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Research Article

A MODIFIED METHOD OF mRNA EXTRACTION AND PURIFICATION FROM FUNGI

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ABSTRACT
An isolation and purification method of mRNA extraction was tried out on the *Trichoderma* alone and *Trichoderma-Fusarium* interactions. The fungi were grown in Petriplates onto PDA, as Trichoderma alone and *Trichoderma-Fusarium*, to made interaction between mycelium of *Trichoderma-Fusarium*. The mycelia of both fungi were harvested from zone of interactions. The RNA extraction process was followed for comparison of hot phenol method and acid phenol-guanidinium-thiocyanate-Chloroform with grinding of mycelium in liquid nitrogen. Then acid phenol-guanidinium-thiocyanate-Chloroform was used for total RNA extraction with slight modifications. While, hot phenol method was used as such as described earlier by various workers. The purification of mRNA was performed using oligo(dT) cellulose chromatography. The results indicates that isolated poly (A)+ mRNA was found to be high quality and most suitable for cDNA-AFLP , suppression subtractive hybridization, Semi quantitative PCR, Real time PCR and Microarray applications. It is a modified and consolidated protocol based on previously described methods for isolated steps from plants and works better for microbes. High yield of poly (A)+ mRNA coupled with its amenability for downstream reactions like RT-PCR, northern blotting and cDNA synthesis for library construction is a key feature of the present method. The modified method should follow acid phenol-guanidinium-thiocyanate-Chloroform method better and amenable products.

Key Words: *mRNA Isolation, Poly (A) + mRNA Purification, Fungus, Trichoderma*

INTRODUCTION
Molecular studies of many interesting phenomena in microbial systems has been hampered due to difficulties encountered in isolation of high quality poly (A)+ mRNA. For isolation of poly (A)+ mRNA from fungal tissues, the single step acid-guanidinium thiocyanate-phenol-chloroform method is generally used. Isolation of high purity and intact nucleic acid are critical step for a number of molecular techniques. High purity and intact RNA are critical for molecular techniques that are designed for gene expression studies such as Reverse Transcriptase-PCR, Northern blot analysis, real time-PCR analysis and Microarray. The problems that are normally associated with nucleic acid isolation in fungi as the presence of high level of polysaccharide contaminants, tough cell walls and high level of inter cellular ribonuclease (Mukhtar *et al.,* 1998; Al-Samarrai and Schmid, 2000; Choudhary and Prasad, 2012).
Generally, the major issue with fungi RNA isolation is the presence of problematic bio-molecules such as lipids, polysaccharides and phenolic compounds which make it difficult to get relatively clean separation of RNA and rest of the cellular debris (Wei *et al.,* 2009).
Such RNA could not be used directly for poly (A)+ mRNA selection using oligo(dT)-cellulose as there might be tracts of poly A in the contaminating DNA also which would cause erroneous results in expression studies (Shukla *et al.,* 2005). Hence, identifying and optimizing the best protocol that produces the highest quality of nucleic acid is essential when working with fungi. Therefore, a protocol was devised using acid-guanidinium thiocyanate-phenol-chloroform method (Sambrook and Russel, 2001) with slight modifications and reducing agent (β-mercaptoethanol) in RNA extraction buffer to overcome these problems. The homogenization step is followed by a LiCl precipitation, phenol extraction and selection of poly (A) + mRNA on oligo (dT)-cellulose column chromatography. This procedure

initially standardized for isolation of poly (A) + mRNA from single fungal i.e. Trichoderma and was applied routinely works well with the co-culture of Trichoderma and Fusarium. Here, we reported a high quality mRNA isolation and purification procedure from fungi.

MATERIALS AND METHODS

Glassware and Solutions

All glasswares and plasticwares were treated by soaking in 0.1% diethylpyrocarbonate (DEPC) water for overnight at room temperature (37°C) and then autoclaved. The stocks solutions were prepared and then desired amount of solutions was treated with 0.1% DEPC before use and 3-morpholinopropane sulphonic acid (MOPS) was filtered through 0.45µm MCE hydrophilic membrane syringe filters (Himedia). Mortars, pestles, and spatulas were soaked overnight in a 0.1% (v/v) solution of DEPC and then autoclaved before use. It is most important for isolation of intact, full length mRNAs to keep an RNase-free environment. The gloves and mask were used throughout working duration.

