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Simple Modifications to Standard TRIzol® Protocol Allow High-Yield RNA Extraction from Cells on Resorbable Materials

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ABSTRACT

Resorbable bioceramics are attractive for medical applications such as bone substitution. Biochemical analysis on cells cultured on these biomaterials is vital to predict the impact of the materials in vivo and RNA extraction is an essential step in gene expression study using RT-qPCR. In this study, we describe simple modifications to the TRIzol® RNA extraction protocol widely used in biology and these allow high-yield extraction of RNA from cells on resorbable calcium phosphates. Without the modifications, RNA is trapped in the co-precipitated calcium compounds, rendering TRIzol® extraction method infeasible. Among the modifications, the use of extra TRIzol® to dilute the lysate before the RNA precipitation step is critical for extraction of RNA from porous α-tricalcium phosphate (α-TCP) discs. We also investigate the rationale behind the undesirable precipitation so as to provide clues about the modifications required for other resorbable materials with high application potential in bone tissue engineering.

Keywords: *Calcium Phosphate, Resorbable Materials, RNA Extraction, TRIzol, Acid Guanidium Thiocyanate - Phenol - Chloroform Extraction, TRI Reagent, TRIsure*

1. Introduction

Resorbable bioceramics with relatively high solubility are attractive materials to be used as bone substitutes since the implanted materials can eventually be replaced by bone newly formed *in vivo* [1-4]. In order to understand the cell-material interaction, gene expression of cells cultured on the materials is often monitored by means of RT-qPCR as several genes can be assessed at the same time. RNA extraction is an important step for RT-qPCR, especially for reliable quantification of gene expression level. TRIzol®, TRI Reagent® and TRIsure™ are commonly used commercial products from different companies for RNA extraction. They are modified versions of the single-step acid guanidium thiocyanate-phenol- chloroform extraction technique developed by Chomczynski and Sacchi, which is particularly useful for processing of large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples [5,6].

Nevertheless, this method cannot be applied to resorbable calcium phosphate ceramics directly as ions dissolved from the ceramics would co-precipitate with the RNA in the alcohol precipitation step, which prevents the RNA from being re-dissolved into aqueous solution in subsequent steps. The silica-gel affinity based method may be a feasible alternative for extracting RNA from resorbable α-tricalcium phosphate (α-TCP) [7,8] but the cost is usually much higher. Besides, TRIzol® gives a yield of 2.4 to 93 times higher than that using silica based protocol as demonstrated in reports using different biological samples [9-11]. High yield in RNA extraction is of particular importance in certain biomaterial studies due to the limited supply of expensive biomaterial scaffolds and the maximization of the number of samples that can be handled in each batch of experiment using cell culture. Furthermore, higher RNA concentration is attainable using this method even for the same yield of RNA compared with silica gel based methods as the pelleted RNA can be dissolved in a small volume (10 μL) of buffer while a relatively large volume (100 μL) of buffer

is required for efficient elution of RNA from the silica gel.

In order to use TRIzol® to extract RNA from cells on resorbable biomaterials, we carried out studies to devise modifications to standard protocol, which allow high quality RNA to be extracted from cells grown on α-TCP. In addition, modifications that may be required for other resorbable materials were suggested based on analysis of the parameters affecting the undesirable co-precipitation.

2. Materials and Methods

2.1. Materials, Cell Culture and RNA Extraction

10mm porous α-TCP discs were synthesized by foaming method using 5% hydrogen peroxide (H_2O_2) solution as described [12] in the National Engineering Research Center for Biomaterials in Sichuan University, China. For cell culture study, 9×10^4 C3H10T1/2 cells (pluripotent mesenchymal stem cells, CCL-226™, ATCC) were plated on the α-TCP disc and subjected to RNA extraction using TRIzol® reagent (Invitrogen) after 6 days of culture according to the manufacturer's instruction except drying the disc with absorbent paper and crushing the discs in TRIzol® with subsequent TCP debris removal by centrifugation during the homogenization step. Without further modifying the protocol, undesirable co-precipitation occurred in the RNA precipitation step.

2.2. Analysis on Precipitation

We studied the chemical composition of the precipitate by energy-dispersive X-ray spectroscopy (EDX) after sputtering with gold and the crystal morphology by scanning electron microscopy (SEM; JSM-6390, JEOL, Japan). To study the parameters affecting the undesirable co-precipitation process, different salts were added during the phase separation step of the TRIzol® protocol using cell free system as shown in **Table 3** followed by studies on effects of dilution, pH and temperature (**Table 4**). Based on results from these experiments, further modifications to the protocol were made and RNA could be extracted eventually.

