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RNA isolation by two different methods in analysing adipose-biomarkers of mouse adipose tissues

Dinh Toi Chu[1, 2], Hue Vu Thi[1, 2*], Ngoc Hoan Le[3]

[1]*Faculty of Applied Sciences, International School, Vietnam National University - Hanoi, 79 Nguy Nhu Kon Tum Street, Nhan Chinh Ward, Thanh Xuan District, Hanoi, Vietnam*

[2]*Center for Biomedicine and Community Health, International School, Vietnam National University - Hanoi, 144 Xuan Thuy Street, Dich Vong Hau Ward, Cau Giay District, Hanoi, Vietnam*

[3]*Faculty of Biology, Hanoi National University of Education, Vietnam National University - Hanoi, 136 Xuan Thuy Street, Dich Vong Hau Ward, Cau Giay District, Hanoi, Vietnam*

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Abstract:

The RNeasy Mini Kit and the TRI Reagent protocol are commonly employed techniques for extracting total RNA and are widely utilised in research pertaining to the expression of adipose biomarkers. This study was undertaken to establish and determine an appropriate method for isolating RNA from frozen adipose tissue, with the objective of analysing adipose biomarkers. Total RNA was extracted from frozen white adipose tissues of mice (-80°C) using both the RNeasy Mini Kit and the TRI Reagent protocol. The concentration and purity of the RNA were assessed using NanoDrop. One-step RT-qPCR was employed to quantify the mRNA expression levels of three adipose biomarkers: MEST, SFRP5, and SCD1. The results of this investigation indicated that the RNeasy Mini Kit yielded a higher RNA concentration when compared to the TRI Reagent method. Furthermore, the RNeasy Mini Kit provided RNA samples with superior purity, as evidenced by a higher 260/230 ratio. Although RNA extracted using the TRI Reagent exhibited slightly elevated mRNA expression levels for the three biomarkers, these differences did not reach statistical significance. In summary, both methods are suitable for total RNA extraction, but the RNeasy Mini Kit is recommended for obtaining higher RNA concentration and purity. The choice of method should be contingent upon the intended downstream application of the RNA.

Keywords: **adipose biomarkers, adipose tissues, RNA isolation, RNeasy Mini Kit, TRI Reagent protocol.**

Classification numbers: **3.4, 3.5**

1. Introduction

To assess the expression of a specific gene, reverse transcription polymerase chain reaction (RT-PCR) is employed to quantify its ribonucleic acid (RNA) concentration from total RNA. Therefore, isolating total RNA from adipose tissue represents one of the most critical steps that can significantly impact the outcomes of subsequent experiments. Given their acknowledged advantages, both the RNeasy Mini Kit and the TRI Reagent protocol are extensively employed in research concerning the expression of adipose biomarkers.

The RNeasy Mini Kit is a well-established commercial kit specifically designed for RNA extraction from adipose tissue [1]. While traditionally viewed as a passive energy storage organ, adipose tissue is now recognized as a complex and highly active metabolic and endocrine organ [2]. Consequently, adipose tissues play a pivotal role in the study of conditions such as diabetes, metabolic syndrome, and obesity, which have garnered substantial attention worldwide due to their epidemic prevalence [3]. Extracting RNA from adipose tissue presents more challenges compared to other tissues, primarily due to its elevated triglyceride levels and lower RNA concentration [4].

Isolating RNA from animal tissues with a high lipid content, such as adipose tissue, can prove to be a challenging task. Obtaining high-quality RNA with a satisfactory yield from adipose tissue is particularly

*Corresponding author: Email: huevt@vnuis.edu.vn

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demanding. Although Pena et al. employed a modified version of the TRI Reagent approach, their RNA integrity values were considerably lower than those achieved using our combined protocol [5]. The TRI Reagent approach exhibited issues related to the purity of the isolated RNA in previous studies. It is widely recognized that all phenol-based procedures leave minimal residual organic solvents in the final separated sample, and these contaminants can potentially impact downstream applications [6-9]. The TRI Reagent approach yields samples that are contaminated with salts and solvents but exhibit better integrity.

