Method Article
Optimization of RNA extraction protocol for rat skeletal muscle samples

ABSTRACT

Aims: The present study aimed to stablish and characterize an optimized protocol conformation to obtain skeletal muscle samples with adequate RNA quality for sequencing studies.
Place and Duration of Study: The in vivo experiments and analyses were performed in the Laboratory of Biochemistry and Gene Expression – LABIEX of the Superior Institute of Biomedical Science – ISCB from the State University of Ceará.- UECE. Between 2017-2020.
Methodology: Were used 23 samples from male Wistar rat soleus muscle. Total RNA extraction was performed using the classic TRIzol® method and commercial kit, merging steps from both.
Results: Capillary electrophoresis in the Bioanalyzer platform was used for RNA quality evaluation. Analyzes of RNA concentration (167,7±75,8 ng/µL), RIN (8,008±0,03981) and rate 28S/18S (1,4833±0,0718) showed satisfactory results. 28S/18S Ribosomal bands appear well defined, without small traces, which indicates RNA with high integrity and without contamination of genomic DNA.
Conclusion: Obtained RNA quality and integrity data satisfied the exigencies for posterior RNA-seq.

Comment [C1]: ?

Keywords: Sequencing, RNA extraction, soleus muscle

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1. INTRODUCTION

Technological advances in sequencing made possible tracking changes in gene expression in different stages of cellular development, disease physiopathology, and in diverse physiological conditions. The functional genome consists in molecular activity analyses to identify functions and interactions between genes and proteins. Some techniques allow functional genomics evaluations, based on mRNA quantitative and qualitative analysis. Among them, we can cite microarray, quantitative polymerase chain reaction (qPCR), and next-generation RNA sequencing (RNA-seq) [1].

The RNA-seq approach was developed for transcriptome analysis, using next-generation sequencing technology (NGS). Transcriptome comprehends the set of transcripts found inside cells, tissues, and organisms, and it reflects general gene expression. Gene activation and inactivation is shown in cellular structures through transcriptome studies, in which genes have their expression levels defined. Messenger RNA (mRNA) encodes proteins, in practice transcriptome were established to embrace mRNA [2] [3].

RNA extraction from specific cells and tissues consists of the first step for gene expression studies and transcript characterization. RNA extraction techniques are commonly used in Molecular Biology labs, and consists of cellular lysis to release nucleic acids, kept soluble,

and separation from the precipitated cellular components. Sample preparation requires caution and specific protocols when the target is RNA. Besides this, NGS transcriptome studies need good quality RNA. RNA evaluation is done in the platform *Bioanalyzer* by measuring concentration, 28S/18S Ribosomal band rate, and RNA Integrity Number – RIN, the latter being of higher relevance [4].

A standard RNA extraction protocol for a diversity of tissues does not exist. Some protocols use common reagents for total RNA extraction, such as *TRIzol®*, or specific kits available in the market with diverse applications, each one with its advantages. *TRIzol®* consists of phenol and guanidine isothiocyanatemonophase solution, which in extraction enables cellular lysis and retains RNA integrity. *TRIzol®* efficiency in acquiring a good quality RNA in extraction has been demonstrated by many studies. Commercial kits are efficient in RNA isolation and purification in columns. In this context, researchers point to combined or adapted methodologies as useful to obtain a high-quality RNA sample from specific tissues or organisms[5] [6].

RNA extraction may be limited by the type of biological material. Skeletal muscle, for instance, is a very dense and fibrous tissue, making cellular lysis difficult and RNA extraction by consequence. Besides this, it is a protein-rich tissue that could contaminate RNA samples. The main objective of this work was to optimize RNA extraction protocol from rat soleus skeletal muscle tissue samples and evaluate obtained RNA quality and integrity, for RNA-seq gene expression analyses.

2. MATERIAL AND METHODS

This study as approved by the Ethics Committee for Use of Animals at UniversidadeEstadual do Ceará (CEUA-UECE) process number 1592060/2014. The study used 23 samples from male Wistar rats muscle soleus, obtained from Instituto Superior de CiênciasBiomédicas, UECE (ISCB-UECE) vivarium. Animals had sixty days of life, weighed between 200g and 250 g, were kept in 12h/12h light/dark cycles, in an environment with a controlled temperature between 22 and 25°C, with water and food ad libitum.

Comment [C2]: ?

2.1 RNA Extraction

2.1.1 Extraction Method 1 - RNeasy Plus Universal Mini Kit (Qiagen®):

RNA extraction protocol following manufacturer recommendations using the correct equipment, RNAse free.

