SHORT COMMUNICATION

Veterinary Research Forum. 2020; 11 (2) 179 – 184
doi: 10.30466/vrf.2018.92133.2230
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

The challenge of getting a high quality of RNA from oocyte for gene expression study

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Article Info

Article history:

Received: 18 August 2018
Accepted: 29 December 2018
Available online: 15 June 2020

Keywords:

Gamete
Pre-implantation embryos
RNA extraction
RT-PCR
TRIzol

Abstract

The extraction of intact RNA from oocyte is quite challenging and time-consuming. A standard protocol using commercial RNA extraction kit, yields a low quantity of RNA in oocytes. In the past, several attempts in getting RNA for gene expression study ended up with a few different modified methods. Extraction of high-quality RNA from oocyte is important before further downstream analyses such as reverse transcription-polymerase chain reaction, quantitative polymerase chain reaction, or northern blot analysis. In this review, the efficiency of RNA extraction methods from all species oocytes was compared between published articles and our research to gather all possible methods of RNA extraction. Two different methods of RNA extraction that were proposed from various experiments were reviewed to determine the best method of RNA extraction from the oocyte. Modified TRIzol method can be concluded as an efficient RNA extraction method especially for good RNA from oocytes. Meanwhile, comparing RNA extraction kits to extract the RNA from oocytes or pre-implantation embryos, the micro RNA extraction kit type is the best. Therefore, an appropriate RNA extraction method is important to obtain high quality of total RNA for gene expression profiling analysis.

Introduction

Each new life begins from a fascinating journey called fertilization, the union of two gametes; an oocyte and a sperm. Upon ovulation, the cumulus-oocytes complex (COC) will be released from the female ovary to the Fallopian tube or oviduct. Sperm will then continue to move towards the oocyte through intact cumulus cells. Once the capacitated sperm attach to oocyte's zona pellucida, a process called acrosome reaction will occur, which releases all the hydrolytic enzymes to enable the sperm to penetrate the oocyte for fertilization. The two-pronuclear zygote will be formed from the fertilization of sperm and oocyte nuclei. Fertilized oocyte will then developed into a 2-cell stage embryo followed with 4-cell, 8-cell, morula, and lastly develops into blastocyst cell before embryo implantation in the uterus.

An oocyte is a female germ cell and one of the largest cells in the body. The oocyte is rich in the cytoplasm that contains yolk granules to support the cell's growth, maturation, and the early development after fertilization. The oocyte is enclosed with a thick glycoprotein shell called the zona pellucida. The main role of the oocyte is to be fertilized and to grow into a fully functional organism. Therefore, the oocyte has to be able to regulate many different cellular and developmental processes such as regulation of the cell cycle progression and cellular metabolism, fertilization, embryo development, activation of the embryonic genome, and formation of body axes.[1] During oocyte growth, a variety of maternally transcribed messenger RNAs (mRNAs) are supplied which represent the maternal contribution to the newly fertilized oocyte, zygote, and early embryo. These mRNAs can be stored in messenger ribonucleoprotein complexes and then translated when needed. These mRNAs also can be localized within a specific region of the cytoplasm or dispersed within the cytoplasm of the entire oocyte.[2]

Oocyte serves as a basis for human or mammalian reproduction. Thus, the quality of oocyte is considered the most important aspect of successful fertilization, embryo

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maturation, and development. One way to determine the quality of oocyte is to explore the genes related using a few methods in gene expression study. In a gene expression study, RNA is extracted as genetic materials for further downstream analysis. However, extraction of high quality of RNA from oocytes and embryos is quite challenging due to limited quantity of cells and consequently, RNA.[3] To overcome the limitation, large pools of up to 300 oocytes or embryos are used for RNA extraction. The major difficulty of using these large pool samples was the time-consumingness to collect a large number of oocytes and embryos.[4] Therefore, various techniques and methods had been proposed to overcome the technical difficulties occurring during RNA extraction from oocytes and pre-implantation embryos as summarized in Table 1.

