RESEARCH ARTICLE

HUMAN URINARY RNA EXOSOME: OPTIMIZING METHODS OF ISOLATIONS AND SAMPLE PREPARATION FOR TRANSCRIPTOME

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ABSTRACT

Introduction: Human urine is potential bio-fluid to study as a diagnostic biomarker method. There are substances that secreted from metabolism residue and damaged cell, including genetic substances that cast away through urine, and RNA (Ribonucleic Acid). Recently, RNA (coding-ncRNAs) has been developed for diagnosis method because it could represent profile expression in the cell. Furthermore, RNA urinary isolation methods are important to be established to get reliable biomarker non-invasive compared to tissue biopsy. In fact, that optimization method for isolation RNA from urine is not clear. So that, the optimization and stability storage study are needed to be used as reference standard protocol. This study performed to know the optimization methods of RNA isolation from urine samples and RNA concentration stability storage for transcriptomic (non-coding RNA) analysis.

Methods: Each sample was collected as many 15 mL in the morning and treated with lysis solution from different manufacturers (Qiagen, Ambion, Geneid, control without buffer). ANOVA Statistic analysis was performed to know significant difference between methods used.

Results: RNA stability measuring of RNA and DNA observed on day 1, 3, 5, 7, 9, 11, and 13-day by P-value > 0.01. at the same time, RNA stability storage is known to decrease consistently by 0.1-1 ng each day. Quantifikasi miRNA could be done from urine samples.

Conclusion: There is no significant difference between all the methods used.

Keyword: urine, lysis buffer, RNA isolation, stability

Introduction

The last few decades study with urine was not much done yet. Urine sample expected can be a non-invasive biomarker on several types of disease so as can replace some gold standard diagnosis like tumour biopsy. Some study has indicated that a heterogeneous population of nanosized structures (microvesicles, apoptotic vesicles and exosomes) secreted and found all cell types of biological fluids (Morrissey et al., 2015; Kind et al., 2007; Bosso et al., 2008).

The exosome is small carrying (30-120 nm) various molecular constituents of their cell of origin, including proteins and RNAs (mRNAs, miRNAs and non-coding RNAs) that represented tissue specific. Many types of cell have capacity to secreting exosome, including reticulosis (Pan, B-T. and Johnstone, R.M.,

1983), dendritic cell (Thery, C et al., 1996), B cell (Raposo, G. et al., 1996), T Cell (Blanchard, N et al., 2002), Mast Cell (Raposo, G. et al., 1997), Epitel cell (Van Niel, G et al., 2001) and tumour cell (Mears, R et al., 2004). miRNAs exosome can be stable in blood, urine, and body fluids, and exosome can reflect tissue or origin cell in the presence of protein specific of the surface (Simmons, M. and Raposo, G., 2009; Mathivanan, S.et al., 2010; and Gross, J.C. et al., 2012).

In this study, we have performed the activity and stability of ribonuclease from the urinary sample. which can be found in almost all living organisms, is quite stable and immediately contaminates RNA degradation samples. RNA is less thermodynamically stable material than DNA because the 2'-OH group is present in the ribose ring, resulting in hydrophilic bonds directed towards the 5'-3 'phosphodiester bond. Temperature rise and elevation of pH can cause RNA damage. Beside it, we have compared the four groups using different commercial lysis buffer ambion (Cat no. 10608970A), GeneAid (Cat no. MK 11107), Qiagen (Cat no. 127154224) and without lysis buffer to know potential methods to isolate and storage.

The activity of ribonuclease, While many consider the temperature of -80o C as the standard in storage. At Biochemistry and Molecular Biology Laboratory of Gadjah Mada University Medical School, we assume that RNA is best kept at ultra-low temperatures, so a temperature of -80o C has been continuously used to preserve Sample RNA. The objective of this study was to know the most optimal method of isolating RNA in urine and to know the stability of RNA concentration on deep freeze storage.

Material and Methods

Sample collection and Exosome Isolation

Urine samples from 16 human urine have been collected in the morning with at the rate 1,1 mL using tube collection 15 mL and centrifuged at 10.000 x g for 5 minutes. Exosome isolation was performed using miRCURRY Exosome isolation kit – cells urine and CSF, 12-80 rxns (cat no. 3001012 Exiqon, Denmark). All the procedures were followed the manufacture recommendation.