Selecting an appropriate RNA extraction method is essential to cater to diverse research objectives, especially within the laboratory settings of Vietnam. Therefore, this study was conducted to establish and identify a suitable RNA isolation method for adipose tissue, with the specific aim of analysing adipose biomarkers.

2. Methods

2.1. Total RNA extraction

In this study, inguinal white adipose tissue (iWAT) was collected from mice, with 10 mice per group. iWAT was collected from both sides of each mouse, and each side was cut and weighed to obtain 100 mg of tissue for each RNA extraction method. Following collection, the samples were promptly frozen in liquid nitrogen and stored in a deep freezer at -80°C. Total RNA was extracted from the frozen mouse white fat using the QIAamp Viral RNA Mini Kit (QIAGEN, Germany) (Total RNA Kit) or the TRI Reagent protocol (TRI Reagent®, Sigma-Aldrich), as previously described [10, 11]. Briefly, each frozen sample was homogenized in 1000 µl of Buffer RLT or 1000 µl of TRI Reagent in a chilled tube using a Tissue Grinder. Subsequently, total RNA extraction was carried out following the procedures outlined in previous studies [10, 11]. During the RNA isolation using the kit, any contaminated DNA was removed using DNase I. Following isolation, total RNA from each sample was diluted to a final volume of 32 µl in RNase-free water, and in this study, we present the comparative concentrations, reflecting the yields of total RNA.

The RNeasy Mini Kit contains a selective binding silica membrane and a specialised buffer with a high salt content, enabling the purification of RNA molecules longer than 200 nucleotides. Consequently, total RNA from biological samples, which typically includes RNA fragments of approximately 18 nucleotides and longer, can be effectively purified using the RNeasy Mini Kit. Alternatively, a whole RNA (>200 nucleotides) fraction and a microRNA (miRNA)-enriched fraction can be extracted separately. Total RNA, inclusive of miRNA, is well-suited for miRNA quantification using real-time RT-PCR. In certain applications where messenger RNA (mRNA) and ribosomal RNA (rRNA) might introduce unwanted background noise, the enrichment of small RNA in a distinct fraction can be advantageous. While the RNeasy Mini Kit approach yields total RNA with excellent purity, quantity, and suitability for PCR applications from adipose tissue, it is somewhat more susceptible to degradation [12].

2.2. RNA measurement using NanoDrop

The concentration and purity of RNA extracted using the RNeasy Mini Kit and the TRI Reagent were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Measurements were taken for the absorbance ratios at 260-280 nm (260/280) and 260-230 nm (260/230), as well as the RNA concentration (ng/µl). The experimental procedure was previously outlined in publications [7, 8].

2.3. One-step RT-qPCR

The mRNA levels of three genes, namely MEST, SFRP5, and SCD1, were determined using the one-step RT-qPCR method. The Rotor-Gene Q system (QIAGEN, Germany) was employed for the PCR reaction. Primers and Taqman probes for the target genes were obtained from previously published research [7, 9]. The mRNA expression of the target genes was normalized against Cyclophilin (Cyclo). The experimental procedure was described in prior publications [7, 9, 10].

2.4. Data analysis

The data were managed and presented as mean ±SEM. Statistical analysis was conducted using Student's t-test for single comparisons with GraphPad Prism 8.0. Significance was established at $p<0.05$, with * indicating $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$, while "n.s" represented not significant ($p\geq0.05$).

3. Results

3.1. The concentration of total RNA

Table 1. Summary of the RNA isolation in the adipose biomarker analysis of mouse fat tissues.