In the past, several attempts that focused on oocyte gene expression profiling by different approaches had resulted in finding a group of genes related to the quality of oocyte or other reproductive functions (Table 2). Oocyte gene expression can be evaluated using mRNA which provides genetic materials and the downstream effect of epigenetic influences mediating oocyte development.[5] The first gene expression analyses in human oocytes was performed by reverse transcription-polymerase chain reaction (RT-PCR) and the latest with microarray technology.[1] In the research done by Zhao *et al*., the authors managed to detect histamine type 2 receptor (H_2) mRNA in mouse blastocysts during pre-implantation embryonic development using the RT-PCR technique. The study confirmed the existence of H_2 in mouse blastocysts, which was believed to bind with histamine the uterus for embryo implantation.[6]

Serotonin 5-HT1D receptor mRNA was expressed in mouse oocytes, zygotes, 2-cell embryos, compacted morulae, and *in vivo* produced expanded blastocysts through RT-PCR in a former study.

The expression of the mRNA 5-HT1D serotonin receptor was also detected in blastocysts cultured *in vitro*. Demonstration of the expression of 5-HT1D serotonin receptor in the mouse oocyte and pre-implantation embryos supports the idea of serotonin 5-HT1D receptor role in the early mammalian development, where cultured with specific serotonin 5-HT1D agonist sumatriptan (1.00 μM) significantly inhibited the development of mouse embryos.[7]

The expression of the α_2C-adrenergic receptor gene in the ovulated oocyte, 8 to 16-cell morulae, and expanded blastocysts using the RT-PCR method has also been demonstrated previously. Exposure of mouse pre-implantation embryos to α_2C-adrenergic receptor agonists led to a significant reduction of the mean embryo cell number. The study suggested that elevated epinephrine and norepinephrine hormones during stress and trauma, could directly affect embryo development via adrenergic receptors. Therefore, from the findings, it is supported that maternal stress can influence embryo development even in the early stage of pregnancy.[8]

Findings showed that gene expression study in the oocyte is important to understand the various mechanisms involving in the development of embryos, fertilization, and other processes related to reproduction. Few methods were applied to study gene expression profiling in oocytes such as RT-PCR, real-time PCR, or northern blot analysis. However, extraction of high-quality RNA from oocyte is crucial before the analysis. In this short communication, a comparison of two common methods of RNA extraction from oocytes; modified TRIzol method and commercial RNA extraction kit, was carried out which could give some guidelines or ideas in extracting high quality of total RNA from oocytes in any species for further gene expression analysis.

Table 1. Summary of various RNA extraction methods from oocyte.

Method	Oocyte	Comment	Reference
Guanidine isothiocyanate procedure	Murine	Large amounts of oocytes used; 200 oocytes.	7
QuickPrep micro mRNA purification kit*	Bovine	The RNA was extracted from pools of 20 oocytes and embryos. Expression of poly(A)-binding protein nuclear-like 1 gene was detected in oocytes and expression of methyl-CpG–binding domain protein 3-like 2 genes were detected in early embryos through RT-PCR.	17
RNeasy Micro Kit†	Ovine	The expression of CD44 gene in sheep oocyte and pre-implantation embryos had been detected through RT-PCR. Micro RNA kit is suggested especially for the extraction of RNA from oocyte compared to commercial RNA extraction kit.	16
Modified TRIzol method	Bovine	In RT-PCR, the DNMT1 gene amplified better using the RNA extracted from this method compared to RNA extracted using other methods.	9
	Bovine & Swine	The amplification of polyadenylated RNA resulted in detectable DNA products ranging from ~ 500 to ~ 5000 nucleotides. The consistency of high RNA quality extracted across samples had demonstrated that the quality was appropriate for single-cell mRNA-sequencing.	11

* Amersham Biosciences, Buckinghamshire, UK, and † Qiagen, Valencia, USA.

Table 2. List of genes involved in oocyte and embryo development in reproduction study.

