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Extraction RNA Protocol from Blood and Testis Tissue of Local Roosters

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ABSTRACT

The (one-step) method has become widely used to isolate total RNA from living organism samples and from different tissues. The aim of this study is to extract RNA from the blood and testicular tissue of male local chickens. The principle behind the method is to separate the RNA from the DNA after extraction with an acid solution containing (Acid guanidinium thiocyanate-phenol-chloroform) with centrifugation, under acidic conditions, the total RNA remains in the upper aqueous phase while most of the acid descends. The DNA and proteins are either in the interphase or in the lower organic phase and then the total RNA is collected by precipitation with isopropanol. Our results showed a significant increase in the concentration of RNA extracted from the blood compared to the testis tissue of local roosters. This protocol made it possible to isolate RNA from cells and tissues in less than 4 hours and was the reason for the great advances in gene expression analysis in plant and animal models, as well as in pathological samples as clearly demonstrated by the huge number of citations the protocol has gained in the past 20 years.

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INTRODUCTION

The expression of the genetic material DNA for itself begins with the first step, which is the conversion of information in DNA into information in RNA and ends with determining the types and quantities of proteins made by the cell. The types of proteins determine the cell type and the amino acid sequence is determined by the sequence of nucleotides in RNA (Al-Shahib et al., 2013). In all types of organisms, a gene is a chain of nucleotides in DNA that code for the RNA sequence. Then this DNA identifies the amino acids that are included in the polypeptide (Tamarin, 2001). Previously, it was thought that RNA was only a mediator in transferring information from DNA to a protein, but as the research progressed, it was found that multiple types of RNA play major roles in many vital activities of the cell, including the regulation of gene expression (Mustafa, 2018).

The important factors for ensuring the accuracy of gene expression analysis and other RNA-based downstream applications RNA quality and quantity, Controlling the accuracy of quantitative values for biological molecules such as nucleic acids and proteins is a critical matter and a critical step for the reliability of biomedical research and research tests and obtaining correct results. Several techniques determine quantitative values assuming absorbance factors or preparing a standard curve after measuring samples at known concentrations (Sasaki et al., 2018; Rodríguez et al., 2020). The challenges in RNA extraction are the imperative RNases that are capable of rapidly

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degrading RNA and which are difficult to eliminate despite caution and appropriate measures (Peirson and Butler, 2007).

RNA degradation results from the presence of additional hydroxyl groups at positions 2 'and 3' in ribose sediments which are more reactive than DNA methylation and which are particularly susceptible to hydrolysis of double ester bonds that bind phosphate and ribose deposits by enzymes RNases, many of these enzymes possess disulfide bonds within the chain making them particularly resistant to denaturation, such as prolonged boiling and deformation factors, being able to rapidly re-folding. Add to that, and unlike DNases, RNases do not require divalent positive ions to be active. Sediments such as EDTA are ineffective in protecting RNA from lysis due to these enzymes (Sulthana et al., 2016).

The tissues that do not directly extract RNA are placed with liquid nitrogen as soon as the tissue is obtained and then stored at a low temperature (-80 ° C) while avoiding freezing and thawing to disrupt the autolysis by RNases and taking into account grinding the hard tissues with a mortar cooled with liquid nitrogen and transferring the powder with a cooled spoon With nitrogen, the tissue is placed in plastic tubes (Eppendorf) to add the genetic material isolate (TRIzol) and the mixture is mixed to increase the homogeneity in the sample, and then it is kept in a cooled place to complete the extraction steps (Carninci et al., 2001).

In a recent study, optimized a three-step protocol for the extraction of high-yield and high-quality total RNA from the liver tissue (LT). Where this method offers an excellent alternative approach that works well across a broad range of animal species for the extraction of NGS-quality RNA (Sharma et al., 2019).

Chomczynski and Sacchi (1987) established the one-step method for isolating RNA with Acid Guanidinium Thiocyanate-Phenol-Chloroform. This substance was later known commercially as TRIzol. The basic principle of this method is the simultaneous isolation of RNA, DNA, and proteins from cell and tissue samples in addition to This is characterized by the possibility of using it in a wide range of tissues of different types and sizes, and the ease of adjusting the size used according to the size of the tissue (Chomczynski and Sacchi, 2006). The results of the study by (Chan et al., 2018) show that the extraction method based on TRIzol is of high quality in terms of accuracy and concentration of the extracted material.