Protocol Steps

i) Acid-Guanidinium Thiocyanate-Phenol-Chloroform Method:

Solutions and Reagents for RNA Extractions:

Denaturing stock solution (solution D): Mix 293 ml water, 17.6 ml of 0.75 M Sodium citrate, pH 7.0, and 26.4 ml of 10% (w/v) N-lauroylsarcosine (Sarkosyl). Add 250 g guanidine thiocyanate and dissolve. Store up to 3 months at room temperature. The working solution was prepared as follows: Add 0.36 ml 2-mercaptoethanol (2-ME) to 50 ml of stock solution. Store up to 1 month at room temperature. Final concentrations of 4M guanidine thiocyanate, 25mM Sodium citrate, 0.5% Sarkosyl and 0.1 M 2-ME are prepared. The 2 M sodium acetate (pH 4.0), Phenol, water-saturated, Chloroform/isoamyl alcohol (49:1),4 M LiCl ,Isopropanol, 75% Ethanol, Water, RNase-free water (pH 7.5) .

The mycelium from co-cultivated three-day old culture of Trichoderma-Fusarium was taken and grind with liquid nitrogen. Fifty mg grinded mycelium powder was taken in 1.5 ml eppendrof tube. The 500 µl of RNA extraction buffer (Solution D) was added in the tube, and then homogenizes the mycelium for 15-30 second at room temperature.

Extraction:

After homogenization, 50 µl sodium acetate (2M), 500 µl phenols (DEPC equilibrated) and 100 µl chloroform: isoamyl alcohol (49:1) was added in the eppendrof tube. Mixed properly and incubated suspension on ice for 15 min, after incubation centrifuge at 9000 rpm for 20 min at 4^0C. After centrifugation the upper aqueous phases containing RNA transfer into another fresh tube. Equal volume of isopropanol was added for RNA precipitation, then place for 60 min at -20^0C and then precipitate were collected by centrifugation 9000 rpm for 30 min at 4^0C.

LiCl Precipitation:

Add 300 µl volume of LiCl (4M) to the aqueous phase, mix well and pellet the RNA by centrifugation at 5,000 rpm for 10 min at 4ºC. Dissolve the pellet in solution D. Add 150 µl ice chilled isopropanol and incubate for overnight precipitation at 4ºC.

Precipitation of Total RNA:

Pellet the total RNA by centrifugation @10,000 rpm for 10 min at 4ºC. Wash the pellet with ethanol (75%) by spinning @10,000 rpm for 5 min at 4ºC, vacuum dry for 15-20 min; and then dissolve the pellet in 40-50 µl DEPC water. Incubate for 10-15 min at 60^0C and then store at -20^0C (Figure: 1 A).

ii) Hot Phenol Method:

Solutions and Reagents:

Extraction buffer (1M Tris-HCl (pH = 8.0) = 5 mL**,** 8M LiCl = 0.625 mL**,** 0.5M EDTA (pH = 8.0) = 1.0 mL, SDS = 0.5 gm**,** DEPC treated water = 43.2 mL**,** Total = 50 Ml), Sodium acetate (3M, pH-5.2), 2 M LiCl, 70% DEPC treated EtOH.

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Protocol:

Set the water bath to 80^0C.Make 50 mL Extraction buffer (in a baked bottle) as above. Add 50 mL phenol (pH = 4.7), in 50 mL extraction buffer (final concentration of 1:1). For small sample add 200 to 300 µl of Extraction buffer:Phenol(1:1). Heat the above mixture to 80^oc for at least 1/2 half. Grind the tissue in 1.5 mL liquid nitrogen and immediately add 15mL (for 1.5gm of tissue), for extraction in eppendorf tubes add 750 µL of 1Extraction buffer: 1Phenol. In case of hard tissues used mortar and pastel. Homogenize by vigorous vortexing. Add 350µL of chloroform to the samples and vigorously vortex again. Centrifuge for 15 min @14000 rpm at room temperature. Transfer the supernatant to 1.5 mL new Eppendorf tube. Add 1/3 volume of 8 M LiCl to the supernatant and mix well by inverting the tubes. Precipitate on ice for at least 2 hrs. Balance the tubes if needed using 2 M LiCl and centrifuge at 10,000 rpm for 30 minutes at 4^0C.

Discard the supernatant and wash the RNA pellet with 500 µL of 2M LiCl (room temp) and centrifuge at 10,000 rpm for 5 min at room temp. Discard the supernatant and wash the RNA pellet with 500 µL of 70% DEPC treated EtOH. Centrifuge again at 10,000 rpm for 5 min at room temp. Discard the EtOH and air dry the pellet. Dissolve the RNA in 100µL of DEPC treated water. If, it does not dissolve immediately, put the tubes in -80^0C and then thaw on ice after 2 hrs or it can also be put the tubes in -80^0C overnight.