Genes	Source	Year	Reference
Histamine type 2 receptor	Mouse blastocysts	2000	Zhao *et al*. [6]
Serotonin 5-HT1D receptor	Mouse oocytes, zygotes, 2-cell embryos, compacted morulae and *in vivo* produced expanded blastocysts	2003	Veselá *et al*. [7]
α_2C-adrenergic receptor	Mouse oocytes, 8 to 16-cell morulae and expanded blastocysts	2007	Cikos *et al*. [8]

Materials and Methods

Extraction of RNA using the modified TRIzol method. Few studies had applied the modified TRIzol method especially in extracting the RNA from oocytes (Table 3). Pavani *et al*. have modified TRIzol protocol from Chomczynski and Sacchi, and used it to extract RNA from bovine oocytes.[9,10] The modifications in the TRIzol protocol were made to the minimum number of bovine oocytes. In 500 µL micro-centrifuge tubes, 100 µL of TRIzol reagent was added with five to 60 oocytes each and vitrified oocytes, 15 to 40 each to different micro-centrifuge tubes. The micro-centrifuge tubes were then vortexed and incubated at room temperature for a few mins, providing the penetration of the TRIzol solution into the cell walls. After incubation for 3 min, 50.00 µL of chloroform was added to the tubes and the tubes were inverted for 15 sec, following incubation for another three min. The micro-centrifuge tubes were then centrifuged at 12,000 *g* for 30 min at 4.00 ˚C. After centrifugation, the aqueous phase was transferred to the new RNAse free tube and isopropanol was added two times of the volume to the aqueous phase recovered.[10] Isopropanol is a very good precipitating agent, which helps in desalting and recovering of nucleic acids. The higher the concentration of isopropanol, the higher will be the recovery of the nucleic acids.[11] After centrifugation at 12,000 *g* at 4.00 ˚C for 30 min, the supernatant was washed with 150 µL of 70.00% ethanol and centrifuged at 7,500 *g* for 5 min. The pellet was dried in an incubator for 30 min at 37.00 ˚C and further dissolved in 20.00 µL of diethylpyrocarbonate (DEPC)-treated water. The quality of the RNA extracted was further quantified using a spectrophotometer (NanoVeu; GE Healthcare, Chicago, USA). From the results, the purity of RNA extracted from the modified TRIzol protocol in Pavani *et al*., study is ranged from 1.50 to 2.11 which nearest to the accepted purity level, 1.80-2.00. The RNA extracted from developed modified TRIzol protocol then was used in further downstream analysis using RT-PCR for gene expression study. In RT-PCR, the amplification of the DNMT1 gene using the RNA extracted from modified TRIzol protocol had clearly shown, which proven its efficiency by attaining better purity and total RNA concentration since the gene amplified better in RT-PCR.[9]

In Kimble *et al*. study, the RNA was extracted from oocytes with the incubation times and centrifugation steps performed as recommended by the TRIzol™ (ThermoFisher Scientific, Waltham, USA) and Phasemaker™ (ThermoFisher Scientific) protocols.[11] The Phasemaker™ tube contains a thick liquid polymer. The polymer in Phasemaker tubes is heavier than the aqueous phase of the TRIzol reagent mix but lighter than the organic phase. After centrifugation, it positions itself between these two layers that will help to remove the aqueous phase containing RNA easily. Few modifications in extracting the RNA from oocytes include the use of Phasemaker™ tubes, a second chloroform wash of the aqueous phase and the precipitation of the RNA with glyclogen in a 200 µL micro-centrifuge tube were made. First, the oocyte was thawed by adding 150 µL of TRIzol and followed by adding 30.00 µL of chloroform. The mixture was transferred to the Phasemaker™ tube for centrifugation at 12,000 *g* for 5 min at 4.00 ˚C. In this experiment, the aqueous solution was mixed for the second time with 20.00 µL of chloroform which followed by second centrifugation at the same parameters. The aqueous solution was collected and mixed with 1.00 µL of glycogen (Glycoblue™; Thermo Fisher Scientific, Austin, USA) and 150 µL of isopropanol in a 200 µL tube and was centrifuged at 12,000 *g* for 10 min at 4.00 ˚C. The use of glycogen in the experiment was to help in nucleic acid recovery in the solution during alcohol precipitations. After the centrifugation, the supernatant was pipetted out and the RNA pellet at the bottom of the tube was washed twice with 75.00% ethanol followed by centrifugation at 7,500 *g* for 5 min at 4.00 ˚C. The pellet was air-dried and eluted in 1.00 µL of nuclease-free water. The purity of the extracted RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) following the manufacturer's protocol. From the analysis, the RNA integrity number values between 5.10 and 7.30.