	RNeasy Mini Kit					TRI Reagent				
	Mean	*SEM*	*Max*	*Min*	*N*	*Mean*	*SEM*	*Max*	*Min*	*N*
RNA concentration (ng/µl)	122.91	10.12	183.78	76.41	10	84.04	12.00	171.37	39.74	10
Ratio 260/280	2.03	0.02	2.09	1.96	10	2.02	0.02	2.09	1.91	10
Ratio 260/230	1.89	0.06	2.05	1.49	10	1.64	0.05	1.99	1.48	10
MEST (mRNA level)	0.10	0.04	0.38	0.01	10	0.17	0.03	0.33	0.06	10
SFRP5 (mRNA level)	0.10	0.04	0.33	0.01	10	0.14	0.03	0.31	0.03	10
SCD1 (mRNA level)	0.07	0.01	0.13	0.02	10	0.09	0.01	0.13	0.06	10

N indicates the number of samples/mice per group (each pair of samples were collected from 1 mouse).

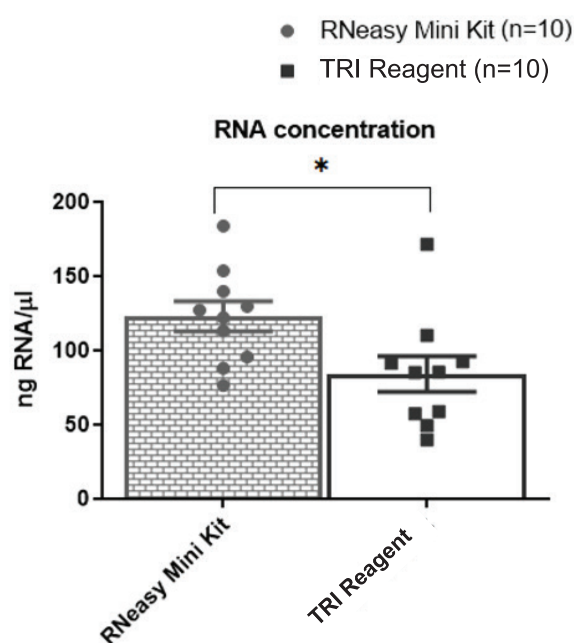

Fig. 1. The concentration of total RNA extracted by two methods. The mean RNA concentration can be described by using the RNeasy Mini Kit and the TRI Reagent method. Student's t-test for single comparisons was used. A significant difference between the two methods was accepted at $p<0.05$ (*).

Total RNA was extracted from frozen adipose tissue of mice, and its concentration (ng/µl) was compared between the RNeasy Mini Kit and the TRI Reagent (Table 1, Fig. 1). The results revealed a significantly higher mean RNA concentration with $p<0.05$ when using the RNeasy Mini Kit (122.91±10.12) compared to the TRI Reagent method (84.04±12.00). The maximum RNA concentration obtained was 183.78 ng/µl for the RNeasy Mini Kit and 171.37 ng/µl for the TRI Reagent, while the minimum RNA concentration was 76.41 ng/µl for the RNeasy Mini Kit and 39.74 ng/µl for the TRI Reagent. These findings suggest that the RNeasy Mini Kit is a more efficient method for RNA extraction, resulting in a higher RNA concentration with less variability in results.

3.2. The quality of total RNA

The quality of total RNA obtained from the two methods was assessed by determining the 260/280 and 260/230 absorbance ratios (Table 1, Fig. 2). There was no significant difference in the mean 260/280 ratio between the RNeasy Mini Kit (2.03±0.02) and the TRI Reagent (2.02±0.02). However, a significant difference was observed in the mean 260/230 ratio between the two methods ($p<0.01$). The mean ±SEM of the 260/230 ratio for the RNeasy Mini Kit was 1.89±0.06, indicating a pure RNA sample. In contrast, for the TRI Reagent, this ratio was 1.64±0.05, suggesting the presence of contaminants in the RNA sample. These results indicate that the RNeasy Mini Kit yields higher purity RNA samples compared to the TRI Reagent, as evidenced by the higher 260/230 ratio.