RNA Isolation

Four methods were used to know the most potential methods for isolation RNA from urine. They were Buffer lyses ambion (Cat no. 10608970A), GeneAid (Cat no. MK 11107), Qiagen (Cat no. 127154224) and without lysis buffer. 100 µL samples were a vortex for 15 seconds. Incubation for 10 minutes and centrifuge for 300 x g for 1 minutes at the temperature 20°C. 100 µL Supernatant was used as a template RNA isolation. Isolation RNA used miRCURY RNA Isolation Kit Bio-fluids (Cat no. 300112 Exiqon Denmark). The Results of total RNA have been measured using Nano-drop GE NanoVue Plus. All the procedures were followed the manufacturer's recommendation.

Synthesis cDNA

Synthesis cDNA used Universal cDNA synthesis kit II, 8-64 rxns (Cat no. 203301, Exiqon Denmark). Total RNA was used as a

template, then cDNA concentration calculated using by Nanodrop GE Nano Vue Plus.

RNA Stability test

To determine the stability of RNA in -80oC storage, concentration RNA samples were calculated on: day 3rd, 5th, 7th, 9th, 11th, and 13th-day using by Nanodrop (NanoVue GE).

qPCR and data analysis

qPCR analysis of miRNA gene expression was performed using qPCR CFX 96 Thermocycler (Bio-Rad). Quantification used SYBR Green miRCURY LNATM microRNA PCR Excellent Each SYBR® Green Master mix concentred (Exiqon, Cat no. 203402). All procedure followed the manufacture's recommendation. All data statistical was determined using SPSS, Graph Pad to know significance difference methods that used.

Results and Discussion

RNA isolation from urine samples

This study using urine samples has not been widely performed. Isolate RNA from urine samples have some advantages for diagnosis and drug development. We have done to approach to look at predictive markers based on non-coding RNA from urine exosome PCA. All type of the cell-secreted microvesicles include: (1) prostasom with size 150-500 nm, produced by epithelial cells of prostate ducts which are the normal components of seminal fluid and play a role in male fertility (Burden et al., 2006); and (2) exosomes of 30-100 nm, special nanovesicles with cup-shaped morphology (Mitchell et al., 2009). Cells can communicate via membrane transfer by secretion of exosomes/microvesicles (Ratajczak J. et al., 2006). Nucleic acids have been identified in exosomes, ie mRNA, microRNA (miRNA) and other non-coding RNA (ncRNA) (Sato-Kuwubara, Y. et al., 2015). Many cells have capacity to release exosome, including reticulocytes (Pan, BT and Johnstone, RM, 1983), dendritic cells (Thery, C et al., 1996), B cells (Raposo, G. et al., 1996) T cells (Blanchard, N et al., 2002), mast cells (Raposo, G. et al., 1997), epithelial cells (Van Niel, G et al., 2001) and tumor cells (Mears, R et al. , 2004). Exosomes miRNAs are stable in blood, urine, and body fluids, and exosomes reflect the tissue or cell origin. (Simmons, M. and Raposo, G., 2009; Mathivanan, S.et al., 2010; and Gross, JC et al., 2012). RNA Exosome is more stable than RNA extracted from whole urine (Kevin C, et al, 2010). Microvesicles from exosome resist to RNase and DNase digestion and still protect the nucleic acids contained within them (Zhou, et al. 2006). Unlike whole cells, microvesicles (exosome) are quite resistant to freeze-thawing, and nucleic acids can be extracted from the urinary exosomes following freeze-thawing (Russo et al., unpublished data). So that isolating RNA from urine exosome is more suitable for transcriptome (RNA) isolation.

miRCURYTM Exosome Isolation Kit (EXIQON) used to isolate exosomes from urine samples. Subsequently, RNA was isolated from urine exosomes with three different lysis buffer (Ambion, Geneaid, Qiagen) as additional buffer and control with no additional buffer. after that RNA was synthesized into cDNA. RNA dan cDNA concentrations measured with nanodrop and analyzed with Anova

Table 1. The results of RNA isolated concentration measurements

Treatment	RNA concentration (ng/µl)
Without lysis buffer	5.0
Ambion	6.8
GeneAid	6.3
Qiagen	8.0

