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RNA Extractions of Mangosteen (*Garcinia mangostana* L.) Pericarps for Sequencing

(Pengekstrakan RNA Perikarpa Manggis (*Garcinia mangostana* L.) untuk Penjujukan)

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ABSTRACT

This study employed several RNA extraction methods for mangosteen pericarps prior to RNA sequencing. The sequencing platform heavily relies on a high quality RNA yield. However, pericarp tissues contain a lot of phenolic compounds that results in low RNA quality. Hence, we studied several RNA extraction methods to obtain the most suitable method for the best RNA quality from the pericarps of mangosteen. Five different methods including Lopez and Gomez, modified hexadecyltrimethyl ammonium bromide (CTAB) method, several commercial kits from TranszolUP, Favorgen and Qiagen RNeasy were compared. By optimising the CTAB method, it was found to be the best method to obtain pure RNA (high A_{260}/A_{280} ratio) with the highest yields (up to approximately 600-800 ng/µL concentration). The QC control of these samples using bioanalyzer validated their suitability for the downstream RNA sequencing. This report details the method for extracting high quality and high yield RNA samples from fruit that are rich in polyphenolic compounds such as mangosteen.

Keywords: Mangosteen; pericarp; RNA extractions; RNA-seq

ABSTRAK

Platform penjujukan bergantung sepenuhnya kepada hasil RNA yang berkualiti tinggi. Walau bagaimanapun, tisu kulit manggis mengandungi banyak sebatian fenol yang menyebabkan kualiti RNA menjadi rendah. Oleh itu, penyelidikan ini mengkaji beberapa kaedah pengekstrakan RNA untuk mendapatkan kaedah yang paling sesuai bagi penghasilan RNA yang berkualiti tinggi daripada perikarpa manggis. Lima kaedah yang berbeza termasuk kaedah Lopez dan Gomez, kaedah yang diubah suai daripada heksadesiltrimetil ammonium bromida (CTAB), beberapa kit komersial daripada TranszolUP, Favorgen dan Qiagen Rneasy telah dibandingkan. Dengan mengoptimumkan kaedah CTAB, ia telah didapati menjadi kaedah terbaik untuk mendapatkan RNA tulen (A_{260}/A_{280} nisbah tinggi) dengan kadar hasil tertinggi (kepekatan sehingga kira-kira 600-800 ng/µL). Kawalan QC sampel ini menggunakan bioanalisis untuk disahkan kesesuaiannya bagi analisis hiliran penjujukan RNA. Laporan ini memperincikan kaedah untuk mengekstrak hasil RNA yang banyak dan berkualiti tinggi daripada buah-buahan yang kaya dengan sebatian polifenol seperti manggis.

Kata kunci: Manggis; pengekstrakan RNA; perikarpa; RNA-seq

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is a tropical climacteric fruit from the family of Clusiaceae (Guttiferae) cultivated in Southeast Asia countries such as Malaysia, Thailand, Indonesia and Philippines (Pedraza-Chaverri et al. 2008). Apart from its sweet pulp with unique taste, mangosteen is also attractive due to natural phenolic compounds such as xanthones and flavonoids that mostly reside in its purple-coloured pericarp (Figure 1). These bioactive compounds are known to have medicinal properties such as anticarcinogenic, anti-inflammatory and antioxidant (Akao et al. 2008; Shan et al. 2011). Interestingly, the molecular mechanism of these compounds in mangosteen has not been elucidated and systems biology approach such as transcriptomics can be utilised to catalogue its gene expression. However, RNA extraction for the transcriptomics approach from mangosteen pericarp samples posed several significant problems. The phenolic compounds and polysaccharides are known to co-precipitate and interact irreversibly with nucleic acids which complicate the downstream molecular analysis such as RNA sequencing (Loomis 1974). Furthermore, the thick and hard layer of the pericarp as well as the presence of latex contributes to the difficulties in handling mangosteen samples for RNA extraction. Moreover, the abundance of anthocyanin can also co-precipitate with nucleic acids which resulted in a coloured RNA pellet, jeopardising its purity.

