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Development of a rapid RNA extraction procedure from urediniospores of the leaf rust fungus, *Puccinia triticina*

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ABSTRACT

Obtaining high quality RNA in good quantities is often a requirement for plant-pathogen interaction studies, so it becomes very essential that a highly efficient method should be deployed to isolate RNA from minute quantities of fungal spores. The methods available to date, either require a high quantity of spores or the use of expensive chemicals. The protocol discussed here for RNA isolation from *Puccinia triticina* pathotype 77–5 urediniospores utilizes TRI Reagent as extraction buffer that is widely used for RNA isolation from plant tissues. Urediniospores have a tough cell wall as compared to other plant cells. Therefore, the protocol was optimized keeping the primary focus on quickly disrupting cell walls. Two different methods, one using a combination of liquid nitrogen and ultrasonic water-bath and the other method using micro-homogenizer were utilized for crushing the spores in the present study. The developed methods do not utilize mortar and pestle, instead they promote direct crushing of urediniospores in tubes; thereby minimizing sample loss and enhancing quality.

1. Introduction

Puccinia triticina, the causal organism for leaf rust in wheat is a biotrophic pathogen. Hence its urediniospores cannot be grown artificially in culture media, *i.e.*, outside its living host. So, samples needed for RNA isolation had to be collected from infected plants, which often was a mixture of both host and pathogen RNA. An efficient RNA isolation method from *Puccinia* spores became necessary when high amount and good quality of RNA is required for strain identification, polymerase chain reaction (PCR)-based studies or small RNA library preparation. Earlier research on pathogen-derived sequences relied mostly on *in silico* methodologies based on transcriptome or genomic data analysis (Kiran et al., 2016; Kumar et al., 2017; Dutta et al., 2017). Validation of pathogen sequences were not possible due to unavailability of suitable technique for isolation of good quality RNA from pathogen spores.

Several methods exist for successful isolation of RNA from different plant and animal tissues, a basic requirement for most molecular biology studies (Tan and Yiap, 2009). Earlier, to obtain RNA free of DNA was a tedious task even after the introduction of Guanidinium thiocyanate in extraction buffers. Later with the development of Guanidinium thiocyanate-phenol-chloroform extraction method, which combines multiple steps, made RNA isolation comparative easy (Chomczynski and Sacchi, 2006; Sambrook and Russel, 2001). Other methods, quite different from the conventional methods, were also developed like chromatography on oligo (dT) columns or magnetic oligo (dT) beads to specifically separate poly (A) containing RNA.

These different techniques developed for RNA isolation using conventional or kit-based methods (Peng et al., 2007) are applicable for a wide range of plant and animal tissues. A few kits are available for yeast cells also, but no method suitable for spores like urediniospores with very hard and tough cell wall are available. Spores have tough cell wall. Therefore, RNA isolation using urediniospores primarily needs a proper technique for cell lysis. A recent report on RNA isolation from *Puccinia striiformis* urediniospores compared several techniques using low cost reagents and the authors suggested a NaCl-based method as the most appropriate for extracting RNA (Li-Jie et al., 2016). But the method required adequate amounts (minimum 0.1 g) of spores to be pulverized in liquid nitrogen using mortar and pestle which is commonly associated with sample loss during transfer processes. In the present study, a method for rapid RNA isolation was developed which requires minuscule quantity of spores but yields good quantity and high quality of