2.3. RNA Quality Check

The quality and yield of the extracted RNA were assessed through gel electrophoresis and UV spectrometry (NanoDrop 1000, Thermo Scientific). The compatibility of the extracted RNA with RT-PCR and RT-qPCR, commonly used techniques for gene expression study, were also tested with the following procedures. DNase I treatment was performed with 500ng RNA using Amplification Grade DNase I (Invitrogen) and reverse-transcribed (RT) into single stranded cDNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) according to the manufacturers' protocol with 5μL and 20μL reaction volumes respectively. PCR was performed with *β-Actin* and *Col1* genes using primer pairs listed in **Table 1** with an annealing temperature of 53°C for both pairs. qPCR was performed with a total reaction volume of 10 μL, containing 1 μL of single stranded cDNA, 5 μL of TaqMan® Gene Expression Master Mix (Applied Biosystems) and 0.5 μL of TaqMan® Gene Expression Assays (Applied Biosystems) in **Table 2**, in 96 well reaction plates (2 μL of cDNA was used for the *Ocn* quantification due to the lower *Ocn* transcript level in the samples).

3. Results and Discussion

3.1. Overview

Figure 1 shows the standard TRIzol® RNA extraction procedures with modifications required for extraction of RNA from cells on resorbable materials. Using the standard TRIzol® RNA extraction protocol as the basis, RNA extraction was started with the porous TCP discs con

Table 1. Primers used in RT-PCR.

Gene (Gene bank ID)		Primer sequence		Genomic amplicon	cDNA amplicon
Mouse *β-Actin* (NM_007393.2)	5'	ATGGATGACGATATCGCTG	3'	1110 bp	569 bp
	5'	ATGAGGTAGTCTGTCAGGT	3'		
Mouse *Col1* (NM_00742.3)	5'	CCCAGAACATCACCTATCAC	3'	639 bp	510 bp
	5'	TTGGTCACGTTCAGTTGGTC	3'		

Table 2. Taqman® Gene Expression Assays used in RT-qPCR.

Gene	Gene symbol	Assay ID	Amplicon length	Reference sequence
β-Actin	Actb	Mm02619580_g1	143bp	NM_007393.3
Cbfa1	Runx2	Mm01269515_mH	74bp	NM_009820.4
Col1	Col1a1	Mm00801666_g1	89bp	NM_007742.3
Alpl	Alpl	Mm00475834_m1	65bp	NM_007431.2
Ocn	Bglap-rs1	Mm00649782_gH	89bp	NM_031368.4

Table 3. Effects of addition of different solutions on the amount of undesirable precipitates formed (indicated by "+"; more "+" indicates more precipitates) during the RNA precipitation step in a cell free system and the explanation of observation.

Tube	Solution	Solution volume (μL)	"Lysate" [a] at 4°C (μL)	Precipitates formed "immediately"?	Observation 1 day later	pH
1	/	-	200	++	+++++	4.5
2	Water	20	200	+	+++++	4.5
3	5M K Ac	20	200	+++++	+++++	5.5
4	5M NH_4Cl	20	200	-	+++++	4.5
5	10M NH_4Ac	20	200	++++	+++++	5.5

In comparison with the samples without any solution added (Tube 1),

- the addition of water (Tube 2) dilutes the ion concentration and reduces the precipitation.
- the addition of KAc solution (Tube 3) increases the pH and increases the precipitation.
- the addition of NH_4Cl solution (Tube 4) dilutes the Ca ion and reduces the precipitation. (The NH_4 ions probably have some effects on reducing the amount of undesirable precipitates but the mechanism is not fully known.)
- the addition of NH_4Ac solution (Tube 5) dilutes the Ca ion in the solution but at the same time increases the pH, thus increases the precipitation.

Precipitates appeared in all of the treatments after 1 day, indicating that there is a delay in the precipitation after the addition of water or NH_4Cl.

a. "Lysate" here refers to the TRIzol® solution which has been incubated with crushed TCP but with TCP debris removed before addition of salt solutions.

Table 4. Effects of dilution, pH and temperature on the undesirable precipitation in a cell free system.