2.1.2 Extraction Method 2 – Trizol® + RNeasy Plus Universal Mini Kit (Qiagen®):

This RNA extraction protocol was adapted with TRIzol® and RNeasyPlus Universal Mini Kit (Qiagen®) steps. The procedure included the following steps:

1. Use of 30 mg from soleus muscle stored in a 2 mL tube with 900 µL of TRIzol®.

2. Samples were homogenized and kept for 5 minutes in environmental temperature.

3. 180 µL of chloroform was added to the samples, each tube passed 5 seconds in Vortex and was maintained for 3 minutes in environmental temperature.

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4. Samples were centrifuged at 1200g / 4°C / 15min. The supernatant (400uL) was transferred to a 1.5mL tube.

5. 400 µL of 70% ethanol was added

6. 700 µL of the resulting mixture was filtrated with a column kit (pink 2 ml tube), the rest of the sample was discarded.

7. Samples were centrifugated 8000g/ 4°C /15s. The material that went through the column was discarded.

8. 500 µL of RPE buffer was added to the column.

9. Samples were centrifuged at 8000g / 4°C / 15s. The material that went through the column was discarded.

10. 500 µL of RPE buffer was added to the column.

11. Samples were centrifuged at 8000g / 4°C / 15s. The material that went through the column was discarded.

12. Columns were put in a new 1.5 ml tube.

13. 40 µL of RNAse free water was added.

14. Samples were centrifugated at 8000g/4ºC/ 1 min.

15. Obtained RNA was stored at -80ºC.

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2.2RNA Integrity and Quality evaluation

RNA Integrity in samples was analyzed by capillary electrophoresis, with the Agilent 2100 Bioanalyzer system (Agilent Technologies®), the standard protocol for RNA. RNA quality was verified by evaluation of RNA integrity number (RIN), RNA concentration, and rate between 28S/18S Ribosomal bands.

2.3Statistical analyses

Results were presented as mean ± standard error of the mean. Student T-test for comparison between two groups was applied, using GraphPad Prism software, and results showing 5% occurrence probability of a null hypothesis ($p<0.05$) were considered statistically significant.

3. RESULTS AND DISCUSSION

RNA extraction adapted protocol with*Trizol®* and *RNeasy Plus Universal Mini kit (Qiagen®)* showed better results regarding RIN (8.0083±0.1379), RNA concentration (466.6667±75.5048), and rate between 28S/18S Ribosomal band (1.4833±0.0718) as presented in table 1.

Table 1. Results of RNA extraction with *TRIzol®* and *RNeasyPlus Universal Mini kit (Qiagen®)*: RIN, RNA concentration, and 28S/18S Ribosomal rate.

***TRIzol®* and *RNeasy Plus Universal Mini kit (Qiagen®)* RNA extraction**			
Sample	**RIN**	**RNA concentration (ng/µl)**	**rRNA ratio [28s/18s]**
12	8.1	359	1.4
13	7.8	421	1.5
14	8.1	493	1.4
15	8.0	416	1.5
16	8.0	465	1.5
17	8.0	527	1.4
18	8.3	377	1.6
19	7.8	540	1.5
20	8.0	438	1.6
21	8.0	633	1.4
22	7.9	455	1.5
23	8.1	476	1.5
Mean	8.0083	466.6667	1.4833
Standard Deviation	0.1379	75.5048	0.0718
Standard Error	0.0398	21.7963	0.0207
Variation Coefficient	2%	16%	5%

The *RNeasyPlus Universal Mini kit (Qiagen®)* RNA extraction protocol yielded RNA with low quality. Hence, the extraction protocol was adapted by adding *TRIzol®* to the samples during the incubation and homogenization first steps, followed by RNA isolation and purification steps using the *RNeasyPlus Universal Mini kit (Qiagen®)*.
Rat soleus muscle RNA extraction with *RNeasyPlus Universal Mini kit (Qiagen®)* was considered unsatisfactory after the evaluation of RNA integrity and concentration. As presented in table 2, RIN mean (4.134±0.4265), RNA concentration mean (167.7093±251.4096), and the rate between 28S/18S Ribosomal band (0.4727±1.0753). These results point to RNA degradation and possible contamination from genomic DNA. Degradation may have occurred through the presence of stable and active RNases from soleus muscle and high concentration of proteins, being the main extraction difficulty, making RNA, which already is a highly unstable molecule, be degraded faster [7].

Table 2. Results of RNA extraction with RNeasyPlus Universal Mini kit (Qiagen®): RIN, RNA concentration and 28S/18S Ribosomal rate.