Table 3. Summary of RNA extraction from oocytes using a modified TRIzol method.

Genes	Source	Year	Reference
GAPDH, β-actin, ribosomal protein L7, 16s ribosomal protein, and histone H2A.Z	Mouse oocytes	2005	Jeong *et al*. [13]
AdipoR1 (132 bp) and AdipoR2 (258 bp) gene	Mouse pre-implantation embryos and oocytes	2010	Cikos *et al*.[12]
DNMT1 gene	Bovine oocytes	2015	Pavani *et al*. [9]

The amplification of polyadenylated RNA resulted in detectable DNA products ranging from ~ 500 to ~ 5000 nucleotides. The consistency of high RNA quality extracted across samples had demonstrated that the quality was appropriate for single-cell mRNA-sequencing.[11]

In another study, the expression of adiponectin receptors in mouse pre-implantation embryos was detected using RT-PCR by Cikos *et al*.[12] In this study, the authors used the RNA extracted from 90-100 mouse pre-implantation embryos and unfertilized oocytes using TRIzol reagents (Invitrogen Life Technologies, Karlsruhe, Germany) method. The RNA extracted using the TRIzol method is proved to have better purity since the authors managed to detect PCR products corresponding to AdipoR1 (132 bp) and AdipoR2 (258 bp) genes in oocytes as well as embryos of all other developmental stages.[12]

While in other experiments done by Jeong *et al*., the authors employed three methods for the extraction of RNA from mouse oocytes. In one of the methods, total RNA of each oocyte or embryo was extracted using the TRIzol reagent (Gibco BRL, Carlsbad, USA).[13] Secondly, messenger RNA was extracted with oligo-dT conjugated bead using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions and lastly, freeze/thaw cycles with the only reverse transcription buffer (Promega, Madison, USA) were carried out for the collection of total RNA.[13] The total RNA extracted was compared for its efficacy by evaluating the expression levels of GAPDH, β-actin, ribosomal protein L7, 16s ribosomal protein, and histone H2A.Z using real-time PCR. The amplification was evident from all the samples prepared with no significant differences of Ct values among the RNA extracted from all the three methods.[13]

Extraction of RNA using RNA extraction kit. Several studies had proposed using an RNA extraction kit in extracting RNA from the oocyte (Table 4). There are many RNA extraction kits offered by different manufacturer companies over the world. As an example, in Steuerwald *et al*. , study, total RNA was extracted from individual oocyte and embryo using Micro RNA Isolation Kit (Stratagene, La Jolla, USA) according to the manufacturer's protocol with the addition of 10.00 µg glycogen as a carrier.[14]

In the experiment done by Zhu *et al*., RNeasy Micro Kit (Qiagen, San Francisco, USA) was employed to extract the RNA from porcine oocyte with some modifications to the samples. Snap frozen samples were incubated at 65.00 ˚C for 5 min to facilitate the lysis of the oocyte and the release of RNA before RNA extraction.[15]

Another experiment also used the RNeasy Micro Kit for RNA extraction from oocytes. In this study, the expression of CD44 gene in sheep oocyte and pre-implantation embryos had been detected through RT-PCR using purified RNA extracted through RNeasy Micro Kit.[16]

In Biase *et al*., study, poly (A)-binding protein nuclear-like 1 and methyl-CpG–binding domain protein 3-like 2 genes had been detected in oocytes and early embryos from the RNA extracted using the QuickPrep Micro mRNA Purification kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.[17]

In addition to the experiments reviewed, the author had also done the experiments to compare the extraction of RNA from mouse oocytes and COC with two methods, using a modified TRIzol method and RNA extraction kit. In the beginning, total RNA was extracted from nine mouse COC and 200 of mouse oocytes using RNeasy Mini Kit (Qiagen, USA). Extractions of total RNA from mouse COC and oocyte were performed according to the manufacturer's recommendations. Additionally, 10.00 µL of β-mercaptoethanol was added to 1.00 mL of RLT buffer solution to help in the process of sample lysis. In the other experiment, total RNA was extracted from snap freezing of 10 COC and 200 mouse oocytes using TRIzol reagents (Ambion, Austin, USA) with some modifications. The purified RNA was dissolved in 30.00 µL of DEPC-treated water followed by incubation at 55.00 ˚C for 10 min.