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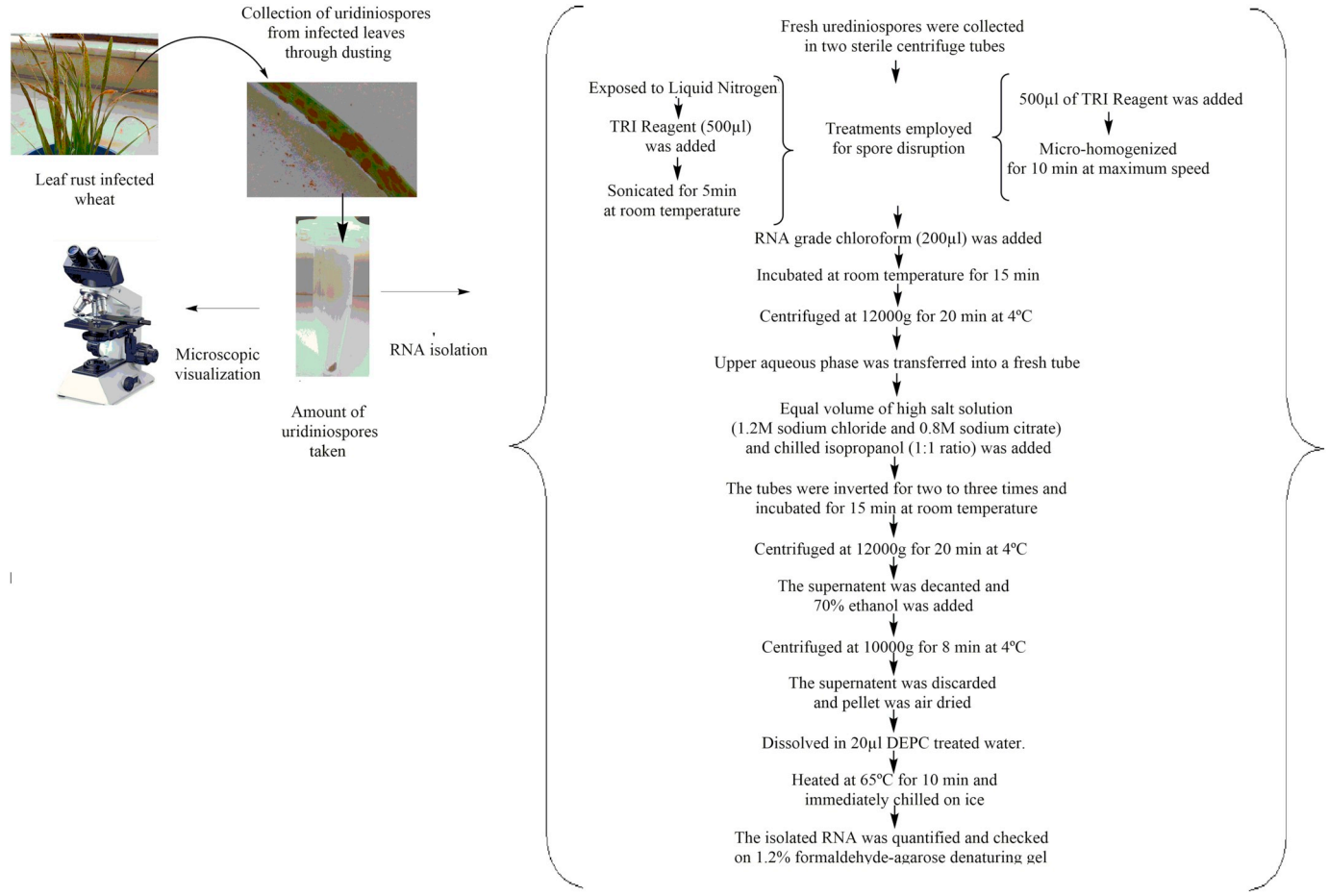

Fig. 1. A brief overview showing the collection of uridiniospore samples from *Puccinia triticina* pathotype 77–5 infected Agra Local wheat plants followed by microscopic examination and RNA isolation. Flow chart at right showing RNA isolation technique from *Puccinia triticina* pathotype 77–7 uridiniospores developed in the present study.

RNA that could be used for almost all molecular biology studies.

2. Materials and methods

2.1. Sample collection

Seeds of highly leaf rust susceptible wheat cultivar Agra Local were soaked overnight in distilled water and sown on soil in pots. The seeds were allowed to germinate and grow in a greenhouse with 80% humidity and 10 h 30 min of natural day light and 13 h 30 min of night duration till plantlets reached two leaf stage. *Puccinia triticina* urediniospores of pathotype 77–5 were mixed with talcum powder (1:20) and used to inoculate leaves of these plants. After inoculation, the plants were watered and covered with sterile autoclavable plastic bags for 72 h to facilitate efficient infection. Pustules of urediniospores appeared approximately after 10 days post inoculation. The brown coloured urediniospores were collected on butter paper through dusting and then transferred to 2.0 ml autoclaved plastic tubes (Li-Jie et al., 2016). The strategies followed in this study are shown in Fig. 1.

