

An Optimised High-Salt CTAB Protocol for Both DNA and RNA Isolation from Succulent Stems of *Hylocereus* sp.

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Abstract—The isolation of high quality DNA and RNA from plant species harboring high levels of polysaccharides and secondary metabolites are typically problematic, especially those in cactus. These compounds often co-precipitate with DNA and RNA thus causes low recovery and quality of the nucleic acids. Six DNA extraction protocols were tested on the sample of *Hylocereus* spp. of which the results were compared and analyzed. For comparison, three manufacturer's protocols from different commercial kits and another three conventional DNA extraction protocols were compared. It was found that conventional method generally produces consistent and higher yield. Among the conventional protocols itself, each has their pros and cons. Therefore, a modified protocol which is concise, quick and simple was developed for *Hylocereus* spp. which is beneficial for further molecular work. This method was proven to be reliable in generating a good quality of DNA from these particular genera. Similarly for RNA extraction, four different extraction protocols were tested on the same sample. The results were analyzed and a modified protocol was developed to obtain a higher quality and yield of RNA for further downstream investigations. The extraction buffer from our modified DNA extraction protocol was tested on the RNA extraction and shows a good yield.

Index Terms— *Hylocereus* sp., DNA, RNA extraction

I. INTRODUCTION

We are currently working on the isolation and characterisation of a resistant gene from the stem of dragon fruit (*Hylocereus* spp.), which belongs to the family of Cactaceae and order of Caryophyllales [1]. To begin with this investigation, obtaining a high yield and good nucleic acids quality from the stem of *Hylocereus* spp. is a crucial pre-requisite step for further molecular biology applications. Due to its existence in dry habitat, these cactus plants naturally produces large quantities of polysaccharides that plays important role in storing huge reserves of water because of its high water-binding capacity of hydroxyl groups in the polysaccharide core [2]. However, the abundance of polysaccharides which give the stems of cacti their sliminess is the major obstacle in molecular work. These long, branched chains of sugars act like a net which entangle or co-precipitate with nucleic acids and form a viscous complex upon

isolation. Further to that, the presence of polyphenols and other secondary metabolites also contributes to the difficulty in DNA and RNA extraction. Therefore, this had led us to develop suitable methods precisely for DNA and RNA extraction for the sample mentioned in order to obtain a good quality of nucleic acids for further downstream investigations.

Although many had venture into DNA extraction, to our knowledge so far, there are none other published reports on DNA extraction from cactus other than Tel-Zur *et al.* [3], Mondragon-Jacobo *et al.* [4], Mihalte *et al.* [5] and Yu *et al.* [6].

Mondragon-Jacobo *et al.* [4] presented a DNA extraction method that was tested on several cacti species namely *Opuntia* sp., *Nopalea* sp., *Stenocereus* sp., *Cleistocactus* sp., and *Echinocereus* sp., in which the sample size has to be adjusted depending on the species as the mucilage content varies among them. In later years, Mihalte *et al.* [5], [7] reported that the protocol of Pop *et al.* [8] was able to generate sufficient DNA yield from cacti of genera *Rebutia*, *Mediolobivia*, *Sulcorebutia* and *Aylostera*, which only requires a small amount of tissue. Yu *et al.* [6] had also reported a reliable DNA extraction method for dragon fruit which is almost similar to the modified protocol by Pop *et al.* [8]. Among all, only Tel-Zur *et al.* [3] and Yu *et al.* [6] had DNA extracted from the *Hylocereus* sp. The research by Tel-Zur *et al.* [3] was based upon the use of roots of cactus as the source tissue in which it has lower viscosity of the extracts relative to that of other tissues. However, our attempt is to extract from the stems which has a relatively higher viscosity.

Therefore, in conducting this current study, six different protocols for DNA isolation from cactus and plants with high concentration of polyphenols and polysaccharides have been tested. The commercial kits that were compared include Vivantis GF-1 Nucleic Extraction Kit and NucleoSpin Plant II Kit with two different lysis buffers. As for conventional method, the protocols tested were from Tel-Zur *et al.* [3], Yu *et al.* [6] and Pop *et al.* [8]. These protocols did not give rise to desirable results with our samples. Hence, we have modified and developed a simpler and efficient method from Tel-Zur *et al.* [3] and Pop *et al.* [8] specifically for these samples of *Hylocereus* sp. and the outcome were compared with that of the rest of the protocols.