RNA degradation may not present problems for many researchers, but it may become apparent and its effect is devastating and causes damage to tissue groups when quantitative measurements of gene expression. In these measurements, the quantity of RNA as well as the quality is necessary to ensure comparable RNA isolation. When examining twice the amount of RNA, it was found that the expression level was higher. Twice the same sample contained only 50% intact RNA hence the level of gene expression obtained was relatively lower (Peirson and Butler, 2007).

The aim of this study is to isolate RNA from the lines of Iraqi domestic roosters beginning of tissue collection to determining the amount of extracted DNA and to provide basic principles for preventing problems associated with RNA contamination.

MATERIAL AND METHODS

Collect sample

Blood was drawn from the lines of Iraqi domestic roosters via a pterygoid vein, 5 ml of blood from each bird was taken and these samples were placed in plastic test tubes (Tube Test) that contain an anticoagulant (EDTA), After collection, the samples were transported in a cooled nitrogen box to the laboratory and the samples were preserved by freezing at -80 ° C. Then RNA was extracted from the samples.

Testicular tissue samples were collected after the roosters were slaughtered, the testes extracted, and testicular tissue samples were taken, and they were placed in plastic test tubes and placed in nitrogen to be transferred to the laboratory and kept at a temperature of -80 ° C, Then RNA was extracted from the samples.

The diagram below represents the main work steps involved in isolating, extracting, assessing the quality, quantity, and preservation of RNA that were followed in this study.

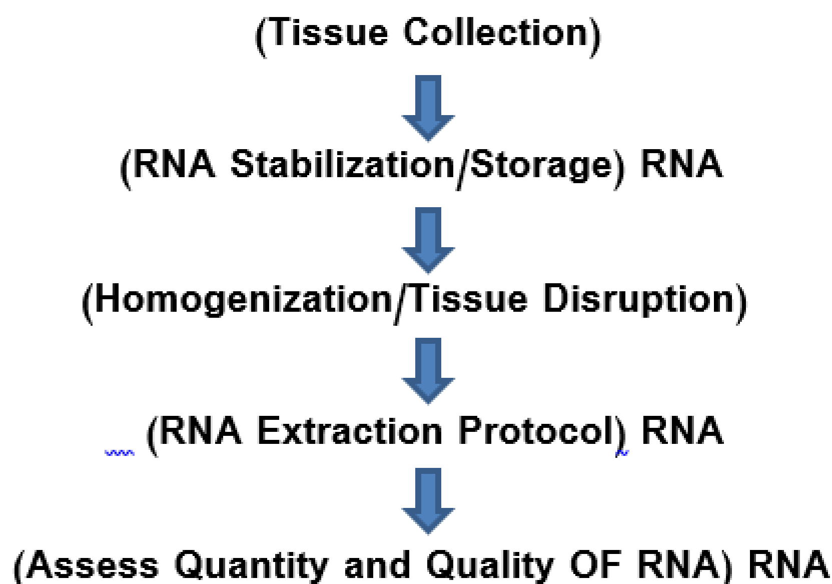

Figure (1) a diagram of the steps for extracting RNA from different tissues

RNA isolation steps

RNA was extracted from the blood and testicular tissue samples using TRIzol isolation method according to the manufacturer's instructions (Invitrogen) according to the following steps

1. The extraction method begins with an incubation period for samples cooled with liquid nitrogen at room temperature (20-25) C. Then 50 ml of the sample is added for every 1 ml of cooled TRIzol.
2. Then 5 ml of chloroform is added to the mixture and left for ten minutes, then it is entered into a centrifuge, cooled to a temperature of 4 ° C for 15 minutes, at (12000) cycles.
3. Then three phases of the sample are formed. The aqueous phase is withdrawn and placed in new, sterile, plastic tubes (Eppendorf) cooled to 4 ° C. Then isopropanol was added at a concentration of 0.5 ml and incubated in a room atmosphere for 10 minutes.
4. The filtrate is discarded, and then 1 ml of 75% ethanol is added. The mixture is withdrawn and pushed in the pipet for more than one time to ensure homogeneity.
5. Samples are placed in a (Vortex) mixer and then centrifuged for 5 minutes at 7500 cycles.
6. Discard the filtrate and leave the samples to dry for 15 minutes to get rid of the ethanol. After that, the RNA granules are re-suspending (pellet) by adding 20-50 ml of (nuclease free water).
7. Finally, the mixture is incubated in a thermal mixture (Maxing Block) at a temperature of 55-60 C for 15 minutes to obtain RNA and then the concentration of RNA is measured to measure its purity.