2.2. Methods for RNA isolation

Different techniques applied in this study were mainly focused on breaking the cell wall as the very first step of RNA isolation from a minute amount of sample. As the spores have very light density, the photographed spores within the tube (Fig. 1) was measured to be only 3 mg. The following two different methods were utilized for crushing the spores (Fig. 1):

2.2.1. Liquid nitrogen and sonication

Liquid nitrogen was slowly poured inside plastic tubes containing spores. Tubes were half closed to prevent spilling of the spores but were not closed completely to prevent breakage. Immediately after a crank sound produced as result of liquid nitrogen, the tubes were opened to add 500 µl of TRI Reagent (Sigma Aldrich, USA) and were placed on ultrasonic water bath (Power Sonic 520) for 5 min at room temperature. The tubes were inverted frequently to get a uniform suspension of spores with TRI Reagent. Then 200 µl of chloroform was added.

2.2.2. Micro-homogenizer

Vacuum created by spinning rotor inside homogenizer (Polytron PT1200E, Kinematica) provides a way between the rotor and the stator's slot for repeated in and out movement of sample which facilitates mechanical shearing of spores. Spores were mixed with 500 µl of TRI Reagent and then homogenization was done for 10 min at maximum speed. Then 200 µl of chloroform was added.

After the above two types of treatments, all tubes were incubated at room temperature (22 °C) for 15 min followed by centrifugation at 12000*g* for 20 min at 4 °C. The upper aqueous phase was carefully transferred into a fresh tube and equal volume of high salt solution (1.2 M sodium chloride and 0.8 M sodium citrate) and chilled isopropanol (1:1 ratio) was added to precipitate RNA. Tubes were gently inverted a few times to get a clear solution and incubated for 10 min at room temperature. Tubes were centrifuged at 12000*g* for 20 min at 4 °C. Supernatant was decanted and pellet was washed with 70% ethanol to remove excess salt followed by air drying. The RNA pellets were allowed to dissolve in 20 µl of DEPC treated water. Details of the protocol

is provided as a flow diagram in Fig. 1. Tubes were heated at 65 °C to linearize the RNA molecules. Quantity and purity of sample was checked using spectrophotometer and 200 ng of sample was loaded onto 1.2% formaldehyde agarose- gels following conventional protocol (Sambrook and Russel, 2001).

2.3. Quality check of cDNA prepared from isolated RNA using conventional and real-time PCR

Two sets of cDNA were prepared using 2.5 μg of isolated RNA. One set was prepared using Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas). The prepared cDNA was checked for Real Time PCR-based amplification of commonly used housekeeping genes like Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Actin to confirm whether the prepared cDNA could be used for gene expression studies. Conventional PCR was also performed using primer pair (5′ ATGCGGTGCAATGTGTTTCC3′ and 5′CGCATGAAGAACGCGATGAG3′) specific for a functional protein domain containing sequence to assure all-purpose use of this cDNA. PCR product obtained was gel purified using Silica Bead DNA Gel Extraction Kit (Roche Diagnostics), cloned in pTZ57R/T plasmids using InsTAclone PCR Cloning Kit (Thermo Scientific, Lithuania). Plasmids were isolated using High Pure Plasmid Isolation Kit (Roche Diagnostics) and were sequenced commercially (Xcelris Labs Limited).

Another set of cDNA was prepared using Mir-X MiRNA First Strand Synthesis kit (Clontech Laboratories, Inc.) specifically designed for stem-loop qRT-PCR to assess the quality of cDNA for the purpose of studying small RNA populations of urediniospores.

3. Results and discussion

3.1. Collection of urediniospores from infected wheat leaves

The highly leaf rust susceptible wheat variety Agra local was used for collection of urediniospores. A clear spot (approximately 1-2 mm diameter) of brown uredia appears after 10 days of pathogen inoculation indicating complete establishment of infection (Fig.1). Using the shaking off method fresh urediniospores were collected.