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There have been many different methods described for RNA isolation. Typically, the manual method of RNA isolation involves the use of CTAB, SDS, phenol and high molarity guanidium salts. Most methods used for RNA extraction from mature fruits which includes phenol or high molarity guanidium salts are not effective in cactus fruits [9]. The composition of the employed tissue affects the efficiency of the applied extraction methods [10]. Isolation of RNA from cactus so far had only been done on the cactus fruit. This was carried out by Valderrama-Chairez and team on prickly pear fruit in the year 2002. Valderrama-Chairez *et al.* [9] described their technique of isolating high quality of RNA from cactus fruit using SDS. Other protocols such as those described in Hu *et al.* [11]; Hou *et al.* [12] is also important to be considered in current study as they are involved in the RNA extraction from recalcitrant plant tissues in which most polyphenols, polysaccharides and other secondary metabolites presence interferes with the isolation. To our knowledge, RNA extraction from the stem of a cactus plant has not been carried out.

In current study, RNA extraction from *Hylocereus* sp. stem was tested using four different protocols on our samples namely, CTAB method from Hou *et al.* [12]; modified CTAB method from Hu *et al.* [11]; Commercial kit from Nacalai Tesque Sepasol-RNA 1 Super G; and SDS method from Valderrama-Chairez *et al.* [9]. In addition, we had modified the extraction protocols to suit our samples. We found that using the same high-salt CTAB extraction buffer as the DNA extraction was efficient for RNA extraction from *Hylocereus* sp. stem.

II. MATERIALS AND METHODS

A. Plant Material

The stems of *Hylocereus* sp. cactus plants are coated with strong waxy layer that helps to retain water as well as to protect the tissue against the sun. In order to obtain the inner tissue for extraction, this waxy layer was first removed.

B. DNA Extraction Protocol

About 0.5-1.0 g of the sample was grinded in mortar and pestle with liquid nitrogen. The frozen powdered sample was then transferred into 1.5 mL of microcentrifuge tube. To each sample, 1mL of pre-heated (60°C) high-salt CTAB extraction buffer [100mM Tris-HCl (pH 8), 4M NaCl, 0.5M EDTA (pH 8), 2.0% (w/v) CTAB, 1% (w/v) Polyvinylpyrrolidone (PVP-40), 2% (v/v) β -mercaptoethanol, and 1% (w/v) sodium sulphite] were added and vortexed for 1 min. The last three components of polyvinylpyrrolidone (PVP-40), β -mercaptoethanol and sodium sulphite were only added to the extraction buffer prior to use. 15 μ L of Proteinase K (10 mg/ml) was then added into the homogenate and mix by vortexing. The sample was incubated at 60°C for 1 hour to allow the proteinase k to react on the sample by breaking down the peptide bonds.

After the sample was cooled to room temperature, it was then centrifuged at 13000 rpm for 20 minutes. The

supernatant was then transferred into a new microcentrifuge tube. 600 μ L of Chloroform:Isoamylalcohol (24:1) was then added to the supernatant and vortexed to form emulsion. This was followed by centrifugation at 11000rpm for 15 minutes. The top aqueous solution was transferred into two separate microcentrifuge tubes equally with about 400 μ L of the solution per tube. To each tube, 600 μ L of 3M sodium acetate (pH 5.2) and 500 μ L of ice-cold absolute isopropanol were added. The tubes were inverted several times after each addition for proper mixture. Then both tubes were allowed to refrigerate at 4°C for 1 hour.

