

A modified protocol for total RNA isolation from different oil palm (*Elaeis guineensis*) tissues using cetyltrimethylammonium bromide

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Extraction of high-quality RNA from oil palm tissues is challenging due to the presence of polysaccharides, polyphenols and other complexes that co-precipitate with RNA. Therefore, isolation of high-quality RNA from oil palm is challenging due to the presence of varying amounts of these constituents in diverse tissues. This communication describes a modified RNA extraction protocol based on the cetyltrimethylammonium bromide (CTAB) method which is useful for extracting high-quality RNA from different oil palm tissues. Total RNA isolation using a modified CTAB protocol was compared with two different methods, a conventional TRIzol method and the method for RNA isolation from palms (MRIP). Both methods were useful for isolating RNA from leaf tissues; however, they were not effective in isolating RNA from other tissues. The current protocol based on a modified CTAB method was efficient in isolating high-quality total RNA from oil palm fruit tissues, including mesocarp and endosperm, stem, root and flower tissues. This modified CTAB-based protocol gave approximately 20–30 µg of total RNA from 150 mg of tissue within 5–6 h.

Keywords: CTAB method, gene expression, oil palm, RNA extraction.

Oil palm (*Elaeis guineensis*) is one of the leading sources of edible oil today¹; it yields tenfold higher oil than soybean². Palm oil is extracted from the fruit pulp (mesocarp) and palm kernel (endosperm) of oil palm. Though 1 ha of oil palm produces about 10–35 tonnes of fresh fruit bunches per year³, there is a huge gap between production and demand of palm oil. It is crucial to improve the quality and yield of palm oil to meet the needs of an increasing population⁴. Worldwide increase in the demand of vegetable oil necessitates an insight into the molecular

mechanism that regulates oil synthesis and fatty acid composition in oil palm fruit. WRINKLED1 (WRI1), a transcription factor (TF) and a key player in seed maturation can regulate glycolysis and fatty acid (FA) biosynthesis in higher plants^{5,6}. However, to study the expression profile of *WRI1* genes in different tissues and to verify the role of regulatory factors and/or target genes in oil palm fatty acid synthesis, extraction of high-quality RNA is required.

The first step in molecular biology experiments (e.g. isolation of the gene of interest by PCR-based technologies, establishment of cDNA libraries and expression studies) is extraction of high-quality RNA. Presence of high level of polyphenols and polysaccharides in most plant materials greatly affects the isolation of high-quality RNA in appreciable quantities^{7,8}. Several RNA extraction methods have been developed over the years to isolate high-quality RNA from diverse species^{9–13}. Besides, multiple commercial kit reagents for RNA isolation from plant tissues are also available, including RNeasy plant kit (QIAGEN, China) and TRIzol (Invitrogen, China). However, these kits are not always effective for the isolation of quality RNA from complex plant tissues (mainly oil palm) due to the presence of high amounts of polyphenols, polysaccharides, and other secondary metabolites^{14–16}. To solve this problem, a modified CTAB method has been developed to extract high quality and quantity of RNA from a wide range of oil palm tissues. The proposed method will help the palm molecular biologists isolate quality RNA from complex palm tissues and utilize it in downstream experiments.

The reagents used in the assay were composed of 0.5 M EDTA stock, 5 M NaOH solution, 1 M Tris HCl stock (pH 8.0), 5 M NaCl and 3 M NaAc (pH 5.2). The extraction buffer was composed of 20 ml 1 M Tris-HCl (pH 8.0), 120 ml 5 M NaCl and 10 ml 0.5 M EDTA, 4 g CTAB and 4 g PVP in 100 ml of water. The final volume of the mixture was adjusted to 250 ml with H₂O. β-mercaptoethanol (βME) (2%) was added to the extraction buffer before use to reduce the possibility of oxidation.

The steps in the extraction of RNA from oil palm tissues consisted of tissue collection and grinding (1–2 h for 10 samples), extraction of RNA from the ground tissue (approximately 2–3 h), RNA precipitation and washing (approximately 1.5–2 h), measuring RNA concentration (15 min for 10 samples) and checking RNA quality by electrophoresis (1.5 h). To isolate high-quality RNA the following steps were carried:

1. The frozen tissue was collected from –80°C freezer in liquid nitrogen to avoid thawing until proceeding to the next step. (In high-quality RNA isolation tissue collection is important. Plant tissues should be frozen in liquid nitrogen immediately after collection and maintained at –80°C prior to step 2.)