Results

Total RNA extracted from the two methods mentioned was analyzed by measuring optical density at 260 nm and 280 nm using Nanodrop 2000 ultraviolet spectro-photometer (Thermo Fisher Scientific). The RNA yielded from mouse COC and oocytes using RNeasy Mini Kit showed a very low quantity of RNA (Table 5).

Table 5. Spectrophotometric readings of RNA extraction using RNeasy Mini Kit.

Sample	Number of COC/oocytes	RNA concentration (ng µL^{-1})	Purity 260/280
Blank	0	0.60	1.04
COC	9	3.80	1.68
CFO	200	1.90	10.50

COC: Cumulus-oocytes complexes, and CFO: Cumulus free oocytes.

Meanwhile, total RNA extracted from mouse COC and oocytes using a modified TRIzol method showed higher quantity of total RNA compared to the RNA extracted using the RNeasy Mini Kit (Table 6). The total RNA solution was also evaluated for RNA integrity through gel electrophoresis. The extracted RNA solution was loaded on 1.00% agarose gel and electrophoretically separated at

Table 4. Summary of RNA extraction from oocytes using commercial RNA extraction kit.

Genes	Source	Year	Reference
β-actin	Human oocyte	1999	Steuerwald *et al*.[14]
PELP1, Myo5b, and CAST	Porcine oocyte	2007	Zhu *et al*. [15]
CD44 gene	Sheep oocyte	2012	Luz *et al*.[16]

100 v for 40 min, separating intact 28S and 18S ribosomal RNA. From the results, RNA extracted using the TRIzol method showed intact 28S and 18S ribosomal RNA with two clear bands shown on the gel compared to RNA extracted using the RNeasy Mini Kit (Fig. 1).

Table 6. Spectrophotometric readings of RNA extraction using a modified TRIzol method.

Sample	Number of COC/oocytes	RNA concentration (ng µL^{-1})	Purity 260/280
Blank	0	0.60	1.04
COC	10	151.00	1.70
CFO	200	126.70	1.68

COC: Cumulus-oocytes complexes, and CFO: Cumulus free oocytes.

The RNA was then used for further downstream analysis through RT-PCR. Total RNA extracted was reverse transcribed to cDNA before PCR amplification for the qualitative analysis. The PCR amplification of control genes, Gapdh and beta-actin were carried out in a total volume of 25.00 µL containing 1.00 µL of cDNA, 0.50 µM of each oligonucleotide primer, 2.00 mM MgCl2, 0.20 mM dNTPs (dATP, dTTP, dCTP and dGTP) and 0.05 U mL^{-1} Taq DNA polymerase (Promega). Amplification was performed for 35 cycles in PCR thermal cycler (ESCO, Singapore). Each cycle included target denaturation for 5 min, at 94.00 ˚C, primer annealing for 30 sec, at 60.00 ˚C and extension for 1 min at 72.00 ˚C. After PCR amplification, the PCR solution was loaded in 1.00% agarose gel for gel electrophoresis by separation at 100 v for 40 min. Clearly, the results showed the amplification of the control gene and beta-actin from PCR analysis using the RNA extracted through the modified TRIzol method. The PCR analysis using the RNA extracted through the RNeasy Mini Kit showed no amplification of the control gene and Gapdh (Fig. 1).

Discussion

Several methods of RNA extraction from oocytes had been proposed for the gene expression profiling study in oocytes. Most of the experiments had employed the TRIzol method with some modifications and micro RNA extraction kit as efficient methods to extract high quality of total RNA from oocytes. TRIzol contains guanidium isothiocyanate, an RNAse inhibitor, which prevents RNAse activity in the environment. The RNA is extremely unstable compared to DNA and this RNAse will degrade the RNA. Therefore, due to the limitation of RNA in the single oocyte, TRIzol seems quite efficient in extracting RNA.[18,19] The lysis process in RNA extraction using TRIzol reagent for certain cells was generally proved to have a higher RNA yield as suggested by Poong *et al.*[20] The authors reported that extraction of RNA in high concentration of lipids and polysaccharides green micro-alga using TRIzol reagent would give a higher RNA yield.