The difficulties of RNA extraction from plant tissues that are rich in polysaccharides such as grapevine, mango and sweet potatoes have been reported in many previous studies (Gambino et al. 2008; Kim & Hamada 2005; López-Gómez & Gómez-Lim 1992). Different conditions/parameters are therefore required to isolate a good quality RNA for different species, different tissues as well as for the same species but grown under different environments

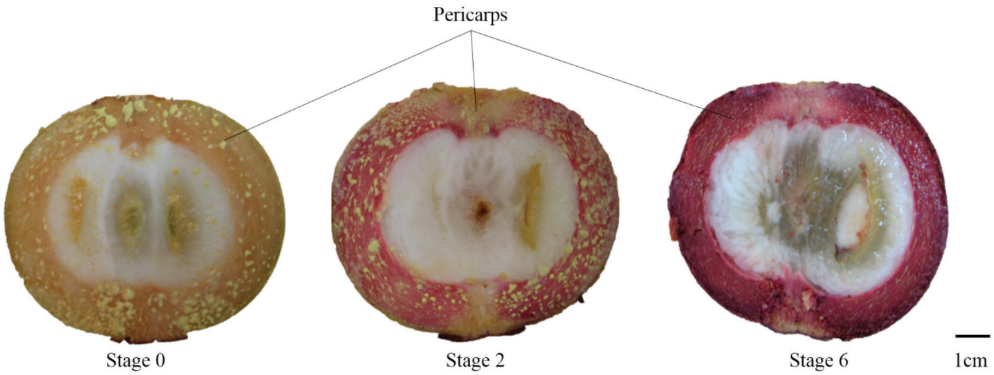

FIGURE 1. Pericarps of *Garcinia mangostana* L. (Stage 0, 2 and 6)

(Soon et al. 1997). The usage of solvents such as phenol chloroform for RNA recovery is one of the oldest methods in RNA extraction introduced by Chomczynski and Sacchi (1987). The method also involved lithium chloride which is used to precipitate RNA at certain concentrations, while sodium acetate is used to remove polysaccharides from the solution. Other methods are also available such as López and Gómez method (1992), CTAB method by Kim and Hamada (2005) and many modern commercial kits for isolating RNA such as Qiagen RNeasy, *TransZol™* Up and Favorgen RNA mini kit.

Nevertheless, a good quality RNA is crucial for next generation sequencing (NGS) which has been implemented in various transcriptomics study including expression profiling. RNA sequencing (RNA-seq) is one of the high-throughput techniques employed to profile RNA molecules of any plant of interest such as mango, blackberry and watermelon to understand its molecular mechanisms (Dautt-Castro et al. 2015; Garcia-Seco et al. 2015; Guo et al. 2015). To date, various researches that employ this technology have been able to overcome the limited dynamic range of detection posed by other more traditional technologies such as microarray (Oszzolak 2011). Hence, more NGS platforms have been developed and commercially available from companies such as Pacific Biosciences, Illumina and Life Technologies (Morozova & Marra 2008; Wang et al. 2009).

However, despite the emphasis on the RNA quality for RNA-seq, there are still limited reports detailing RNA extraction method and quality control (QC) of the sample for sequencing. A good RNA quality is determined by the A260/A280 absorbance between 1.8 and 2.0. The integrity of RNA can be validated using gel electrophoresis based on two distinct 28S and 18S rRNA bands with no genomic contamination. Furthermore, QC based on bioanalyzer for RNA integrity number (RIN) is of paramount importance. Typically, a good RIN suggested for RNA sequencing is about 7 to 10 (Jordon-Thaden et al. 2015; Yockteng et al. 2013).

In this study, we conducted experiments to determine the best RNA extraction protocol from different methods such as López & Gómez method (1992), modified CTAB method by Kim and Hamada (2005), as well as using several abovementioned commercial kits. The most suitable method of RNA extraction was then chosen based on their RNA purity ratio (A260/A280) and RIN prior to RNA-seq. Samples from three different ripening stages of mangosteen were analysed in this study.