Effect of dilution [a]				Effect of pH [c]		Effect of temperature [d]	
Ratio	" Lysate" [b] (μL)	Extra TRIzol® (μL)	Precipitation	Solution added	Precipitation	Immediate observation	
1:0	100	/	+++	-	+++	0°C	-
4:1	80	20	-	Water	++	25°C	+++
3:2	60	40	-	2M HCl	+	Observation after 1 h	
2:3	40	60	-			0°C	-
1:4	20	80	-			25°C	+++

a. Extra TRIzol® was added after TCP debris removal before chloroform addition.
b. "Lysate" here refers to the TRIzol® solution which has been incubated with crushed TCP but with TCP debris removed before extra TRIzol® addition.
c. 20μL of solution was added to 200μL of "Lysate" after TCP debris removal before chloroform addition. HCl stands for hydrochloric acid.
d. It refers to the temperature of TRIzol® during crushing of TCP discs (0°C was maintained by putting the tube on ice while 25°C indicates that the crushing of TCP was performed at room temperature).

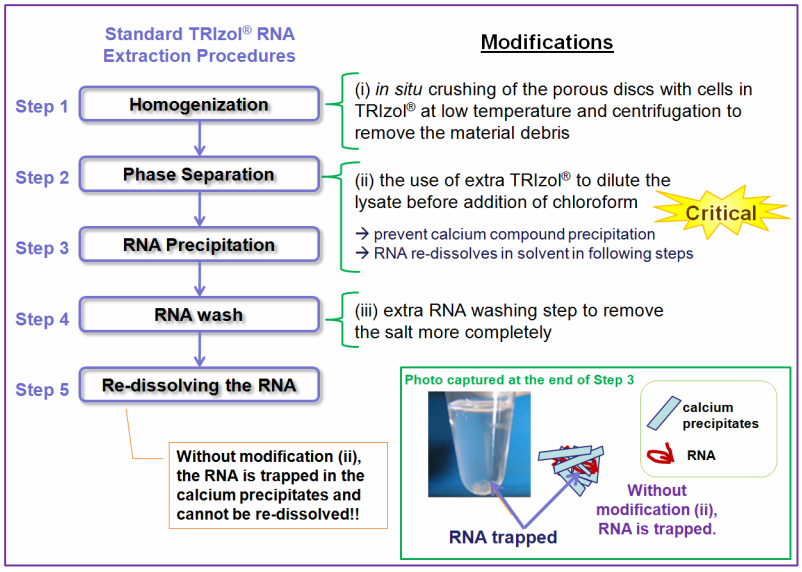


Figure 1. Schematic showing the standard TRIzol® RNA extraction procedures shown on the left with modifications required for RNA extraction from cells on resorbable materials denoted on the right.

taining the attached cells crushed in TRIzol®. TCP debris was removed by centrifugation in Step 1 (Homogenization). Step 2 (Phase separation) and Step 3 (RNA precipitation using isopropanol) were performed as described by the manufacturer. Using this procedure, undesirable co-precipitate appeared, making the precipitated RNA unable to be dissolved in Step 5. Thus no RNA could be extracted. Analysis of the undesirable precipitation which eventually leads to the suggested modifications will be explained in the following.

3.2. Identification of the Undesirable Precipitate

To identify the components of the undesirable precipitate, we studied the morphology and the composition of the precipitate by SEM and EDX. SEM examination shows that the precipitate is in the form of clustered crystals (**Figure 2(a)**). From the EDX analysis **(Figures 2(b)** and **2(c)**), the precipitate was found to be mainly composed of Ca, O, C and N. Calcium thiocyanate ($Ca(SCN)_2$) and calcium acetate ($Ca(C_2H_3O_2)_2$) may be the possible constituents of the precipitate, leading to the interpretation of having the precipitate coming from the dissolution of the calcium containing biomaterials during the TRIzol® mediated cell lysis step, rather than being the direct precipitation of components present in the TRIzol® reagent.