Centrifuge at 10,000 rpm for 10 min at 4^0C and transfer the supernatant into a new tube. This step used to throw away any un-dissolved debris. Add $1/10^{th}$ vol of 3M Sodium acetate (pH-5.2) and 2.5vol of 100% EtOH (room temp). Sodium acetate (0.1) and EtOH (2.5) vol were used to precipitate the RNA at -80^0C for 20 min and centrifuge at 10,000 rpm for 15 min at 4^0C. Discard the supernatant and wash the pellet (room temp) with 70% EtOH and centrifuge again at 10,000 rpm for 5 mins at 4^0C. Air dries the pellet properly. Dissolve the RNA in 50 µL of DEPC treated water and store it in deep freezer at -80^0C. Treat the samples with 2 µL DNase and incubate at 37^0C water bath for 1/2hr quickly run normal 1.5% agarose gel to see the quality and quantify the RNA.

Extract RNA by adding equal volume of phenol:chloroform(1:1), mix well and then centrifuge at 14000 rpm at room temperature for 10min. Add 1/10 vol of Sodium acetate (pH 5.2) and 2.5 vol of 100% ethanol, mix well and centrifuge at 14000 rpm for 10 min. Wash the pellets with 70% ethanol (DEPC water) and centrifuge at 14000rpm for 5min, Drain the ethanol and air dry the pellets, depending upon the size of pellet /quantification dissolve in DEPC treated water and store at -80^oC. Proceed for cDNA synthesis.

Quantitative Analysis:

Quantities analysis of RNA was done using UV visible spectrophotometer by measuring the OD at 260nm and 280nm. One unit of absorption at 260nm represents 50µg/mL of RNA.

Formaldehyde-Agarose Gel Electrophoresis:

Formaldehyde-agarose gel (1.2%) was prepared using 1x MOPS buffer pH 7.0 and formaldehyde 1.8% v/v. A mixture of 4µL sample (2µl RNA and 2µl loading dye) and this mixture were heated at 65°C for 10 min and keep it on ice for 5 min and, then load into the gel. The electrophoresis was run at 5 V/cm in the running buffer (1x F buffer, 6% formaldehyde). **Note:** The 6x loading dye was prepared and used without bromophenol blue.

mRNA Purification:

This protocol separates poly (A) + RNA from the remainder of total RNA, which is largely rRNA and tRNA. Total RNA is denatured to expose the poly(A) (polyadenylated) tails. Poly (A)-containing RNA is then bound to oligo(dT) cellulose, with the remainder of the RNA washing through. The column can then be repeated to remove contaminating poly(A) RNA.

Solutions and Reagents:

The following chemicals and items were used. Diethylpyrocarbonate (DEPC);10N NaOH; Oligo(dT) cellulose; 5M NaCl; 3M Sodium Acetate; Ethanol; 2X Poly(A) Column loading buffer (40mM Tris-Cl,1M NaCl, 2mM EDTA,0.2% sodium dodecyl sulfate (SDS); Elution buffer (10mM Tris-Cl,1M NaCl,

1mM EDTA,0.05% SDS; Dispocolumn(BioRad); Oligo(dT)-cellulose(invitogen); wash buffer- High salt buffer (2 mM EDTA/0.1% sodium dodecyl sulfate (SDS); Medium salt buffer; Low salt buffer and RNase-free DEPC water.

Purification Steps

Oligo(dT)-Cellulose Preparation and Column Packing:

For equilibration of 5 mg oligo(dT)-cellulose was kept for swelling overnight in 50 µl 0.1N NaOH with loading buffer by aspirating out the overlaying loading buffer and addition new loading buffer (150 µl) next day to make final volume 200 µl. Pour this equilibrated oligo(dT)-cellulose into the columns and leave for 30 min. Provide a spin pulse in the mini-centrifuge to wash out the loading buffer. Wash the column with 200 µl high salt buffer.

Poly (A)+ mRNA Selection and Elution

Heat the total RNA aliquots (100 µl each) at 65ºC for 15 min to relax the secondary structures, cool immediately on ice for 5 min, add equal volume (100 µl) of loading buffer to each and load onto an oligo(dT)-cellulose column. Collect the flow through and load it on the column once again. Repeat this process once more for a total of three loading steps. Wash the loaded column first with 200 µl high salt buffer and then with 200 µl medium salt buffer. Elute the poly (A)+ mRNA bound to the oligo(dT)-cellulose column using 200 µl low salt buffer.