METHODS

PLANT MATERIALS

Mangosteen (*Garcinia mangostana* L.) fruit was obtained from UKM mangosteen plot, UKM Bangi, Selangor, Malaysia. Seven ripening stages of mangosteen (Stage 0 until Stage 6) were identified according to the Malaysian Mangosteen maturity indices (Osman & Milan 2006). A minimum of six random biological replicates of initial (stage 0), middle (stage 2) and fully ripened (stage 6) mangosteen were harvested (during May until July 2014) and their pericarp were isolated (except Stage 0 due to inseparable aril and pericarp) and ground in liquid nitrogen before stored at -80ºC (Details on the preparation of materials and RNA handlings can be found in Appendix 1).

METHOD 1

This method is based on López-Gómez and Gómez-Lim (1992). The frozen powders of stage 6 pericarps were transferred to the extraction buffer containing 10 mM Tris-borate pH 7.5, 50 mM sodium chloride (NaCl), 1% sodium dodecyl-sulphate (SDS), 6% polyvinylpyrolidone (PVP), 1 mM *ethylenediaminetetraacetic* acid (EDTA) pH8.0 and 14 mM β-mercaptoethanol. The mixture was shaken for 1 min on ice and 1.0 mL of phenol pH4.5 were added, followed by 0.25 (v/v) ethanol and 0.11 (v/v) 3 M sodium acetate (NaOAc). Solution was centrifuged at 13,300 rpm for 10 min at 4ºC. The supernatant was collected and an equal volume of chloroform: isoamyl alcohol (49:1) was added and centrifuged again. Liquid-liquid portioning was followed by using phenol: chloroform: isoamyl alcohol and 1/3 volume of 12.0 M lithium chloride (LiCl) were added for precipitation and incubated overnight. The RNA

was collected by centrifugation (13,300 rpm, 90 min, 4ºC), followed by washing using 3.0 M LiCl, before dissolved in NaOAc and absolute ethanol. The dissolved pellet was incubated at -80ºC for 2 h. RNA was recovered by centrifugation for 10 min and washed with 70% ethanol before air dried and dissolved in DEPC-treated water.

METHOD 2

Ground frozen tissue (100 mg) was added to 1.0 mL *TransZol™* Up. Tube was shaken vigorously for 30 s and incubated at room temperature for 3 min. Then, 0.2 mL of chloroform were added followed by centrifugation (13,300 rpm, 90 min, 4ºC). Aqueous phase was collected and an equal volume of absolute ethanol was added to the solution. The resulting solution was transferred to a spin column and washed using a clean buffer (CB9) that is provided with the kit. RNA was eluted in RNase free water and kept at -80ºC until further analysis.

METHOD 3

This method was done using Qiagen RNeasy Plant Mini Kit (Qiagen, Mississauga, Ontorio). All the protocols were followed according to manufacturer's protocols.

METHOD 4

This method was done by combining Method 1- López-Gómez and Gómez-Lim (1992) followed by RNA spin column (silica matrix) from Favorgen RNA Mini Kit (Favorgen Biotech Co., Ping Tung, Taiwan). After obtaining and quantifying the RNA obtained from Method 1, the RNA was precipitated and washed using wash buffers and columns from the Favorgen RNA Mini Kit.

METHOD 5

Total RNA from the ground tissues of mangosteen were extracted using modified CTAB RNA extraction method by Kim and Hamada (2005). One gram of frozen tissues were added into 10 mL of CTAB extraction buffer that contains 2% hexadecyltrimethyl ammonium bromide (CTAB), 100 mM Tris-HCl pH8.0, 2.0 mM EDTA pH8.0, 2.5% PVP-40 and 2% of β-mercaptoethanol. The mixture was incubated for 1 h in 65ºC water bath and swirled gently for every 10 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 13,000 × g for 10 to 12 min at 4ºC. After that, the supernatant was collected and the previous step was repeated once before adding 1/3 of lithium chloride. After overnight incubation at -20ºC, the sample was centrifuged (13,000 × g, 4ºC) for 90 min. The resulting RNA pellet was dissolved in 1.0 mL of RNase-free water and liquid-liquid portioning (24:1 chloroform:isoamyl) was repeated as previously mentioned with 1/3 of 12 M LiCl and overnight incubation at -20ºC. Finally, the solution was centrifuged (13,000 × g, 30 min at 4ºC) and the pellet obtained was washed using 70% ethanol before resuspended in RNase-free water.