3.3. Analysis of mouse adipose-biomarkers

From the total RNA obtained from the two methods, the mRNA expression levels of three biomarkers (MEST,

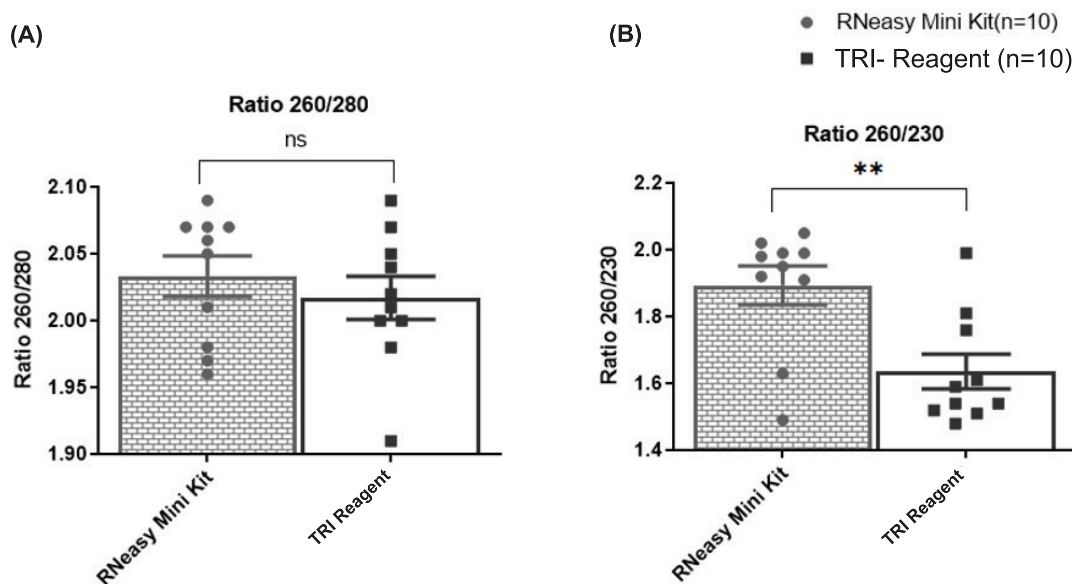

Fig. 2. The quality of total RNA extracted by the two methods. The quality of total RNA obtained from the two methods was compared by determining **(A)** the 260/280 ratio and **(B)** the 260/230 ratio. Student's t-test for single comparisons was used. "ns" indicates a non-significant difference and ** indicates $p<0.01$.