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2. Mortar and pestle were pre-cooled by pouring liquid nitrogen on them. The frozen tissues were placed in the mortar containing liquid nitrogen and ground with a pestle to get the fine powder. Next, the powdered frozen sample was carefully transferred into a 2 ml polypropylene tube with the help of pre-cooled spatula (pre-cooled in liquid nitrogen). (Do not let the tissue thaw during grinding. If necessary, add more liquid nitrogen slowly and continue until the sample becomes a fine powder. Secondly, evaporate all the liquid nitrogen before sealing the tube. Sealed tube with liquid nitrogen may pop open, resulting in loss of sample).
3. The powdered microfuge tubes were immediately placed in liquid nitrogen.
4. The sample was maintained at -80°C until proceeding to step 5.
5. Extraction buffer was pre-heated to 65°C in a water bath.
6. Next, 1 ml of preheated extraction buffer was added to 150 mg of powdered tissue, followed by vigorous shaking/vortexing for 15–20 sec at room temperature (RT).
7. Equal volume of chloroform : isoamyl alcohol (24 : 1 v/v) was added to the mixture and vortexed for 5 min.
8. The mixture was centrifuged at 13,000 g for 10 min at 4°C .
9. The supernatant (not $>700\text{--}800\ \mu\text{l}$) was transferred into a new 2 ml microfuge tube.
10. Next, 500 μl chloroform : isoamyl alcohol (24 : 1 v/v) was added to the supernatant and vortexed for 15–20 sec at RT.
11. The mixture was centrifuged at 13,000 g for 10 min at 4°C .
12. The supernatant was transferred (not $>600\text{--}700\ \mu\text{l}$) into a new 2 ml microfuge tube.
13. Next 500 μl of phenol : chloroform : isoamyl alcohol (25 : 24 : 1 v/v) was added to the supernatant and vortexed for 5–10 sec at RT.
14. The phases were separated by centrifuging the mixture at 13,000 g for 10 min.
15. The upper aqueous phase (not $>500\text{--}600\ \mu\text{l}$) was carefully transferred to a new 2 ml microfuge tube.
16. Next, 500 μl of chloroform : isoamyl alcohol (24 : 1 v/v) was added to the supernatant of the previous step to remove traces of phenol.
17. The tube was gently vortexed and centrifuged at 13,000 g for 10 min at 4°C .
18. The supernatant (not $>400\ \mu\text{l}$) was transferred to a new 1.5 ml microfuge tube.
19. Next three volumes of cold absolute EtOH (4°C) and 0.1 volume of 3 M sodium acetate (pH 5.2) were added to the recovered aqueous phase and nucleic acid was precipitated.
20. After gently mixing by inverting the tube, the sample was stored till frozen or stored overnight at -80°C .
21. Frozen tubes were centrifuged at 13,000 g for 20 min at 4°C .
22. The supernatant was discarded and 1 ml of 75% EtOH was added to wash out the pellet and centrifuged as described in step 21.
23. The supernatant was carefully removed without disturbing the pellet. The pellet was air-dried for 10 min to remove residual alcohol.
24. The pellet was then dissolved in 40–50 μl of RNase free water.

RNA concentration was determined spectrophotometrically by taking absorbance readings at 230, 260 and 280 nm to determine the presence of organic impurities (polysaccharides and polyphenolics).

The quality of RNA was determined by agarose gel electrophoresis.

- (i) Total RNA was electrophoresed in 1.2% (wt/vol) agarose gel.
- (ii) The mixture was heated until agarose completely dissolved in the buffer; it was then cooled to 50°C . Next ethidium bromide ($0.5\ \mu\text{g}\ \text{ml}^{-1}$) was added and the gel poured into a gel mould held in a cassette with the comb. The gel was allowed to harden for about 20 min. Then approximately 5 μg of total RNA mixed with 2 μl of 6 \times loading buffer was loaded into each well.
- (iii) The gel mould was transferred into a container with 0.5 \times TBE buffer and the tank was connected to the power supply. The gel was electrophoresed at 120 V for 20 min and finally RNA bands were visualized using gel imaging system.

To utilize total RNA in downstream applications, 3 μg of total RNA from each oil palm sample was reverse transcribed with the TaKaRa PrimeScript II 1st strand cDNA synthesis kit and oligo dT according to the manufacturer's instruction. Semi-quantitative RT-PCR was performed to check the quality of cDNA using TaKaRa PrimeScript II 1st strand cDNA synthesis kit as follows: 10 μl 5 \times buffer *Taq* buffer, 1 μl of both sense and anti-sense gene-specific primers (10 μM each), 1 μl dNTP mix (10 mM), 0.1 μl *Taq* polymerase (5U/ μl ; Real *Taq*) and 36.4 μl H_2O . PCR amplifications were performed with an TaKaRa thermocycler, using the following conditions: 3 min at 94°C (denaturation); 25–32 cycles with each cycle for 30 sec at 94°C ; 30 sec at 55°C ; 30 sec at 72°C (annealing); 10 min at 72°C (extending). For WR11 amplification primers were designed according to sequence information of the *Elaeis guineensis* cDNA libraries from the literature^{17,18}. PCR amplification for WR11 was performed using the following primers: forward: 5'-ATGACTCTCATGAAGAACTCT-3' and