Table 2. The measurements of cDNA concentrations from RNA synthesis results

cDNA			
Without lysis buffer	Lysis Buffer		
	Ambion	Genaid	Qiagen
345			
338.5	371.5	357.5	337.5
351.5	364.0	359.5	381.0
307.5	379.0	379.0	358.5

Table 3. One Way ANOVA Analysis optimization of RNA isolation method results

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Without Buffer	3	15.1	5.033333	0.723333
Ambion	3	21.2	7.066667	2.443333
Genaid	3	19	6.333333	5.453333
Qiagen	3	23.9	7.966667	2.843333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13.83333	3	4.611111	1.608995	0.262309	7.590992
Within Groups	22.92667	8	2.865833			
Total	36.76	11				

Based on result Qiagen has been a higher mean score of RNA concentration (8.0 ng/µl), then second is ambion with RNA concentration 6,8 ng/µl, Genaid 6,3 ng/µl and without additional buffer 5,0 ng/µl RNA. The mean of concentration data analysis has been performed with ANOVA.

Anova statistical analysed indicating P-value (>) 0.01, its mean that the concentration showed at each group of RNA isolation method using 4 treatments have no different significant value. A lysis buffer is a buffer solution used for the purpose of breaking open cells for use in molecular biology experiments that analyze the compounds of the cells. Most lysis buffers contain salts (e.g. Tris-HCl or EDTA) to regulate the acidity and osmolarity of the lysate. Sometimes detergents (such as Triton X-100 or SDS) are added to break up membrane structures (Posch, 2014). Lysis buffer is an important thing in isolating RNA step because get an optimal lysis buffer to break up the membrane in exosome, can get a better concentration of total RNA.

We performed quantification miRNA using miR-21, miR-22, miR-19a and miR-19b. The results of miRNA expression can be seen in figure 1 and 2. Quantification expression of miRNA can be able to detect from urine samples.

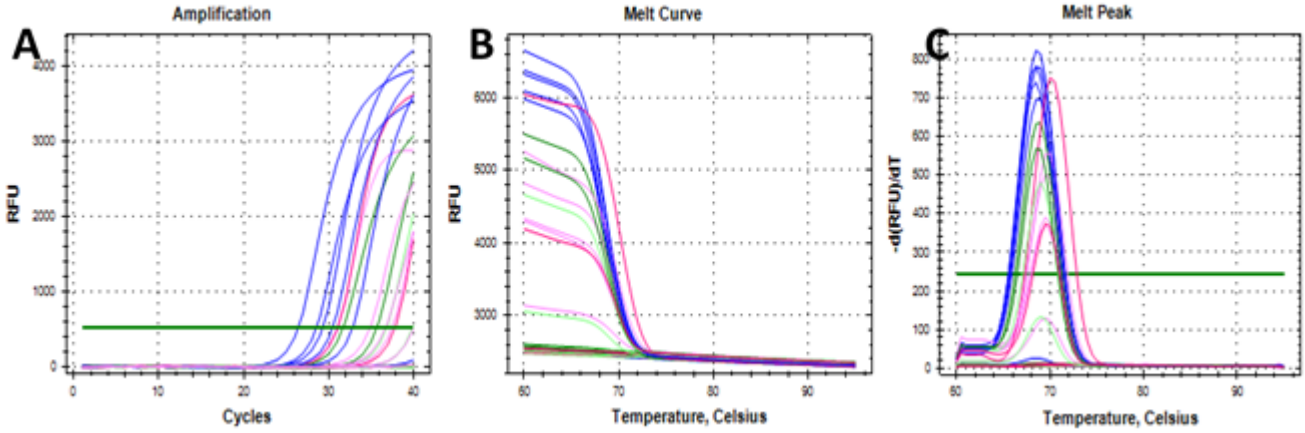
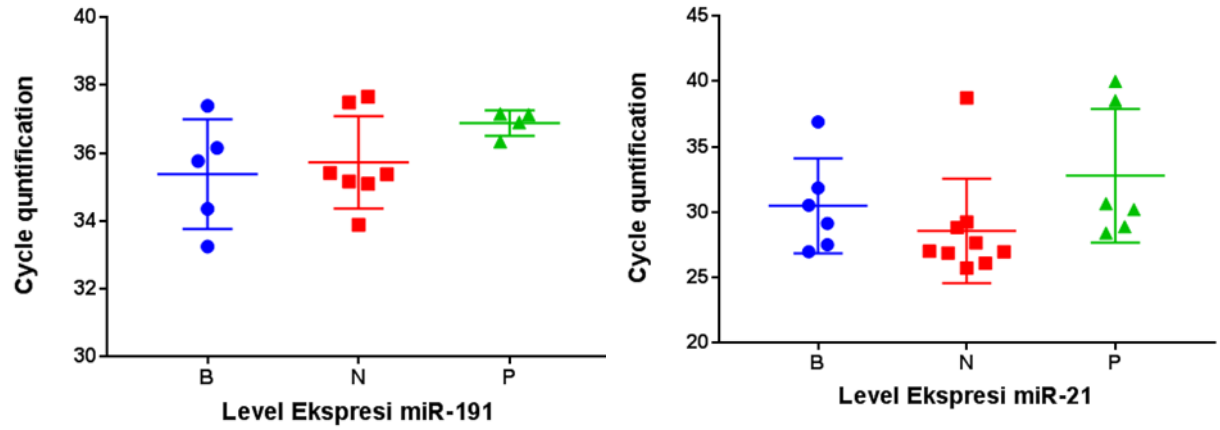