3.3. Parameters Affecting the Undesirable Co-Precipitation

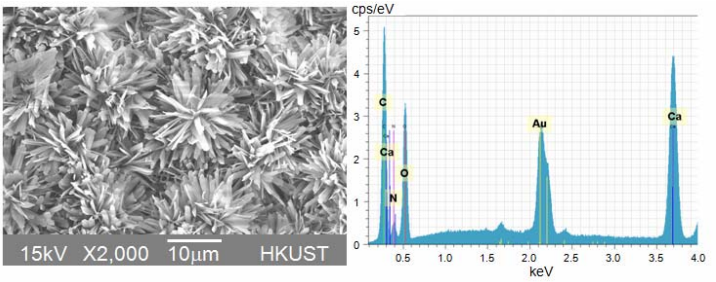
Different approaches to get rid of the undesirable co-precipitates were attempted and the key experiments leading to the modifications are highlighted in this report. In molecular biology, precipitation with ethanol is the standard method to recover nucleic acids from aqueous solutions and ammonium acetate is frequently used to reduce the co-precipitation of unwanted contaminants such as dNTPs or oligosaccharides with nucleic acids [13]. This ethanol precipitation step has high similarity to the RNA precipitation step using isopropanol in the TRIzol® protocol. And having known that most ammonium, potassium, acetate and chloride compounds have high solubility in aqueous solutions [14], we attempted to avoid the undesirable co-precipitation by adding different salt solutions in the phase separation step (Step 2 of **Figure 1**). Cell-free system was used to simplify the analysis and the results together with the explanation of observation are summarised in **Table 3**. The addition of ammonium chloride solution eliminates the undesirable precipitation but more importantly the results revealed that dilution and pH are critical parameters in the precipitation process. Besides, we proposed that lower temperature would reduce the precipitation by slowing down the dissolution of calcium ions from TCP. Hence, we carried out experiments to investigate the effects of these three parameters: dilution, pH and temperature.

3.3.1. Effect of Dilution

From **Table 3**, the addition of water reduces the precipitation but it also dilutes other salts in the TRIzol® which may affect the phase equilibrium in the phase separation step and hence affects the normal RNA precipitation process. Thus we have experimented using TRIzol® to dilute the Ca ions after TCP debris removal. Surprisingly, the addition of as little as 1/4 volume of TRIzol® already eliminates the unwanted precipitation (**Table 4**), probably by preventing the solution from being saturated in one of the insoluble calcium compounds. For more soluble materials, larger extra TRIzol® to lysate ratio may be required.

3.3.2. Effect of pH

Besides, the addition of HCl can reduce the precipitates formed (**Table 4**). This observation indicates that lower pH increases the solubility of the insoluble compounds, thus less precipitates would be formed. Therefore we can reduce the amount of precipitates by lowering the pH during the RNA precipitation step. On the other hand, increasing the pH would lower the solubility of the calcium phosphate materials [15], which in turn may reduce the dissolution of Ca ions from materials that are more soluble than TCP. By using "self-made TRIzol® reagent"



(a) (b)

Element	Atomic % (average ± SD)	
O	40.59	±0.05
C	29.53	±0.85
N	16.78	±0.62
Ca	11.35	±0.27
Au	1.76	±0.02

(c)

Figure 2. (a) SEM photo of the precipitate, (b) EDX spectrum and (b) Atomic concentration of the precipitate in elemental analysis using EDX (average ± SD).

(with all the components in TRIzol® reagent except sodium acetate) at neutral pH to lyse the cells on the resorbable materials and adjusting the pH after material debris removal by using sodium acetate (2 M, pH 4) (50 μL added per 1 mL), the undesirable co-precipitates can probably be reduced.

3.3.3. Effect of Temperature

Since most chemical reactions are slower at lower tem--perature, we have proposed that lower temperature would reduce the undesirable precipitation by slowing down the dissolution of Ca ions from TCP. This is confirmed by experiments summarized in **Table 4**. Hence, it is better to use chilled TRIzol® and keep the TRIzol® with α-TCP on ice during the crushing of materials so as to reduce the amount of Ca ions dissolved from the α-TCP.

3.4. Suggestions on Modifications

From the above experiments, we summarized the factors affecting the undesirable precipitation in **Table 5**. Based on this, we proposed the modifications required for RNA extraction from cells on porous α-TCP discs as shown on the right of **Figure 1**. (A sample protocol was attached to the end of this article). On the other hand, TCP is only one of the promising resorbable materials, while other resorbable materials may have high application potential in the future. Different materials differ greatly in solubility in aqueous solution (**Table 5**) [16]. Analysis in **Table 5** also suggests that increasing the extra TRIzol® to lysate ratio, increasing the pH during crushing of the materials and lowering the pH during the RNA precipitation step can be used for materials which are more soluble than TCP. These aim to minimize the dissolution of ions from materials and the precipitation of the undesirable co-precipitates. On the other hand, TRI Reagent® and TRIsure™ have similar extraction principles and procecures as TRIzol®. Thus similar modifications can be made to their RNA extraction protocols to extract RNA from cells on resorbable materials in high yield.