RNA extraction with *RNeasy Plus Universal Mini kit (Qiagen®)*			
Sample	**RIN**	**RNA concentration (ng/µL)**	**RNA ratio [28s/18s]**
1	2.6	3.75	0

2	2.6	271	0.1
3	5.5	1.33	0.1
4	3.7	1.092	0.1
5	2.6	1.239	1
6	6.0	1.204	0.1
7	5.1	1.175	0
8	5.5	1.012	3.6
9	3.0	391	0.1
10	3.2	467	0.1
11	5.7	705	0
Mean	4.1364	167.7093	0.4727
Standard Deviation	1.4144	251.4096	1.0753
Standard Error	0.4265	75.8029	0.3242
Variation Coefficient	34%	150%	227%

Comparing obtained RIN from the two tested protocols, an adapted *Trizol®* and *RNeasyPlus Universal Mini kit (Qiagen®)* methodology (8.008±0.03981) had a significant increase regarding the use of commercial Kit (4.134±0.4265). Regarding RNA concentration, the adapted methodology (466.7±21.8 ng/µl) also increased concerningthe commercial kit (167.7±75.8 ng/µl), with $p<0.05$, as presented in figure1.

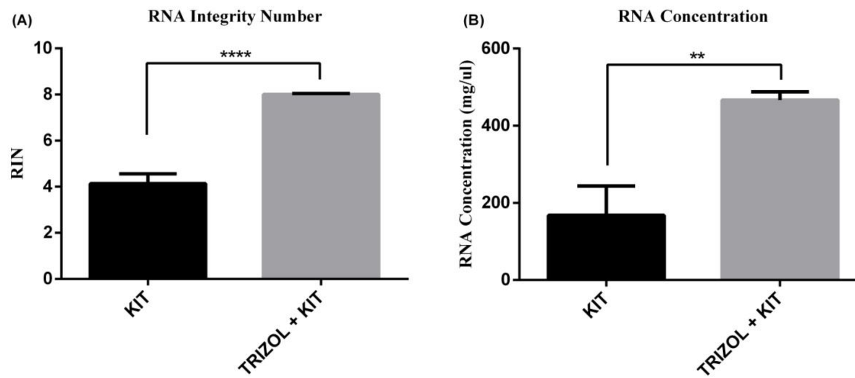

Fig. 1. a) RNA integrity number for *RNeasy Plus Universal Mini kit (Qiagen®)* (4.134±0.4265) vs *TRIzol®* and *RNeasy Plus Universal Mini kit (Qiagen®)* (8.008±0.03981), p<0.0001; b) RNA extraction concentration for *RNeasy Plus Universal Mini kit (Qiagen®)* (167.7±75.8 ng/µl) vs *TRIzol®* and *RNeasy Plus Universal Mini kit (Qiagen®)* (466.7±21.8 ng/µl), p<0.01.

Capillary electrophoresis methodology with *TRIzol®* and commercial kit showed homogeneity in intensity between 28S/18S Ribosomal bands, while electrophoresis samples of extracted RNA using only the commercial kit presented small bands with different

intensity, indicating degraded RNA (figure 2). Thus, the adapted protocol with *TRIzol®* and commercial kit showed good quality RNA, once 28S and 18S bands in eukaryotes are Ribosomal RNA integrity markers. RNA is considered of good quality when the 28S/18S Ribosomal bands are well defined in agarose or capillary electrophoresis (*Agilent),* without the presence of less striking small bands, and 28S peak is stronger than 18S.

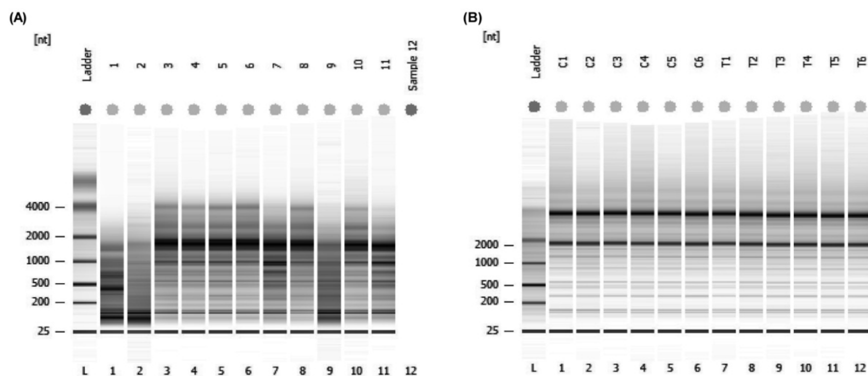

Figure 2. Capillary Electrophoresis of RNA samples: (A) RNeasy Plus Universal Mini kit (Qiagen®) (samples 1 to 11);(B) TRIzol® and RNeasy Plus Universal Mini kit (Qiagen®) (samples 1 to 12).