QUANTITATIVE AND QUALITATIVE ANALYSIS

RNA samples were quantified using Nanodrop spectrophotometer (NanoDrop ND-1000 UV) for measuring the concentration and the purity of RNA based on the ratio of absorbance at 260 and 280 nm. The presence of intact 18S and 28S rRNA bands without genomic DNA contamination was visualised using agarose gel electrophoresis.

RNA QUALITY CHECK (QC) ANALYSIS

Prior to RNA sequencing, RNA must go through quality check analysis to measure the integrity of RNA by using microcapillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Foster City, California). Bioanalyzer provides the basis for automated RNA-seq to estimate the integrity of RNA by determining the electrophoregram data. The RIN algorithm software provides a numbering systems from 1-10, of which number 1 being the most degraded profile and 10 being the maximum intact RNA (Schroeder et al. 2006).

RESULTS & DISCUSSION

RESULTS

The results of the five RNA extraction methods are shown in Table 1. Fully ripened (stage 6) mangosteen pericarp was used for the comparison of RNA isolation methods. Stage 6 was chosen particularly because it contains a lot of bioactive compounds such as phenolics and anthocyanins that can influence RNA quality. TransZol-Up Kit and Qiagen RNeasy Kit as well as the combination of conventional and commercial kits (López-Gómez and Favorgen) failed to produce sufficient detectable RNA (Table 1). Only López-Gómez and Gómez-Lim (1992) and the modified CTAB methods yielded high amount of RNA (413.6 and 622.3 ng/μL, respectively). However, extracted RNA from López-Gómez and Gómez-Lim (1992) method showed low A260/A280 and A260/A230 absorbance ratio (0.77 and 0.22, respectively) suggesting it is not suitable for mangosteen RNA extraction. Only modified CTAB gave good quality RNA with values of 2.0 and 2.2 absorbance ratio for A260/A280 and A260/A230, respectively.

Based on the gel electrophoresis result (Figure 2), Methods 2 and 3 did not produce any nucleic acid bands. Meanwhile, Methods 1 and 4, produced a sharp distinct bands with a smear of genomic DNA contaminations. Method 5 produced an intact and bright 18S and 28S rRNA bands without significant DNA contamination. This demonstrates that the total RNA extracted from modified CTAB method (Method 5) was pure and intact. Hence, this method was chosen to isolate the RNA from stage 0, 2 and 6 of mangosteen pericarp (The detailed bench-ready protocols can be found in Appendix 2).

RNA samples with good quality were successfully extracted from all three ripening stages. The RNA concentration was high for most samples, ranging from 160 to 800 ng/μL. The RNA yield per sample fresh weight

TABLE 1. Different methods for RNA extraction of ripened mangosteen (*Garcinia mangostana* L.) pericarps

Method	Column Based	RNA Concentration (ng/μL)	A260/A280 Ratio	A260/A230 Ratio	Total Time Required (day/s)
Method 1 Lopez-Gomez	No	413.6	0.77	0.22	1-2
Method 2 TransZol-Up Kit	No	null	-	-	Half day
Method 3 Qiagen RNeasy Kit	Yes	0.8	1.12	0.37	Half day
Method 4 Lopez-Gomez & Favorgen	Yes	0.6	1.46	1.03	1
Method 5 Modified CTAB	No	622.3	2.03	2.20	3