One of the tubes was first centrifuged at 4°C and 13000 rpm for 10 minutes and the supernatant were discarded. The second tube of solution was transferred into the first tube that consists of visible pellet and centrifuged once again under the same settings. The supernatant was then discarded carefully. The DNA pellet was washed with 600 μ L of cold 76% Ethanol followed by centrifugation at 13000 rpm for 5 minutes. This washing step was repeated twice. Ethanol was carefully poured off and the tube was left inverted on Kimwipes tissue at room temperature for about 20 minutes to completely remove the remaining ethanol without drying the pellet. Finally, the DNA pellet was rehydrated in 100 μ L of TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. In order to eliminate possible RNA contamination, 2 μ L of RNase solution (10mg/mL) was added to the DNA sample and incubated at 37°C for 60 minutes.

C. RNA Extraction Protocol

About 1mL of high-salt CTAB extraction buffer was added to a liquid nitrogen-grinded sample of 0.5-1 g in a microcentrifuge tube and vortex to mix thoroughly. The mixture was divided into two tubes equally with about 750 μ L in each tube. 10 μ L of Proteinase K (10 mg/mL) was added into each tube followed by vortex. The tubes were then incubated in 60°C water bath for 15 minutes and cooled to room temperature. 60 μ L of 5M potassium acetate and 100 μ L cold absolute ethanol were added.

Once again, the tubes were vortex and the suspension that appeared flocculent was incubated in ice for 15 minutes. 600 μ L of Phenol:Chloroform:Isoamylalcohol (25:24:1) were added and vortex before incubating in ice for 30 minutes. The tubes were then centrifuged at 16000xg for 20 minutes. The top layer was transferred into new microcentrifuge tube and 600 μ L of Chloroform:isoamylalcohol (24:1) were added. After vortexing, the tubes were brought to be centrifuged again at 16000xg for 15 minutes. The top layer in both tubes was recovered into one new microcentrifuge tube. 8M of lithium chloride was added to the final concentration of 3M. The tube was inverted a few times for proper mixture. It was then incubated overnight at -20°C. After that, the tube was centrifuged at 13.3rpm at 4°C for 20 minutes. The RNA pellet was then washed with chilled 76% ethanol twice and air-dried. 100 μ L of DEPC-treated water was added to resuspend the RNA pellet. The sample was stored at -80°C.

III. RESULTS AND DISCUSSION

The DNA quantity and quality was estimated with standard molecular biology techniques using agarose gel electrophoresis (Fig.1) as well as spectrophotometrically by NanoPhotometer (Implen, UK).

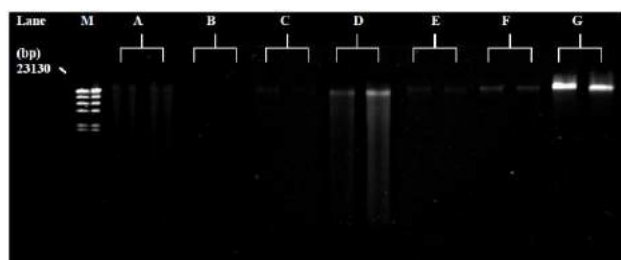


Figure 1. Genomic DNA by 1.0% agarose gel electrophoresis from A (Vivantis GF-1 Nucleic Extraction Kit); B (NucleoSpin Plant II Kit – Lysis Buffer PL1); C (NucleoSpin Plant II Kit – Lysis Buffer PL2); D [3]; E [6]; F [8]; G (Modified protocol); with M as standard marker, λ HindIII DNA ladder.

Based on the results obtained from gel electrophoresis, DNA extracted using Vivantis GF-1 Nucleic Extraction Kit produces higher yield as compared that of NucleoSpin Plant II Kit using two different lysis buffers, but has a relatively low DNA purity. NucleoSpin Plant II Kit, on the other hand produces better DNA purity which falls within the acceptable range. Lysis Buffer PL2 used in protocol C which is based on SDS-lysis method produces a slightly higher yield compared to Lysis Buffer PL1 that is based on CTAB-lysis method.

However, the conventional methods generate a more consistent and generally higher yield of DNA from *Hylocereus* sp. as compared to the commercial kits. The method described by Tel-Zur *et al.* [3] produces an average yield of about 350ng/ μ L with acceptable purity but the gel image shows smearing of sample. Both protocol E and F [6], [8], respectively produces intact bands without smearing but with a low yield of less than 300ng/ μ L which will eventually limit the success for downstream application such as PCR. Low DNA purity in protocol E was also observed as the ratio of A_{260}/A_{280} reading was higher than the purity range with suspected contamination of RNA.