Electropherograms (figure 3) graphically showed the intensity of ribosomal bands in capillary electrophoresis, with fluorescence by second peak (FU). Two peaks relate to RNA ribosomal bands. Electropherograms from extracted RNA samples with *TRIzol®* and *RNeasyPlus Universal Mini kit (Qiagen®)* satisfied the required sequencing quality parameters. Besides, samples purified with *RNeasyPlus Universal Mini kit (Qiagen®)* did not show satisfactory results, which is observed by the absence of defined electropherograms, indicating the presence of degraded RNA, implying low-quality RNA for sequencing.

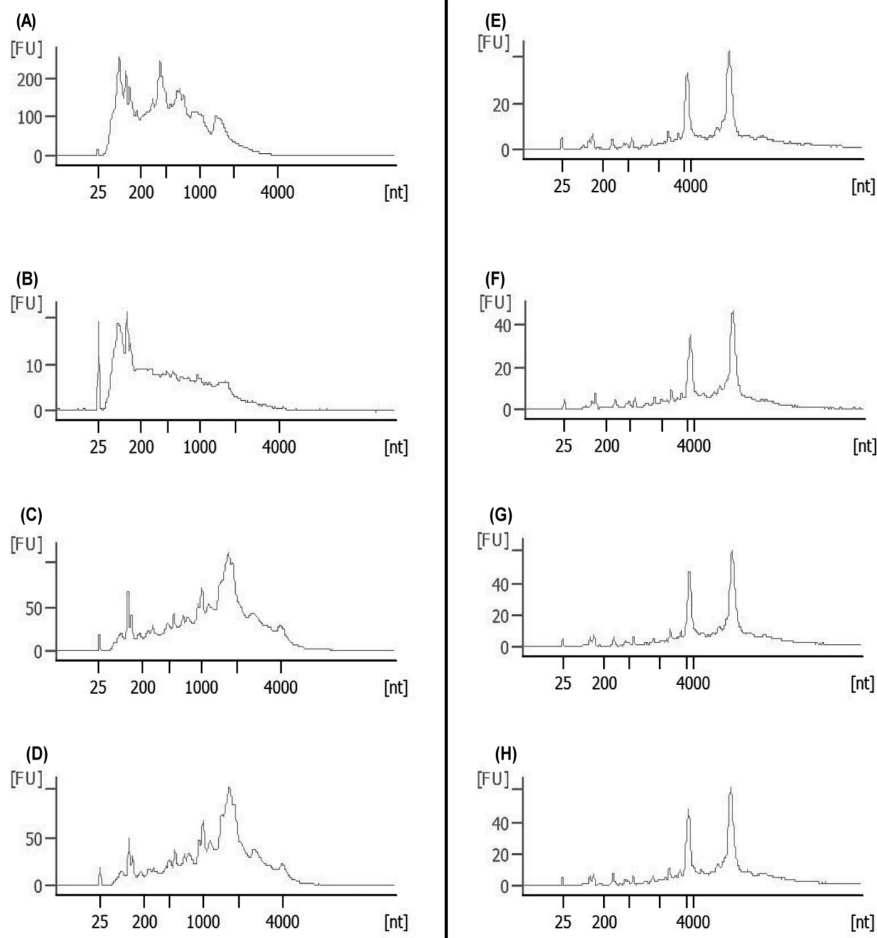

Figure 3.Electropherograms. (A), (B), (C) and (D): RNA samples extracted with *TRIzol®* and *RNeasyPlus Universal Mini kit (Qiagen®)*; (E), (F), (G) and (H): RNA samples extracted using only *RNeasy Plus Universal Mini kit (Qiagen®)*.

Results indicate that the adapted protocol, with steps from the classic *TRIzol®* method added to the commercial kit for RNA extraction from rat skeletal muscle, contributed to increasing the integrity and concentration of obtained RNA. *TRIzol®* incubation provided good tissue stabilization and efficient cellular lysis. Column kits showed efficiency in RNA precipitation and purification. The combined methodology, starting with a modified protocol and after the initial steps following manufacturers' instructions, yielded better quality RNA for sequencing.

Next Generation Sequencing technologies need high concentration and quality of extracted RNA. Therefore, specific studies for certain organisms or tissues may be limited, once they do not have a standardized RNA extraction protocol for diverse types of tissues

and species. Manufacturers' protocols and published methodologies combined or adapted may improve gene expression analyses from RNA extraction results (GAYRAL *et al*, 2011).

4. CONCLUSION

A combination of the classic *TRIzol®* method and *RNeasyPlus Universal Mini kit (Qiagen®)* was efficient for RNA extraction from rat skeletal muscle. This methodology contributed to increased RNA integrity and concentration, ensuring sample approval for sequencing (RNA-seq). RNA extraction with *RNeasyPlus Universal Mini kit (Qiagen®)* did not present satisfactory results.

ETHICAL APPROVAL

All procedures of this study described were reviewed and approved by theEthics for Animal Use Committee of the State University of Ceará – UECE, with the protocol number: 1592060/2014.

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