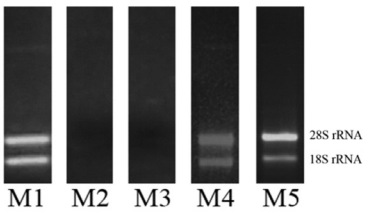

FIGURE 2. Agarose Gel Electrophoresis image for Method 1 (M1, Lopez-Gomez), Method 2 (M2, TranszolUp Kit), Method 3 (M3, Qiagen RNeasy Kit), Method 4 (M4, Favorgen Kit) and Method 5 (M5, CTAB)

(FW) was also satisfactory for all samples, ranging from 5.0 to 25.0 μg/g FW (Table 2). Moreover, the purity of most of these samples were 1.90-2.00 for the A260/A280 absorbance. The gel electrophoresis result also showed two distinct bands with no genomic contamination (Figure 3). Furthermore, RIN scores for all samples were greater than 8 and their corresponding electrophoregrams are shown in Figure 4.

DISCUSSION

Method 1 was done following the method of López-Gómez and Gómez-Lim (1992). This method was originally developed to isolate RNA from samples that are high in phenolic compounds. Thus, Tris-Borate and ethanol precipitation were used in this method to purify and concentrate RNA. Theoretically, β-mercaptoethanol and PVP used in this extraction is important to prevent oxidation problems (Shu et al. 2014). β-mercaptoethanol is also useful as a reducing agent for eliminating RNase during cell lysis (Jaakola et al. 2001). Meanwhile, PVP plays an important role in removing phenolic compounds and secondary metabolites from nucleic acid extracts and preventing the browning effects of polyphenols (Chan et al. 2007). Chloroform: isoamyl alcohol is then used to remove PVP before the addition of phenol: chloroform: isoamyl alcohol (PCI). This is because phenol is not compatible with PVP and hence chloroform: isoamyl alcohol is used as an alternative solvent (Vasanthaiah et al. 2008). This method produced sharp and distinct bands indicating intact RNA products (Figure 2). However, the purity measurement indicated that there was high degree of contamination potentially from protein impurities that can affect its A260/A280 ratio. Genomic DNA band was also present in the gel electrophoresis image (Figure 2). Furthermore, the pellet obtained in this extraction method also appeared brown colour suggesting remnants of colour pigments (data not shown). This suggests that the method is not able to remove impurities from the RNA of mangosteen pericarp and hence not suitable for RNA-seq.

The extraction method 2 (TranZolUp Kit) yielded a brown and insoluble pellet, right after ethanol precipitation (data not shown). The absorbance ratio reading was out of linear range (Table 1) because the insoluble pellet gave

TABLE 2. Quantitative analysis for Stage 0, Stage 2 and Stage 6 of mangosteen pericarps using Method 5 (CTAB)

Stage	Samples	RNA Concentration (ng/μL)	RNA Yields (μg/g FW sample)	A260/A280	A260/A230	RIN
0	GmS0-1	829.19	24.8	2.04	2.08	8.30
	GmS0-2	234.64	7.03	1.95	1.99	8.30
2	GmS2-1	260.79	7.80	1.98	1.77	8.40
	GmS2-2	369.56	11.08	1.96	1.53	8.20
6	GmS6-1	161.80	4.85	1.90	1.64	8.30
	GmS6-2	622.26	18.66	2.03	2.20	8.10

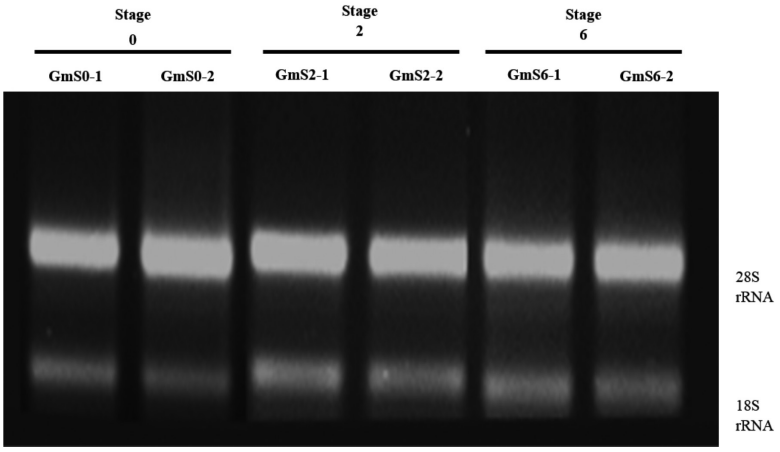

FIGURE 3. Gel electrophoresis image for Stage 0, 2 and 6 of *Garcinia mangostana* L.