3.2. Effect of isolation method on quality and quantity of RNA

Cell lysis is the first step of isolation of any kind of biomolecule which brings all the cellular component outside the bound cell wall into the extraction buffer. Extraction buffer facilitates protection of RNA by inactivation of nucleases present in the cell lysate in addition to buffering. All the conventional methods used for RNA isolation to date included either Guanidinium Thiocyanate-Phenol-Chloroform based extraction or use of detergents like Sodium Dodecyl Sulphate (SDS), Cetyltrimethylammonium Bromide (CTAB) or salts like Sodium Chloride (NaCl) or Lithium Chloride (LiCl) in extraction buffer (Sambrook and Russel, 2001; Li-Jie et al., 2016). These reagents efficiently isolate RNA from 100 or 200 mg of tissue samples but provided no solution for measurably low quantity of starting material. Even the earlier proposed method had utilized mortar-pestle with a handsome quantity (30 mg) of spores (Li-Jie et al., 2016).

Here we avoided the use of mortar and pestle for grinding and instead took spores directly in plastic tubes. In the first treatment, liquid nitrogen was directly poured inside the spore (3 mg) containing tube for instant freezing which provide easy fracture. This was followed by addition of TRI Reagent which prevented enzyme activity as the temperature rises. The cracking sound produced by the fragile spores was followed with incubation in an ultrasonic water bath to ascertain proper breakage of the spore cell walls. In the second strategy using micro-homogenizer at maximum speed for 10 min provided mechanical sheering of spores suspended in TRI Reagent.

Both the treatments reduced the chances of sample loss during transfer from mortar to centrifuge tubes. Here, TRI Reagent (containing Guanidine thiocyanate and phenol), was used as extraction buffer which helped in phasing, denaturation of protein and also made aqueous phase acidic to reduce the chances of DNA contamination (Chomczynski and Sacchi, 2006; Sambrook and Russel, 2001). Chloroform aided phasing, an old technique, was employed here also, in both the tubes presumed to contain ruptured spores (Doyle and Miles, 1996). Total RNA was recovered by precipitation with isopropanol.

TRI Reagent has earlier been employed for RNA isolation from sufficient amount of tissue samples but in the developed method it has been utilized for a minuscule quantity of sample. Also, TRI Reagent has not been reported earlier to be used for RNA isolation from spores. Therefore, for unusual samples like leaf rust urediniospores that are available in limited quantity, use of TRI Reagent mediated RNA isolation makes the procedure further unique. In addition, the developed technique, utilizes comparatively less time (~2 h) since it does not require prolonged incubation steps as by most conventional methods.

The concentration of RNA isolated using first method was 2232 μg ml^{-1} with 260/280 ratio of 1.64 while for the second method was 1762 μg ml^{-1} with 260/280 ratio of 1.59. Gel image showing well resolved RNA bands (28 and 18S) are indicative of good quality of isolated RNA (Fig. 2, 2.1).

3.3. Quality check of cDNA prepared from isolated RNA using PCR and real-time PCR

The concentration of cDNA prepared using RNA isolated by first method was 1539 μg ml^{-1} while for that prepared using second method was 2424 μg ml^{-1}.

PCR amplification using a set of primer pair specific for a functional polynucleotide kinase containing sequence provided a band of approximately 700 nucleotides (Fig. 2, 2.2). Sequencing of the eluted band revealed the presence of a 798 nt (submitted to GenBank with Accession no.: MH427367). Presence of polynucleotide kinase domain was confirmed using Conserved Domain- search of NCBI (www.ncbi.nlm.nih.gov). These kinases have dual property of serving as phosphorylating 5′ OH of nucleotide while having phosphatase activity at 3′ end. Its role has been anticipated in DNA repair. When there are several breaks in the DNA strands both kinase and phosphatase activity are needed simultaneously (Zhang et al., 2013). A similar function of this gene in *Puccinia* might help in enhancing pathogenicity inside wheat by repairing its DNA damage caused by host nucleases and small RNAs.

Real-time PCR for housekeeping genes β-Actin and GAPDH provided normal amplification plots that can be used for gene expression analysis. Real-time PCR amplification plot using U6 primer pair for cDNA prepared from Mir-X miRNA First Strand Synthesis kit which generally serves as reference for small RNA also displayed normal amplification pattern (Fig. 3). This implies good quality of cDNA for the purpose of sRNA population study.