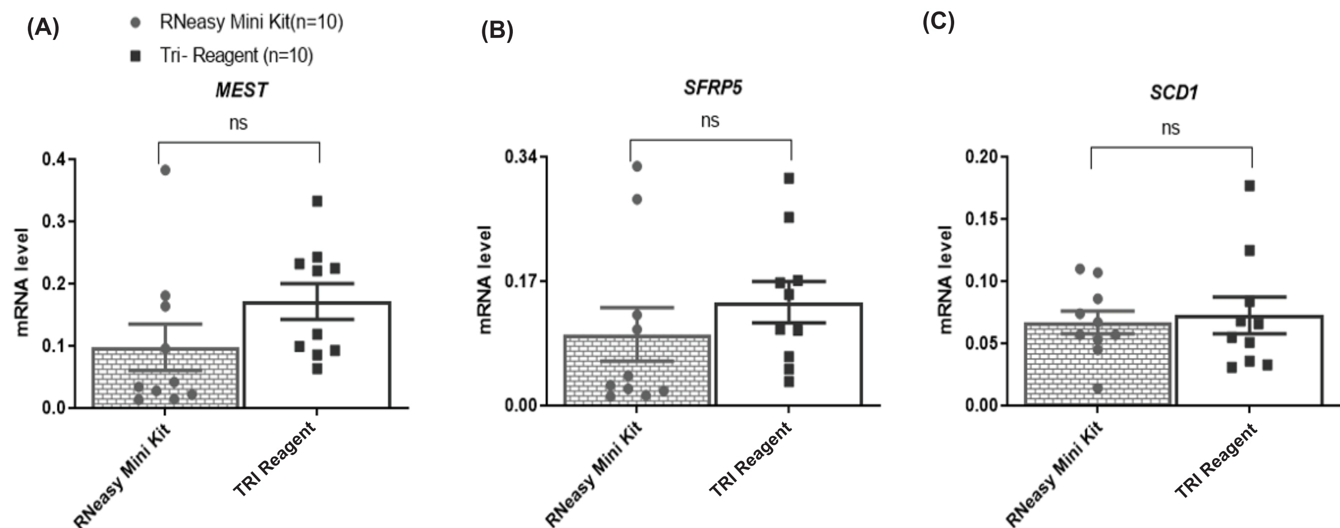

Fig. 3. The RNA expression of some adipose biomarkers using total RNA extracted by the two methods. mRNA expression levels of three biomarkers **(A)** MEST, **(B)** SFRP5, and **(C)** SCD1 using RT-PCR. Student's t-test for single comparisons was used. "ns" indicates a non-significant difference between the two methods.

SFRP5, SCD1) were measured using RT-PCR (Table 1, Fig. 3). The results indicated that the expression levels of MEST, SFRP5, and SCD1 appeared to be slightly higher when using RNA extracted with TRI Reagent compared to the RNeasy Mini Kit. Specifically, the MEST expression level was 0.17±0.03 with the TRI Reagent method, slightly higher than 0.10±0.04 with the RNeasy Mini Kit method. Similarly, the expression of SFRP5 was higher with RNA extracted using TRI Reagent (0.14±0.03) than with the RNeasy Mini Kit (0.10±0.04). Likewise, the SCD1 expression using TRI Reagent and RNeasy Mini Kit was 0.09±0.01 and 0.07±0.01, respectively. However, none of these differences reached statistical significance ($p>0.05$). These results indicate that the expression levels of critical biomarkers in adipose tissue remained consistent after RNA extraction using both methods.

4. Discussion

The isolation of high-quality RNA from adipose tissue presents challenges due to its high lipid content and relatively low cellular density. Researchers frequently turn to the RNeasy Mini Kit method and the TRI Reagent protocol as they are among the most commonly used techniques for total RNA isolation, with other methods often considered time-consuming and requiring larger input amounts [6]. The RNeasy Mini Kit, which employs phenol-chloroform (organic extraction), offers the advantage of isolating RNA from a single sample. However, it can be costly and yield lower quantities of samples, making the TRI Reagent protocol a viable alternative [3].

Researchers typically assess the quantity and quality of isolated RNA by examining absorbance ratios, specifically the 260/280 and 260/230 ratios, using a Nanodrop spectrometer. Ratios closer to 2.0 indicate better RNA quality. In line with Susanna Cirera's study, a combination of both methods yielded excellent isolate purity, with a 260/280 ratio of 2.00±0.049 and a 260/230 ratio of 1.73±0.189 [6]. Our study's results align with these findings. The RNeasy Mini Kit excels in terms of purity compared to the TRI Reagent protocol. This can be attributed to the RNeasy Mini Kit's use of silica film-based bonding technology, effectively removing contaminants such as proteins, genomic DNA, and polysaccharides. Consequently, researchers are more likely to obtain cleaner RNA samples for subsequent experiments. However, the TRI Reagent method yields a higher percentage of RNA samples compared to the RNeasy Mini Kit. Therefore, combining both methods can produce ample amounts of high-quality total RNA from adipose tissue, suitable for downstream applications such as RT-qPCR, microarrays, and high-throughput sequencing [6].

Despite their advantages, both methods have drawbacks, notably their cost. Commercial kits, although commonly used, tend to be expensive. However, the inherent automation capabilities of silica spin column extraction methods offer significant advantages. In the case of TRI reagents, RNA purity needs to be considered, as other substances like proteins and salts may still be present [6]. Hence, a flexible approach involving the combination of both methods can ensure efficient and cost-effective RNA isolation while maintaining high quality.