3.5. Quality of the Extracted RNA

To evaluate the quality of RNA extracted using the modified procedures, we used agarose gel electrophoresis analysis and UV spectrometry to check the samples. Two distinct bands showing the 28S and 18S ribosomal RNA were observed and degradation of RNA was not detected after the agarose gel electrophoresis (**Figure 3(a)**). By UV spectrometry, the A260/A280 ratios were consistently

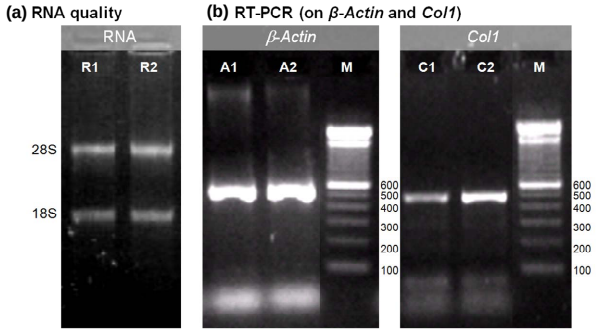


Figure 3. (a) Agarose gel image of RNA in 2% GelRed pre-stained gel (b) Conventional RT-PCR of *β-Actin* (A1 and A2) and *Col1* (C1 and C2), M: 100bp DNA ladder.

Table 5. Factors affecting the dissolution of materials in the homogenization step and the precipitation of undesirable precipitates in the RNA precipitation step.

	Dissolution of materials	Precipitation of undesirable precipitates
Nature of compounds	Different materials differ greatly in solubility. Relative solubility [a]: MCPM ~ MCPA > DCPD > DCPA >> α-TCP > β-TCP > TTCP >> CDHA > OCP > HA [16]	Most ammonium, potassium, acetate and chloride compounds have high solubility in aqueous solutions while many calcium compounds are insoluble [14].
Volume of TRIzol®	A smaller TRIzol® volume reduces the total amount of ions dissolved from the materials but the volume should be large enough for effective lysis of the cells on materials.	A larger volume of extra TRIzol® reduces the concentration of ions which may contribute to the undesirable precipitation.
pH	For many calcium phosphates (such as OCP, α-TCP, β-TCP, HA and TTCP) [a], a higher pH reduces the dissolution rate within pH range of 4 to 7 [15].	A lower pH increases the solubility of the undesirable precipitates, thus reduces the precipitation.
Temperature	A lower temperature reduces the dissolution rate.	A higher temperature can probably increase the solubility of the undesirable precipitates but a low temperature is preferred to prevent RNA degradation.

a. MCPM: monocalcium phosphate monohydrate; MCPA: monocalcium phosphate anhydrate; DCPD: dicalcium phosphate dihydrate; DCPA: dicalcium phosphate anhydrate; OCP: octacalcium phosphate; α-TCP: α-tricalcium phosphate; β-TCP: β-tricalcium phosphate; CDHA: calcium-deficient hydroxyapatite; HA: hydroxyapatite; TTCP: tetracalcium phosphate

close to 2.0 and the A260/230 ratios were higher than 1.0, indicating that the RNA samples extracted by this method are not carrying contaminating proteins and salts (**Table 6**).

Finally, the quality of the RNA was confirmed by conventional RT-PCR and RT-qPCR. After DNase I treatment, reverse transcription and PCR amplification with primer pairs specific to the transcripts from *β-Actin* gene (a housekeeping gene as control) and *Col1* gene (a chondrogenic gene), specific amplicons with the corresponding molecular sizes were observed (**Figure 3(b)**). Furthermore, consistent Ct values from RT-qPCR analysis on *β-Actin*, *Col1* and other bone cell differentiation-markers such as *Cbfa1*, *Alpl* and *Ocn* (**Table 7**) were noted, indicating that the RNA quality of these samples is compatible with protocols for gene expression study. In addition, this protocol allowed us to isolate 2 μg of total RNA from cells grown on 10 mm α-TCP disc, an amount enough for performing expression evaluation on 40 different target genes. This good yield allows the protocol to be used for large scale analysis, when cell supply is limited because of the lack of large volume of biomaterials used in the study.

4. Conclusions

For RNA extraction from cells on porous α-TCP, the following three modifications are sufficient to obtain high quality RNA: 1) *in situ* crushing of the porous discs with cells in TRIzol® at low temperature 2) the use of extra TRIzol® to dilute the lysate after TCP debris removal and 3) extra RNA washing step to remove the salts more completely. Without modifications, the RNA was often trapped in the undesirable precipitates which prevent effective recovery and render TRIzol® extraction method incompatible with studies using α-TCP.