Protocol F by Pop *et al.* [8] had described a simple method of DNA extraction that produces a pure product but low yield, and conversely, protocol D by Tel-Zur *et al.* [3] shows average yield but low purity. Protocol D uses high-salt CTAB buffer in which is said to be able to separate DNA from other substances such as proteins more efficiently. Hence, from these two methods, we aimed to use small amount of sample as the starting material while going through a simple and quick protocol with the addition of high-salt CTAB buffer to generate a good yield.

In our modified protocol, we adopted the use of high-salt CTAB buffer containing 4M of NaCl as in the protocol stated by Tel-Zur *et al.* [3]. We have also included the addition of Proteinase K with sufficient concentration to breakdown the cell walls and dissolving the cell membranes. Sodium sulfite was used as a reducing agent for polyphenol oxidase which acts by preventing the production of polyphenolic compounds

that will cause the degradation of DNA [13]. According to the gel image of DNA extracted using our modified protocol, it produces favorable yield which is >500 ng/ μ L and high purity that falls within the range of 1.8-2.0. The absence of smears also indicates high purity of DNA were isolated (Table I).

TABLE I. YIELD AND PURITY OF DNA EXTRACTED FROM DRAGON FRUIT STEMS BY DIFFERENT PROTOCOLS.

Protocol	Average DNA purity (A_{260}/A_{280})	Average DNA yield (ng/ μ L)
A	1.57	240
B	1.73	51
C	2.10	63
D	2.00	362
E	2.23	110
F	1.98	213
G	1.83	608

The RNA extraction protocols tested in current study involved the use of CTAB, SDS and commercial kit. We applied the mentioned protocols as these methods are designed for extracting RNA from recalcitrant tissues or tissues containing high interferences such as polyphenols and polysaccharides compounds. The first protocol applied was as described by Hou *et al.* [12] where CTAB with 2M of NaCl was used to extract RNA from a Chinese medicinal plant, *Fritillaria unibracteata* that contains considerable amounts of secondary metabolites. The second protocol from Hu *et al.* [11] also uses CTAB but they have modified to extract RNA samples from fruits of kiwi, apple, and peach which contain high level of polyphenol and polysaccharide compounds. A third protocol was carried out according to Nacalai Tesque Sepasol-RNA 1 Super G kit's instruction manual whereas, the fourth protocol was performed according to Valderrama-Chairez *et al.* [9]. The method was developed for RNA isolation from cactus fruit, of which expected to be the best method for isolating RNA from cactus stem. The results were shown on Fig. 2.

Based on Fig. 2, the RNA extracted using protocol 1 and 2 (CTAB method) produces two RNA bands each with low intensity and slight DNA contamination. As for protocol 3 using a commercial kit from Nacalai Tesque, no bands could be observed on the 1% agarose gel as the concentration of RNA extracted could be too low. Valderrama-Chairez *et al.* [9] using SDS method (protocol 4) as shown in lane 4 of Figure 2 appeared faint visible RNA bands which showed less intensity as compared to the CTAB methods and with DNA contamination. In addition, this method has less advantage as the extracted sample itself has thick consistency and appeared gluey while being pipette out. It was suggested that SDS extraction gives less purification of the sample comparatively which is a drawback.

Hence, we modified the extraction buffer as well as the protocol steps from Valderrama-Chairez *et al.* [9]. First, we replaced the SDS extraction buffer with CTAB extraction buffer with high salt. CTAB is a strong detergent that helps to break plant cell walls and able to separate nucleic acids from polysaccharides [14]. The addition of salts helps to dissolve polysaccharides. Thus,

the complex of CTAB-RNA provides an efficient removal of polysaccharides. Beta-mercaptoethanol acts as a strong reducing reagent that can irreversibly denature RNases. Further addition of PVP is important as it helps to prevent the oxidation of polyphenols in cell walls and extracellular matrices because oxidised polyphenols will co-precipitate with nucleic acids. We decided to try the same extraction buffer which was used in our modified DNA extraction protocol as they consist of the same composition. Furthermore, it would be an advantage to use the same buffer as less time would be needed to prepare different buffers for different nucleic acid extractions. We have also scaled down to the use of less amount of starting sample from 4 grams as stated by Valderrama-Chairez *et al.* [9] to 1 gram and the amount of reagents added was adjusted accordingly.