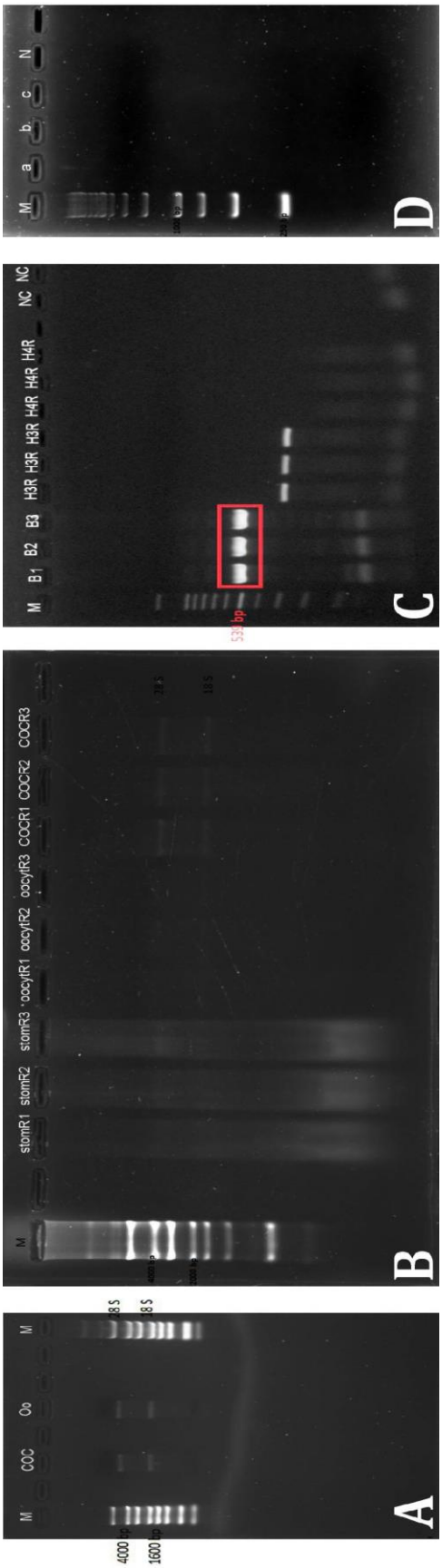

Fig. 1. Agarose gel electrophoresis (1.00%). **A)** Showing RNA extracted using the modified TRIzol method; **B)** Showing RNA extracted using RNeasy Mini Kit. M: Molecular weight marker; COC: Cumulus oocyte complexes; Oo: Cumulus free oocytes; Oocyt: Cumulus free oocytes; stom: Stomach. **C)** Amplification of the control gene and beta-actin using RNA extracted through the modified TRIzol method. M: Molecular weight marker; B1, B2, and B3: Beta-actin gene; H3R: Histamine H3 receptor; H4R: Histamine H4 receptor; NC: Negative control. **D)** Amplification of the control gene, Gapdh using RNA extracted through RNeasy Mini Kit. M: Molecular weight marker; a, b and c: Gapdh; N: Negative control.

Most of the experiments using RNA extraction kit employed a micro RNA extraction kit to successfully extract total RNA from oocytes.[21] This corresponds with the results from the author's study where a low quantity of RNA was obtained using the RNeasy Mini Kit for RNA extraction from mouse oocytes. Yet, the RNeasy Micro Kit is a high-cost method for RNA extraction. Therefore, the modified TRIzol method will be suggested as a low-cost and practical guide for RNA extraction in oocytes.

Acknowledgments

The authors is gratefully acknowledging the University of Malaya Research Grant (UMRG-RG310/14AFR) and University of Malaya Postgraduate Research Grant (PG110-2014B) for the funding of this project. Immense gratitude to Interactome Laboratory, High Impact Research, University of Malaysia, Kuala Lumpur, Malaysia for technical support, and usage of facilities. Also, special thanks to Kamilatulhusna Zaidi for technical guidance.

Conflict of interest

The authors declare that there is no conflict of interest.

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