Measuring the purity and concentration of extracted RNA

The concentrations and purity of the extracted amino acids (RNA) from the samples were measured by adding (1 ml) of the extract, then adding (0.5 ml) of (QuantiFluor ONE) dye with (199 ml) of the buffer solution (TE Buffer solution). Then we mixed a fast mix and put it in the dark for 5 minutes and then put it on the Quantus screen and the concentrations were recorded. The problem of intron-rich RNA pieces on its triple ends, or introns that are among the pieces or introns on its five-pointed ends, which pose serious problems in quality and cost, can be overcome by converting the extracted RNA into cDNA complementary DNA that can be stored as a genetic library for the organism's tissue through a group of the use of a transcriptase enzyme that polymerizes dNTPs with an mRNA molecule as a template to initiate the polymerization process (Carninci et al., 2001).

Figure (2) Quantus device with conversion kit

Statistical Analysis

Statistical analysis was performed in the statistical model (CoStat) and significant differences (ANOVA) were compared using Duncan's (1955) multinomial test at the level of significance ($P < 0.05$). (full random design).

RESULTS AND DISCUSSION

1. RNA concentration in blood tissue (Circular RNAs (circRNAs))

Table (1) shows a comparison between the average of RNA concentrations in blood tissue that were extracted from the blood of Iraqi local rooster's lines, and the results showed a significant difference for each of the White (W), Black (BL) and White and Black (BA) lines at averages of 500, 500 And 460.75 ng/µl, respectively, compared to the White Naked Neck line (WN), and the Brown Naked Neck line (BRN) and the Brown line showed no difference compared to the rest of the lines.

Table (1) the average of RNA concentrations in blood tissue (Circular RNAs (circRNAs)) in µg/µl unit

Lines	average of RNA concentrations in blood tissue
BA	460.75 ±125.69 a
WN	308 ±80.30 b
BRN	414 ±70.22 ab
W	500 ±0.0 a
BR	415 ±69.4 ab
BL	500 ±0.0 a

The different letters in the columns mean that there are significant differences at ($P \leq 0.05$). BA: White and Black Roosters. WN: White naked-neck roosters, BRN. Brown naked-neck roosters. W: White roosters. BR: Brown roosters. BL: Black roosters.

2. Concentration of RNA in testicular tissue

Table (2) shows the concentration averages of RNA extracted from testis tissue for Iraqi local roosters, and the results showed a significant difference for the White and Black (BA) and Black (BL) lines at a concentration of 59.25 and 59 (ng/µl), respectively compared to the rest of the lines. The results also showed a significant difference for the White (W), Brown (BR), and Brown Naked Neck lines (BRN) with concentrations of 55, 53 and 52.57 (ng/µl) respectively, compared to the White Naked Neck line (WN).

Table (2) The average of RNA concentrations in testis tissue in (ng/µl) unit

Lines	average of RNA concentrations in testis tissue
BA	59.25 ±0.3 a
WN	51 ±0.28 c
BRN	53.75 ±0.71 b
W	55 ±0.94 b
BR	53 ±0.92 b
BL	59 ±1.11 a

The different letters in the columns mean that there are significant differences at ($P \leq 0.05$). BA: White and Black Roosters. WN: White naked-neck roosters, BRN. Brown naked-neck roosters. W: White roosters. BR: Brown roosters. BL: Black roosters.

The results of a comparative study with (Jeon et al., 2019), that the efficiency of methods for measuring the purity and concentration of nucleic acids in the (Quantus Fluorometry) method has more accurate quantitative estimates than the (TapeStation) method and that the measurement of the concentration of nucleic acids by (Quantus) depends on the intensity of the fluorescent dye flashes that are directly related to the nucleic acid. The device absorbs dissolved materials with wavelengths ranging from 160-900 nm.

3. Comparison of extracted RNA concentrations between tissues

Table (3) shows the results of measuring the concentrations of RNA that were extracted from two types of tissues (blood and testis tissue) for Iraqi local roosters as a comparison table between the averages of the concentrations of two different types of tissues for the same animal by following the protocol for extraction and measuring the concentration of one RNA.

We have found that roosters RNA concentration varies by as much as tenfold among rooster tissues, and the concentration of RNA extracted from blood (Circular RNAs (circRNAs)) for all local lines with a difference between 250-450 (ng/µl), and the concentration of RNA extracted from the same tissue varied between samples according to the strain with a significant difference.