Figure 1. miRNA quantification used qPCR Biorad CFX-96 manager including miR-19a, miR-19b, miR-21 and miR-22 from urine samples. A. Amplification cycle, B. Melt Curve, C. Melt Peak.

Four types distribution of miRNA cycle quantification (cq) (figure 2) indicated that consistently stable in each type of samples. miR-191, miR-21, miR- 22 and miR-19b formed a spread pattern with mean cq between 30-34.



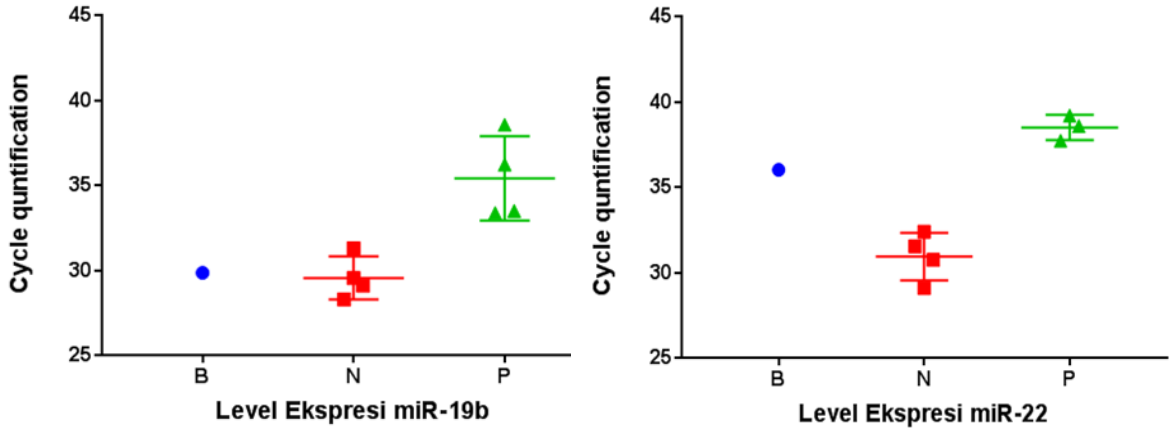


Figure 2. cycle quantification distribution of miRNA used qPCR with targeting miRNA-191, 21, 19b and miR-22. The results indicated that mon-coding RNA (miRNA) can be detected from urine samples.