Analysis of various physical parameters affecting the formation of undesirable co-precipitates suggests that a combined protocol of increasing the extra TRIzol® to lysate ratio, increasing the pH during crushing of materials and lowering the pH during the RNA precipitation step would make extraction of RNA from cells cultured on other more soluble materials possible.

In summary, this study introduces to the field some easy-to-perform and low cost modifications to extract RNA from cells grown on α-TCP in high yield. It also offers a direction for further modifications of the procedures in extracting nucleic acid samples from cells cultured on soluble ceramic materials. The impact on biomedical development and applications could be tremendous.

Table 6. UV absorbance readings using Nanodrop of the RNA extracted from cells on porous α-TCP disc, indicating the extracted RNA is of high quality.

	A260/280 [a]	A260/230	conc. (ng/mL)
average	2.00	2.05	223.23
SD	0.08	0.14	40.98

a. Using other spectrometric equipments for the UV absorbance measurements often requires a dilution of the samples and may result in readings of A260/280 deviating from 2.00 but the RNA may still be suitable for RT-PCR. In such case, the RNA quality should be confirmed by gel electrophoresis.

Table 7. Ct values of *β-Actin*, *Cbfa1*, *Alpl*, *Col1* and *Ocn* of RT-qPCR with the RNA samples extracted using the modified procedures.

	β-Actin	*Cbfa1*	*Alpl*	*Col1*	*Ocn*
average	20.6	25.2	31.6	18.8	35.7
SD	0.6	0.4	0.2	0.3	0.5

5. Acknowledgements

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6. Sample Protocol for RNA Isolation from Cells Grown on Porous α-TCP Discs[a]

Materials used: 10mm porous α-TCP discs with 9×10^4 cells plated and cultured for 6 days.

1) HOMOGENIZATION

- At the day of analysis, wash the bioceramic disc (containing cultured cells) with PBS twice to remove trapped cell culture medium. Briefly dry the disc on absorbent paper between washes.
- Briefly dry the disc on absorbent paper, gently break (not crush) the disc into smaller pieces and transfer the pieces to a 1.5 ml microcentrifuge tube.
- Add 0.5 ml of TRIzol® (Invitrogen) to the tube and crush the disc into powder with a steel rod with the tube placed on ice.
- Incubate the tube for 5 min at room temperature to lyse cells.
- Centrifuge the tube at 12,000 x g for 5 min at 4°C and transfer the clear orange lysate to a new tube. Repeat this step if TCP debris is observed at the bot-

[a]All reagents and consumables involved in RNA experiments should be free of RNase (Critical!). Besides, caution should be taken while using TRIzol® (specifically phenol) and Chloroform.

tom of the tube.[b]

2) PHASE SEPARATION

- Add another 0.5 ml of TRIzol® to the clear lysate and mix the content.
- Add 0.2 ml of chloroform[c] to the lysate and shake vigorously by hand for 15s and incubate the tube for 2 min at room temperature.
- Centrifuge the tube at 12,000 x g for 15 min at 4°C.

3) RNA PRECIPITATION

- Transfer the aqueous supernatant to a new tube and gently mix it with 0.5 ml of isopropanol by inverting the tube several times.
- Incubate the tube for 10 min at room temperature and centrifuge the tube at 12,000 x g for 10 min at 4°C

4) RNA WASH

- Remove the supernatant, add 0.5 ml of 75% ethanol to the tube and centrifuge at 7,500 x g for 5 min at 4°C. Repeat this washing step once.

5) REDISSOLVING THE RNA

- Remove the ethanol in the last wash[d] and air-dry the RNA pellet (until no observable liquid droplet was found).
- Resuspend the RNA in RNase-free water / buffer and incubate at 60°C for 10min. Then proceed to gene expression studies after RNA quality check and quantitation.

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[b]For a large number of materials being tested with cells plated in one batch, the cells may be lysed in TRIzol® at the same time and stored in a –80°C freezer after material debris removal before subsequent extraction procedures which require relatively longer time. Besides, the samples can be splitted into several extraction batches for easier handling if required.

[c]Bromo-chloropropane may be used instead of chloroform as a safer and equally efficient phase-separation agent [17].

[d]When the amount of RNA is small, the RNA pellet may be difficult to be observed by naked eye. In such case, the orientation of the tubes during centrifugation should be noted so that the position of the tiny RNA pellet is known. During this step, it is important to avoid pipetting the tiny precious RNA pellet together with the ethanol which would then be discarded.

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