Table (3) RNA concentration averages in blood and testicular tissue for local roosters' lines in (ng/µl) unit

Lines	RNA concentration averages in blood and testicular tissue	
	in testis	in blood
BA	59.25 ±0.3 b	460.75 ±125.69 a
WN	51 ±0.28 b	308 ±80.30 a
BRN	53.75 ±0.71 b	414 ±70.22 a
W	55 ±0.94 b	500 ±0.0 a
BR	53 ±0.92 b	415 ±69.4 a
BL	59 ±1.11 b	500 ±0.0 a

The different letters in the columns mean that there are significant differences at (P ≤0.05). BA: White and Black Roosters. WN: White naked-neck roosters, BRN. Brown naked-neck roosters. W: White roosters. BR: Brown roosters. BL: Black roosters.

RNA abundance may play a role in translational control of highly expressed, tissue-specific genes via their codon usage. Transcriptional control of RNA genes may therefore play a role in the function of rooster tissues or possibly in cellular development and differentiation, determination of RNA abundance and charging levels for differentiating cells or cells undergoing adaptation may reveal previously unseen connections between translation and other cellular processes. Given the central role of RNA in protein synthesis, this wide variation of RNA abundance may reflect translational control via the availability of certain RNAs (Dittmar et al., 2006).

Recent studies have revealed that though non-coding RNAs do not code for proteins directly, they do play a regulatory role in the transcription and translation of protein-coding genes, Non-coding RNAs have been considered the “dark matter” of the genome due to their great diversity and unclear functions (Zhao et al., 2017).

Circular RNAs (circRNAs) are derived from the reverse splicing of exons, introns, or both to form a closed continuous loop5, 6. Because circRNAs do not have 5′ or 3′ ends, they are free of exonuclease-mediated degradation and more stable than most linear RNAs (Cech and Steitz 2014).

At different stages of biological development the types and expression levels of circRNAs are vary significantly, CircRNA expression is tissue-specific and time-specific and the biogenesis of circRNAs is an integral, conserved, and regulated feature of the gene expression program (Salzman et al., 2013;You et al. 2015). Substantial amounts of circRNAs are widely distributed in the cytoplasm, the nucleus and a variety of body fluids, including saliva and serum exosome (Li et al., 2015; Lin et al. 2015).

CONCLUSION

This study established that TRIzol-based RNA extraction provides simple, fast, reliable, and feasible sample using different poultry tissues (blood, testes). The original single-step method described here is expected to yield the whole spectrum of RNA molecules, including small RNAs. So, the amount of RNA isolated will depend on the tissue used for extraction.

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بروتوكول استخلاص الحامض النووي RNA من نسيج الدم والخصية للديكة المحلية

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الكلمات المفتاحية:
الحمض النووي RNA, الدم, نسيج الخصية, الديكة المحلية, TRIZOL

الخلاصة

أصبحت طريقة (الخطوة الواحدة) مستخدمة على نطاق واسع لعزل الحامض النووي RNA الكلي من عينات الكائنات الحية ومن انسجة مختلفة، والهدف من هذه الدراسة استخلاص الحامض النووي الرايبي من دم ونسيج الخصية لذكور الدجاج المحلي. المبدأ الذي تقوم عليه الطريقة هو فصل الحمض النووي RNA عن الحمض النووي DNA بعد الاستخلاص بمحلول حامضي يحتوي على (Acid guanidinium thiocyanate-phenol-chloroform)مع الطرد المركزي، في ظل الظروف الحامضية يبقى إجمالي الحمض النووي RNA في الطور المائي العلوي بينما ينزل معظم الحمض النووي DNA والبروتينات إما في الطور البيني أو في الطور العضوي السفلي ثم يتم جمع إجمالي الحمض النووي RNA عن طريق الترسيب باستخدام الأيزوبروبانول (isopropanol). وقد اظهرت نتائجنا زيادة معنوية في تركيز RNA المستخلص من الدم مقارنة بنسيج الخصية لديكة المحلية.أتاح هذا البروتوكول عزل الحمض النووي RNA من الخلايا والأنسجة في أقل من 4 ساعات وكان سبب في التقدم الكبير في تحليل التعبير الجيني في النماذج النباتية والحيوانية وكذلك في العينات المرضية كما ظهر بوضوح من خلال العدد الهائل من الاستشهادات التي اكتسبها البروتوكول في الـ 20 سنة الماضية.