RNA Stability in Storage

Subsequently, total RNA was storage deep freeze storage (-800C). Then to compare stability of the RNA concentration from 4 different treatment method, RNA concentration was measured every two days for thirteen days

Table 4. Result of RNA concentration measurement

Average of RNA concentration measurements per 2 days							
Treatments	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Day 13**
Without *lysis buffer*	5.0	6.2	5.5	5.4	5.0	5.0	3.9
Ambion	6.8	8.27	5.3	5.8	5.0	5.7	6.1
GeneAid	6.3	7.0	5.6	6.8	3.8	7.4	3.2
Qiagen	8.0	10.3	7.4	6.9	5.1	5.7	4.5

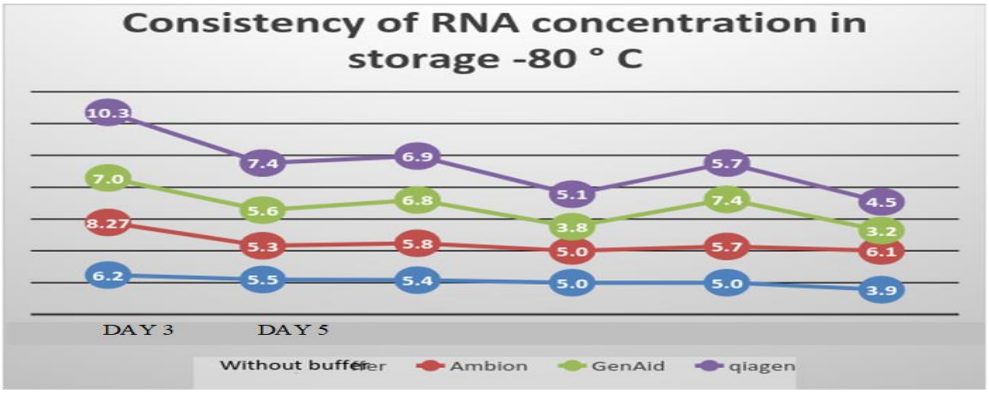


Figure 3. Graphic consistency of RNA concentration on the duration of shelf life

Table 4 and Figure 1 show that RNA samples that stored at -80° C little bit decreased in concentration in all treatment. RNA has bad stability compared to DNA, because of 2'Hydroxyl (-OH) group of RNA nucleotides that make it easily degraded. (sumber ?). Deep freeze storage can inactive any endogenous enzyme degradation of RNA, such as RNase, Helicase, etc (Houseley, et al., 2009).

Table 5. Decreased score of RNA concentration

Difference in RNA Concentration on Storage -80°C				
Day	**Without *lysis buffer***	**Ambion**	**GeneAid**	**Qiagen**
Hari 5	-0.7	-2.97	-1.4	-2.9
Hari 7	-0.1	+0.5	+1.2	-0.5
Hari 9	-0.4	-0.8	-3	-1.8
Hari 11	0.0	+0.7	-3.6	+0.6
Hari 13	-1.1	0.4	-4.2	-1.2

Table 6. One Way ANOVA Analysis, reduction of RNA concentration from each treatment

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Without Buffer	5	-2.3	-0.46	0.203		
Ambion	5	-2.17	-0.434	2.35478		
GenAid	5	-11	-2.2	4.7		
Qiagen	5	-5.8	-1.16	1.743		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Between Groups	10.30714	3	3.435712	1.526851	0.2459	5.292214
Within Groups	36.00312	16	2.250195			
Total	46.31026	19				

Based on One-Way ANOVA test shows that degradation of RNA concentration from 4 different methods almost smilar, has no significant value (p-value > 0.01) (Table 6). This result shows that deep freeze storage cannot totally stop RNA degradation but it can slow down RNA degradation. So this study recommend that RNA isolation method without additional lysis buffer treatment is better way, it will be cheaper and efficient to isolate RNA from urine samples

Conclusion

RNA isolation without additional lyses buffer is successfully conducted without buffer treatment. There are no different results between buffer and without buffer treatment showed by RNA concentration and degradation score in deep freeze storage. Therefore, isolation exosome RNA from urine sample can be better conducted without additional lysis buffer, it is more efficient and economical .

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ABBREVIATIONS ORIGINALITY AUTHOR CONTRIBUTIONS

Study design: Tirta Wardana, Sofia Mubarika Haryana and Indwiani Astuti, Data acquisition, management and sample collection: R Danarto. Data Analysis: Angga Dwi Prasetyo, Joni Kristanto, Richardus Hugo, Santoso Pradana Putra. Manuscript writing: Angga Dwi Prasetyo, Joni Kristanto, Richardus Hugo, Santoso Pradana Putra, Tirta Wardana. Project supervisors : Sofia Mubarika Haryana and Indwiani Astuti.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest in this study.

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