The modified RNA extraction protocol 5 as visualised in the 1.0% agarose gel electrophoresis shown in Fig. 3 was proved to be efficient. The corresponding bands of 28S and 18S rRNA was intense and no DNA contamination was observed.

Further quantitation of the RNA was done using spectrophotometer whereby RNA absorbs UV light at 260nm and protein contaminants absorbs at 280nm. Hence, the RNA sample purity is indicated by the ratio of A_{260}/A_{280} . The sample reading with ratio values that falls in the range of ~1.8 – 2.0 indicate good purity of the extracted RNA [14].

The absorbance reading obtained from the extracted samples is shown in Table II. It was observed that both CTAB methods (Protocol 1 and 2) gave a slightly higher RNA yield as compared to commercial kit (Protocol 3) and SDS method (Protocol 4). However, protocol 1 gives lower purity of 1.57 absorbance ratio with protein contamination as compared to protocol 2 of 1.73 absorbance ratio. This is because protocol 2 consists of additional sample purification step which is more tedious compared to the rest of the protocols. Commercial kit produces a good purity of 2.1 but has a very low concentration to be considered. Protocol 4 of SDS extraction produced both low purity and yield. The improvised method results as stated in protocol 5 showed better yield of 164ng/μL and acceptable sample purity of 1.79 absorbance value which is close to the 1.8~2.0 range. The readings suggested that the sample obtained from protocol 5 is less contaminated by polysaccharides and polyphenols which are sufficient for downstream applications.

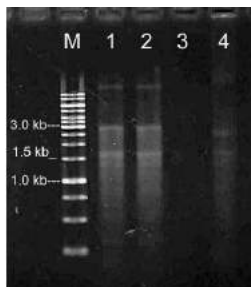


Figure 2. Extracted RNA by 1.0% agarose gel electrophoresis from protocol 1 [12]; 2 [11]; 3 (Nacalai Tesque Sepasol-RNA 1 Super G kit); 4 [9]; with M as standard marker, 1kb ladder.

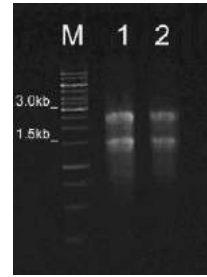


Figure 3. Extracted RNA by 1.0% agarose gel electrophoresis from modified protocol 5 with M as standard marker, 1kb ladder.

TABLE II. YIELD AND PURITY OF RNA EXTRACT FROM *HYLOCEREUS* STEMS BY DIFFERENT METHODS

Protocol	Average RNA purity (A_{260}/A_{280})	Average RNA yield (ng/μl)
1	1.57	64
2	1.73	51
3	2.10	22
4	1.56	48
5	1.79	164

IV. CONCLUSION

Although there were many presented protocols for nucleic acids extraction, it is necessary to develop an optimised protocol for DNA and RNA isolation specifically as different cacti species has variable mucilage content levels [4]. A simple and reliable DNA extraction procedure for Genera *Hylocereus* was developed via modification of a method from Tel-Zur *et al.* [3] and Pop *et al.* [8] and has been successfully used to extract superior DNA in terms of quality and quantity for downstream applications. The same goes for RNA isolation from the same sample wherein Valderrama-Chairez *et al.* [9] protocol was improvised to allow for better yield and purity. The advantage is that only a small amount of tissue is required for extraction and fewer chemicals were used in this protocol compared to the rest. In addition, this method is inexpensive, quick and simple to be carried out without the need for further purification. Furthermore, the same extraction buffer which can be used for both DNA and RNA isolation helps to reduce time-consuming preparations of different buffers.

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