Poly (A)+ mRNA Precipitation:

Divide the eluted poly (A)+ mRNA sample from each of the four columns into four parts (50 µl aliquots). Precipitate the poly (A) + mRNA by addition of 1/25 volume NaCl (5M) and 2 volumes ethanol (100%) followed by overnight/2 hr incubation at –20ºC. Pelleted the poly (A)+ mRNA by centrifugation at 20,000 rpm for 1 hr at 4ºC, vacuum dry the pellet for 15-20 min and dissolve in RNase free water (20 µl per aliquot) for downstream applications. If required for further use, store the dry poly(A) + mRNA pellet at this stage in ethanol (100%) at –80ºC instead of dissolving in water.

RESULTS AND DISCUSSION

The methods described was used successful for isolation of RNA from fungi, which are rich in polysaccharides, for which other methods failed to deliver RNA suitable for reverse transcription and Northern analysis, cDNA-AFLP , suppression subtractive hybridization, Semi quantitative PCR, Real time PCR and Microarray applications. The hot phenol method is more suitable for isolation of plant RNA while guanidinium thiocyanate (GTC) method is best methods for RNA extraction from fungi and plant (Figure 1.a). Hot phenol method also be more time consuming process than guanidinium thiocyanate (GTC) method (Figure 1.b). The guanidinium thiocyanate (GTC) based method with some modifications are highly successful for fungal RNA isolation. The guanidinium thiocyanate (GTC) method reported here is, therefore, simple and efficient for the isolation of RNA from fungi that possess a wide range of properties that can interfere with RNA extractions and analysis. The mRNA was synthesized enzymatically into a single strand complementary DNA. The results of same cDNA was successfully amplified with a gene-specific primer pair i.e. chitinase (Figure1.c) having approx. 175 bp amplicon size. Furthermore, our method is not complicated and does not require long ultracentrifugation but is straightforward. The value of this method is that it should be suitable for isolating RNA from other

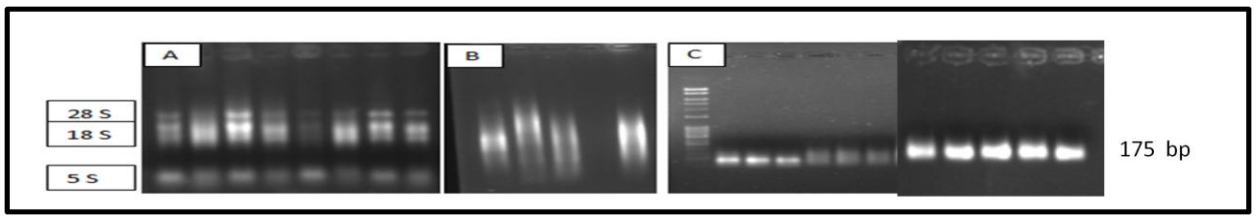

Figure: 1: A - Total RNA isolation using guanidine thiocyanate method. B - RNA Isolation using hot phenol method. C - cDNA Amplified with Chitinase gene ~175bp.

Filamentous fungi and this protocol have been routinely used in our lab for isolation of RNA from different species rich in polysaccharides.

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REFERENCES

Al Samarrai TH and Schmid J (2000). A Simple method for Extraction of Fungal Genomic DNA. *Letters in Applied Microbiology* **30** 53–56.

Chomczynski P and Sacchi N (1987). Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate Phenol Chloroform Extraction. *Analytical Biochemistry* **162** 156–159.

Choudhary V and Prasad L (2012). Morpho Pathological, Genetic Variations and Population Structure of *Sclerotinia sclerotiorum. Vegetos* **25** (1) 178-183**.**

Kohrer K and Domday H (1991). Preparation of High Molecular Weight RNA. *Methods Enzymol* **194** 398-405.

Mukhtar M, Parveen Z and Logan DA (1998). Isolation of RNA From the Filamentous Fungus *Mucor Circinelloides*. *Journal of Microbiological Methods* **33** 115–118.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular Cloning. a Laboratory Manual. Second Edition, Cold Spring Harbor Laboratory Press. New York

Sambrook J and Russel DW (2001). Molecular Cloning: a Laboratory Manual. Third Edition, Cold Spring Harbor Laboratory Press. New York.

Shukla AK, Shasany AK and Khanuja SPS (2005). Isolation of Poly (A)+ mRNA for Downstream Reactions from Some Medicinal and Aromatic Plants. *Indian Journal of Experimental Biology* **43** 197-201.

Sokolovsky V, Kaldenhoff R, Ricci M and Russo VEA (1990). Fast and Reliable Mini-Prep RNA Extraction from *Neurospora Crassa*. *Fungal Genetic Newsletter* **37** 41–43.

Wei Ye, Lu Liu, Wenlong Zheng, Juan Lin and Jingyuan Yi (2009). Comparison of RNA Extraction Methods Applied to Gene Cloning of the Taxol Producing Fungi. *African Journal of Microbiology Research* **3**(10) 632-636.