The above three amplification experiments demonstrated the suitability of the prepared cDNA from the isolated RNA from urediniospore. The RNA isolation technique from minute amounts of spores with hard cell walls may be considered appropriate for all molecular biological studies.

4. Conclusion

In order to study pathogen genes during their dormant stage within spores, a rapid protocol for RNA isolation becomes prerequisite to check up- or down-regulation of genes during establishment of infection and stages of disease development. The protocol described here does not use costly reagents or kits but utilizes the basic principles of RNA isolation used for plants. The technique differs chiefly in the processes for crushing and breaking the hard cell wall of spores. Thus, the developed technique provides an easy, cost effective and time saving

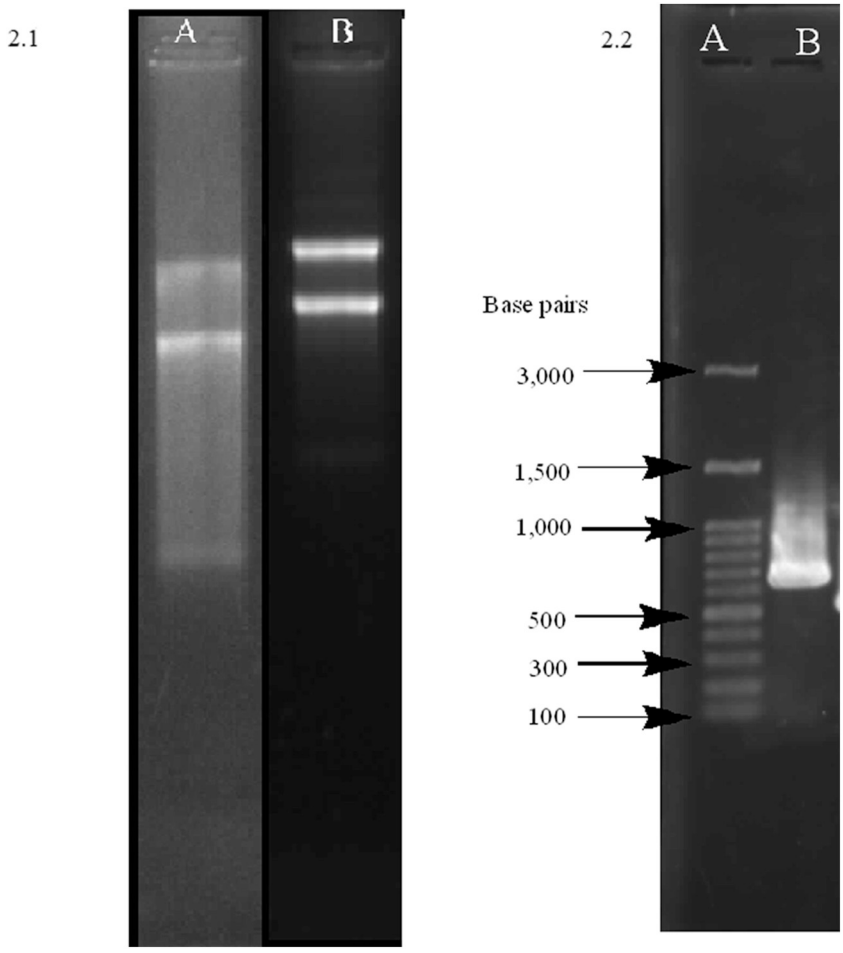

Fig. 2. Gel images showing 2.1. Separation of isolated RNA in 1.2% denaturing formaldehyde agarose gel; Lane A: RNA isolated using micro homogenizer method while lane B using liquid nitrogen method, 2.2. PCR amplification from cDNA prepared from RNA isolated using Liquid Nitrogen method. Lane A: Molecular weight marker (NEX-GEN DNA Ladder; 100 bp; PUREGENE); Lane B: PCR amplification using primer pair specific to polynucleotide kinase domain with approximately amplicon size of 700 bp.

method of RNA isolation from spores.

Author contribution statement

KM conceived and designed the research, MK supervised the research, SD executed the research. SD and KM wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Not required.

Conflict of interest

The authors declare that they have no conflict of interest.