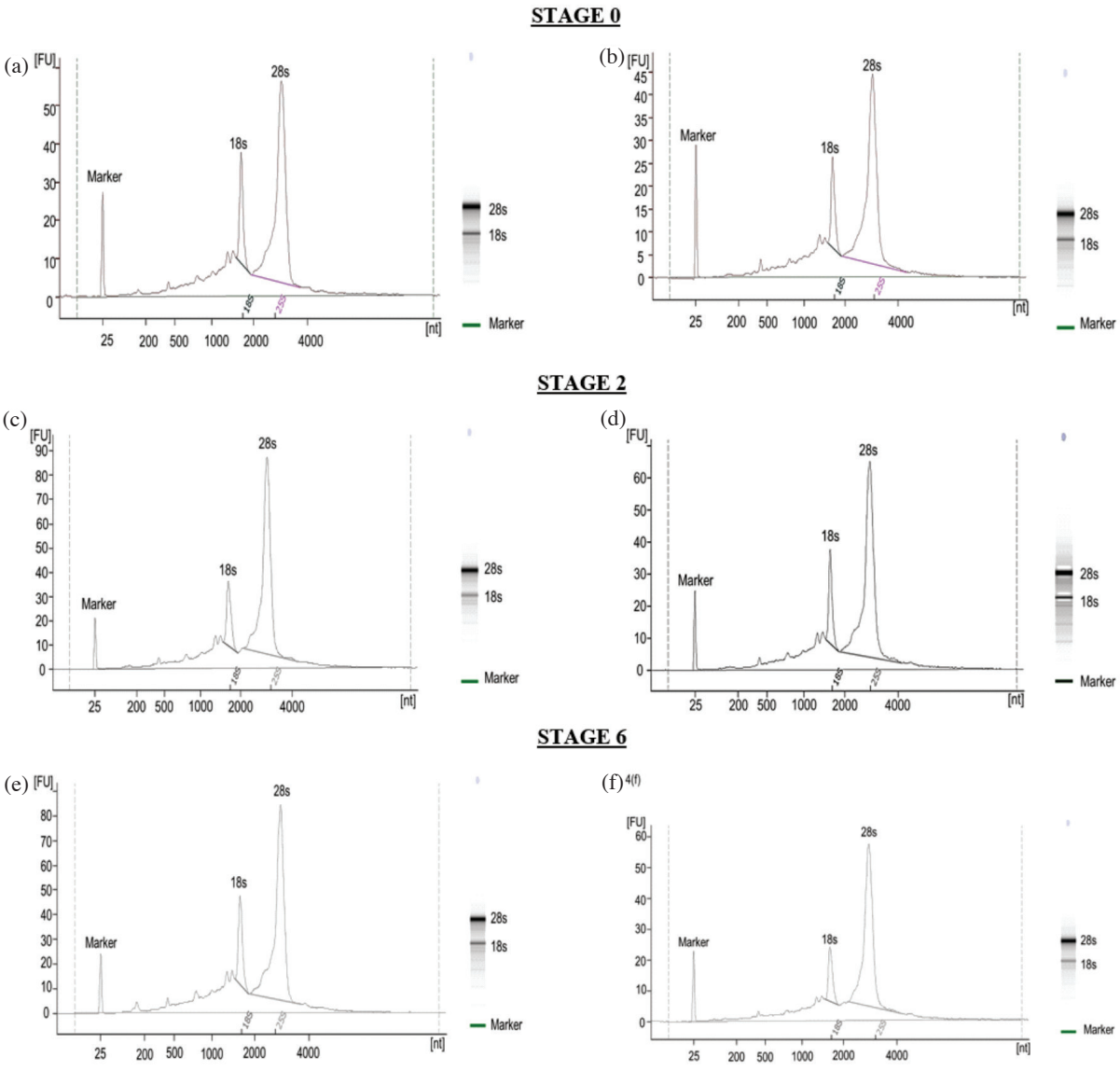

FIGURE 4. Electropherogram summary using Agilent 2100 bioanalyzer for RNA samples extracted from Stage 0 (a and b), Stage 2 (c and d) and Stage 6 (e and f) of mangosteen pericarps