Previous research employing microarray analysis has identified several candidate genes as potential biomarkers for adipose tissue, including MEST, SFRP5, and SCD1 [10]. MEST and SFRP5-encoded proteins have been found to inhibit Wnt signalling, thereby suppressing mitochondrial respiration and promoting lipid accumulation in fat depots. In a mouse model of diet-induced obesity, higher expression levels of MEST and SFRP5 were significantly correlated with adiposity in both subcutaneous and visceral adipose tissues [10]. However, the mRNA and protein levels of MEST and SFRP5 were found to be sensitive to the age of adipose tissue, being severely suppressed in aging mice despite continued adipose expansion [11]. Although their expression levels decrease after reaching a plateau, MEST and SFRP5 have been identified as biomarkers for healthy adipocytes [10, 11]. SCD1 protein plays a crucial role in catalysing the biosynthesis of monounsaturated fatty acids, regulating lipogenesis. Due to its high expression during adipogenic differentiation, SCD1 is considered an adipogenesis biomarker. Like MEST and SFRP5, SCD1 did not exhibit significant changes in response to aging in mice with high adiposity [11].

D.T. Truong, et al. (2019) [11] conducted a study on C57BL/6J and 129S mouse models to investigate the impact of developmental phases on the function and expression of key adipose biomarkers. TRI reagent was used to extract total RNA from RP, EP, ING, and iBAT tissue, and RNA Mini Kit was employed in several independent experiments [11]. Another study established a positive correlation between SCD1, SFRP5, and MEST expression and the adiposity of mice subjected to a high-fat diet [10]. For cost-effectiveness reasons, a different study employed the TRI reagent protocol instead of the RNeasy Mini Kit for total RNA isolation. The authors reported similar results, with increased expression of SFRP5 and MEST in obese mice correlating with fat depot expansion, suggesting the use of SFRP5 and MEST as biomarkers for healthy adipocytes [10]. Concerns regarding the purity of products extracted by the TRI reagent led R.P.A. Koza, et al. (2016) [13] to use the RNeasy Mini Kit for RNA purification before measuring MEST and SFRP5 expression. They also observed significant correlations between MEST and SFRP5 expression and adiposity in inbred mice. Thus, the findings regarding the expression of these adipose biomarkers remain consistent irrespective of the RNA isolation methods. However, various modifications, such

as additional ethanol washes or reduced reagent volumes, can be applied to optimize the quality and quantity of total RNA isolated through standard protocols [14]. Our study's results indicate that the mRNA expression levels of MEST, SFRP5, and SCD1 in adipose tissue are not affected by the RNA isolation methods employed.

5. Conclusions

This study conducted a comparative analysis of total RNA extraction from frozen adipose tissue of mice using the RNeasy Mini Kit and the TRI Reagent method. The study assessed the concentration and quality of the extracted RNA and measured the mRNA expression levels of MEST, SFRP5, and SCD1 through RT-PCR. The findings indicated that the RNeasy Mini Kit yielded a higher RNA concentration with less variability in results when compared to the TRI Reagent method. Furthermore, the RNeasy Mini Kit provided RNA samples of superior purity, as evidenced by a higher 260/230 ratio. Despite slightly elevated mRNA expression levels of the three biomarkers observed in RNA extracted using the TRI Reagent method, these differences did not reach statistical significance. In summary, both the RNeasy Mini Kit and the TRI Reagent method are viable options for total RNA extraction. However, the RNeasy Mini Kit is recommended when aiming for higher RNA concentration and purity. The choice of method should be tailored to the specific downstream application of the RNA, taking into consideration the research objectives and priorities.

CRediT author statement

Dinh Toi Chu: Conceptualisation, Methodology, Validation, Formal analysis, Data curation, Visualisation, Supervision, Writing - Reviewing and Editing; Hue Vu Thi: Methodology, Data curation, Writing - Original draft preparation; Ngoc Hoan Le: Data curation, Formal analysis, Writing - Reviewing and Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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