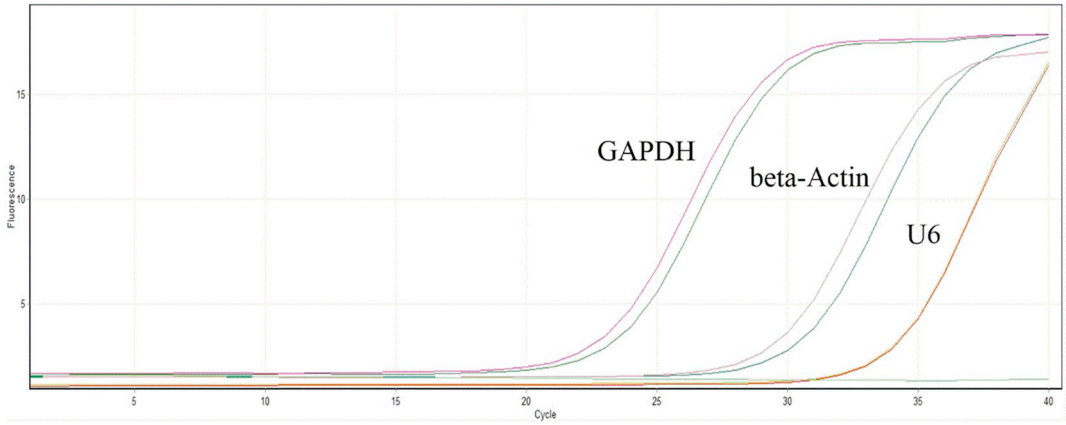

Fig. 3. Real-time PCR plot showing amplification of housekeeping genes with cDNA as template prepared using RNA isolated from urediniospore of *Puccinia triticina* pathotype 77–5.

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References

Chomczynski, P., Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat. Protoc. 1, 581–585.

Doyle, K., Miles, J., 1996. Promega Corporation (Madison). Protocols and Applications Guide. Promega Corporation.

Dutta, S., Kumar, D., Jha, S., Prabhu, K.V., Kumar, M., Mukhopadhyay, K., 2017. Identification and molecular characterization of a trans-acting small interfering RNA producing locus regulating leaf rust responsive gene expression in wheat (*Triticum aestivum* L.). Planta 246, 939–957.

Kiran, K., Rawal, H.C., Dubey, H., Jaswal, R., Devanna, B.N., Gupta, D.K., ... Balasubramanian, P., 2016. Draft genome of the wheat rust pathogen (*Puccinia triticina*) unravels genome-wide structural variations during evolution. Genome Biol. Evol. 8, 2702–2721.

Kumar, D., Dutta, S., Singh, D., Prabhu, K.V., Kumar, M., Mukhopadhyay, K., 2017. Uncovering leaf rust responsive miRNAs in wheat (*Triticum aestivum* L) using high-throughput sequencing and prediction of their targets through degradome analysis. Planta 245, 161–182.

Li-Jie, M.A., Qiao, J.X., Kong, X.Y., Wang, J.J., Xu, X.M., Hu, X.P., 2016. An improved method for RNA extraction from urediniospores of and wheat leaves infected by an obligate fungal pathogen, *Puccinia striiformis* f. Sp. *tritici*. J. Integr. Agric. 15, 1293–1303.

Peng, L., Meinan, W., Chen, X.M., Garland, C.K., 2007. Construction and characterization of a full-length cDNA library for the wheat stripe rust pathogen (*Puccinia striiformis* f. Sp. *tritici*). BMC Genomics 8, 145.

Sambrook, J., Russel, D., 2001. Molecular Cloning: A Laboratory Manual, 3rd edition. Vol. 3 Cold Spring Harbor Laboratory Press, New York, NY, USA.

Tan, S.C., Yiap, B.C., 2009. DNA, RNA, and Protein Extraction: The Past and the Present. BioMed Research International.

Zhang, L., Zhao, J., Zhang, H., Jiang, J., Yu, R., 2013. Double strand DNA-templated copper nanoparticle as a novel fluorescence indicator for label-free detection of polynucleotide kinase activity. Biosens. Bioelectron. 44, 6–9.