brown solution after elution. Thus, this method is also not suitable to isolate RNA from the mangosteen pericarps.

RNA extraction using Qiagen kit in Method 3 also failed to give satisfactory result as the yield was too low (0.8 ng/μL) (Table 1). The column technologies employed in this kit creates other problems including complicated manipulation of buffers while loading and washing the columns, sensitivity problems due to non-specific binding and finally, 'drop-out' issues where no nucleic acid is detected. Subsequently, no RNA band was observed in the gel electrophoresis (Figure 2).

Method 4 was an attempt to purify the low purity but intact RNA obtained from Method 1 using the column from Favorgen. However, this method failed to produce high yield of RNA, though it showed slightly increased A260/A280 absorbance ratio, but the yield was too low. This suggests that the column used is not efficient enough to preferentially purify RNA from other contaminants.

In Method 5, CTAB RNA extraction was used with slight modification from original CTAB RNA extraction by Kim and Hamada (2005). Several changes were made such as all centrifugation steps were carried out at 13,000 × g for 10 to 12 min at 4°C, the concentration of PVP-40 and LiCl were increased to 2.5% and 12 M, respectively. The incubation period time were also added up to 1 h at 65°C instead of directly mixed with pre-warmed extraction buffer as described by Kim and Hamada (2005). The longer time of incubation period was needed due to the firmness of the mangosteen pericarps that are often difficult to disrupt in the extraction buffer. Even though this method is time consuming (3 days), but it produced the best RNA yield and quality compared to other methods (Table 1). This method also produced good RNA quality for all stages of mangosteen fruit (Table 2, Figure 3). This suggests that this method is able to purify RNA samples from various stages of mangosteen fruit ripening despite the differences in their chemical compositions (Palapol et al. 2009) and the presence of higher amount of latex in early ripening stages (stage 0 and stage 2) (Figure 1). The RIN for these RNA samples were also more than 7 (Table 2, Figure 4) indicating a good RNA quality for the downstream RNA-seq analysis.

Conclusion

In conclusion, this paper details the method for extracting high quality and high yield RNA samples from pericarp tissues which are rich in polyphenolic compounds such as that of mangosteen.

Acknowledgements

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APPENDIX 1

RNA precautious and handlings

- Preparation of materials/apparatus
  - All apparatus needed were immersed in 1% DEPC water overnight. For examples:
    - Schott Bottles
    - Pipette Tips
    - Falcon Tubes
    - Microfuge Tubes
    - Mortar and Pestle
    - Spatula
  - These apparatuses were then autoclaved

- Preparation of buffers and solutions
  - 0.1% DEPC water was used for making all buffers and these were stored at room temperature overnight before used. (autoclaving solutions and buffers is unnecessary once DEPC is used)

- RNA Extraction precautions (Jordon-Thaden et al. 2015)
  - *Workplace preparation*: Workplace during RNA extraction needs to be properly clean in order to reduce any RNase contamination from environment

  - *RNA extraction*:
    - Throughout the extraction protocols, samples were kept on ice
    - All reagents or solutions used for liquid-liquid portioning, precipitation and washing were chilled (4°C) before extraction
    - Cool conditions is required to reduce any chances of RNA degradation

  - *RNA storage*: pure RNA were kept in -80°C freezer

APPENDIX 2

Bench-ready protocol for Method 5 (Modified CTAB method). Prepared by
Azhani Abdul-Rahman and Wan Adibah Zakaria

Reagents:

- CTAB Extraction Buffer
  - 100 mM Tris-Hcl pH8.0
  - 25 mM EDTA pH8.0
  - 2M Nacl
  - 2% CTAB

  Add freshly before use:
  - 2.5% PVP-40
  - 2% β-Mercapthoethanol

- Other reagents
  - Chloroform
  - Isoamyl alcohol
  - 12M LiCl
  - 70% ethanol
  - 0.1% DEPC water

  *Use 0.1% DEPC water for making all buffers and these were stored at room temperature overnight before used (autoclaving solutions and buffers is unnecessary once DEPC is used)

  *Add 2.5% PVP-40 and 2% β-Mercapthoethanol FRESHLY before used

Procedure:

Day 1

- Preparation for extraction
- Pre-warm 10 mL extraction buffer (including 2.5% PVP-40 and 2% β-ME) in water bath at 65°C
- Pre-chill the centrifuge to 4°C

- RNA Extraction
  - Add 1.0 gram of sample into 10 mL pre-warmed extraction buffer
  - Gently shake the mixture
  - Incubate the sample in 65°C waterbath for 1 h (swirl the sample gently for every 10 min)
  - Add equal volume of 24: 1 choroform : isoamyl alcohol
  - Centrifuge the sample mixture for 12 - 15 min (13000 g, 4°C) (gently put the centrifuged samples in ice to prevent any accidental mixing)
  - Transfer the supernatant into a new falcon tube
  - Add equal volume of 24: 1 choroform: isoamyl alcohol
  - Centrifuge the sample mixture for 12 - 15 min (13000 g, 4°C) (gently put the centrifuged sample in ice to prevent any accidental mixing)
  - Transfer the uppermost layer into a new tube (oakridge tube as Sorvall Revolution Rc will be used for the next centrifugation step)
  - Add 1/3 of 12 M LiCl and swirl to mix
  - Precipitate overnight at -20°C freezer

Day 2

- Centrifuge the sample for 90 min (13000 g, 4°C) (Thermo Scientific, Sorvall Revolution Rc Centrifuge)
- Observe the pellet carefully (the pellet can be colourless, transparent and very tiny). Gently remove all aqueous phase
- Dissolve the pellet in 1 mL 0.1% DEPC water
- Transfer the sample into a 2 mL microfuge tube
- Add equal volume of 24: 1 chloroform: isoamyl alcohol
- Centrifuge the sample for 12 -15 min (13000g, 4°C)
- Transfer the supernatant layer into a new microfuge tube
- Add 1/3 of 12 M LiCl and swirl to mix
- Incubate overnight at -20°C freezer

Day 3

- Thaw the sample in ice (make sure sample is completely thawed before proceed to the next step)
- Centrifuge sample for 30 min (13000 g, 4°C)
- Observed the pellet carefully, remove all the aqueous (pellet can be small, droplet-like and colourless)
- Add 300-500 µL of 70% ethanol
- Centrifuge again for 12-15 min (13000 g, 4°C)
- Remove the aqueous and observed the pellet
- Again, add 300-500 µL of 70% ethanol
- Remove the aqueous and observed the pellet
- Air-dry the sample for 5 min (optionally, any excessive aqueous left in the tube can be carefully pipetted out)
- Dissolve the resulting pellet in 30 µL of 0.1% DEPC water (make sure the pellet is completely dissolved)

- Check for RNA concentration
  - Immediately measure the RNA concentration using Nanodrop
    - The A_{260}/A_{280} ratio should be 1.8 - 2.0 (accepted as a good RNA purity)

- Check for RNA integrity
  - Run RNA in 0.1% agarose gel (90V, 400MA, 1 h)
  - Store RNA at -80°C freezer
  - After 1 h, obtain gel for 28 s and 18 s band with no DNA contamination (2 sharp distinct bands)

- RNA integrity quality check (Agilent Bioanalyzer 2100)
  - RNA with value of above 7 is generally accepted for next generation sequencing
  - rRNA Ratio of 28 s/18 s need to be higher than 2 (Jordon-Thaden et al. 2015)