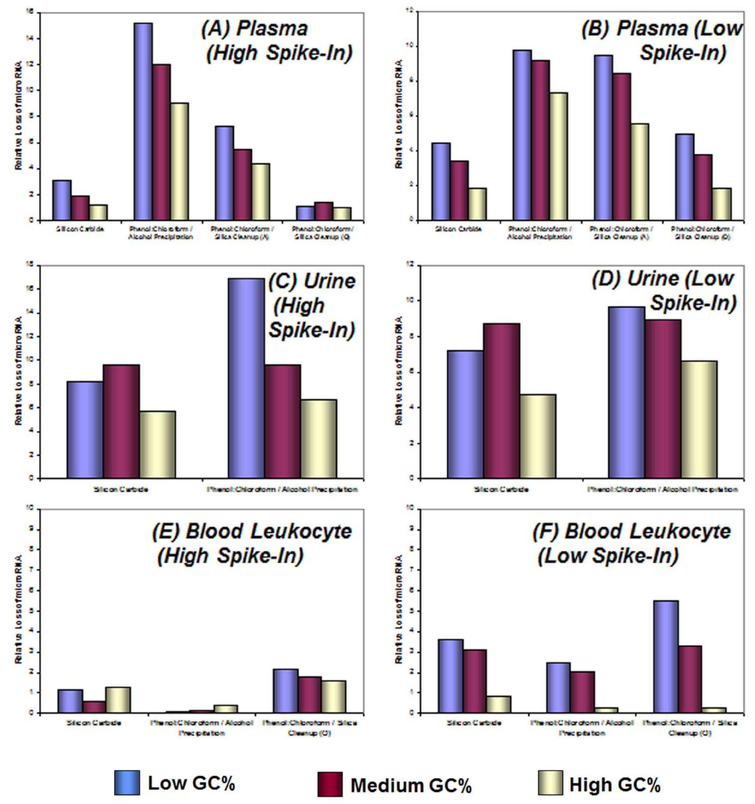


**Figure 4. Difference in Recovery Efficiency of microRNA with Different GC Content using biological fluids (blood, plasma and urine) with medium to low intrinsic RNA content**

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

Davis et al. 2008. Computational Biology and Chemistry. 32:222–226

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