Table 1. Yield and purity of RNA isolated from different oil palm tissues

Oil palm tissue	Absorbance ratio		Concentration (ng/ μ l)
	260/280	260/230	
Leaf	1.95 \pm 0.02	1.89 \pm 0.01	730.6 \pm 100.45
Shoot	1.89 \pm 0.05	1.83 \pm 0.06	671.1 \pm 128.0
Root	1.96 \pm 0.03	1.93 \pm 0.06	676.4 \pm 84.6
Female flower unopend	2.02 \pm 0.02	2.02 \pm 0.02	616.2 \pm 15.9
Female flower opend	1.92 \pm 0.06	1.94 \pm 0.06	835.5 \pm 128.0
Male flower unopened	1.84 \pm 0.06	1.85 \pm 0.09	1352.3 \pm 231.3
Male flower opened	1.87 \pm 0.05	1.84 \pm 0.06	839.4 \pm 171.9
Mesocarp young	1.96 \pm 0.03	1.98 \pm 0.04	579.9 \pm 42.2
Mesocarp mature	1.86 \pm 0.06	1.81 \pm 0.06	529.1 \pm 54.7
Endospem mature	1.98 \pm 0.06	1.92 \pm 0.07	1016.4 \pm 311.7

Values are means of three replicates with \pm indicates SD.

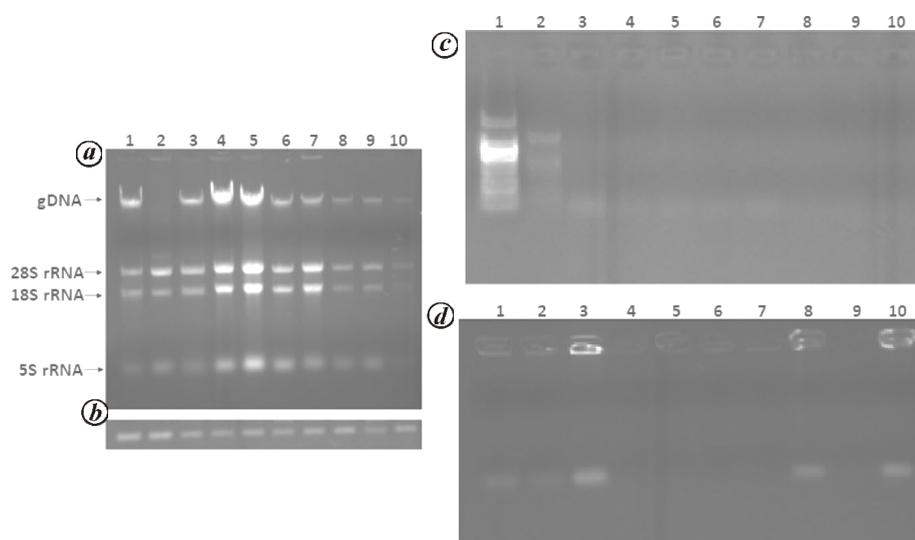


Figure 1. Isolation of RNA from various tissues of oil palm using different methods. (a) Total RNA (1 μ g) extracted at once from different oil palm organs by modified CTAB method; (b) RT-PCR for beta-actin reference gene. (c, d) Total RNA (1 μ g) extracted at once from different oil palm organs using (c) MRIP buffer and (d) TRIzol reagent. Numbers indicate different organs, i.e. (1) leaf, (2) stem, (3) root, (4) female unopened flower, (5) female opened flower, (6) male unopened flower, (7) male opened flower, (8) mesocarp young, (9) mesocarp mature and (10) endosperm mature.

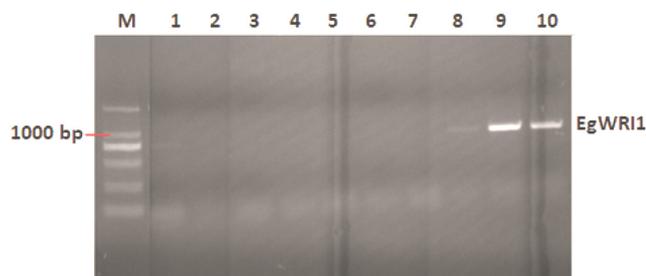


Figure 2. Agarose gel electrophoresis of RT-PCR analysis. RT-PCR amplification of EgWR11 fragment with EgWR11F1 and EgWR11R1 primers using total RNA isolated by modified CTAB method from different oil palm organs: (1) leaf, (2) stem, (3) root, (4) female unopened flower, (5) female opened flower, (6) male unopened flower, (7) male opened flower, (8) mesocarp young, (9) mesocarp mature and (10) endosperm mature. M, DNA ladder.

reverse: 5'-CTAGGCACCTTTGCTTGCA-3'. Beta-actin (forward: 5'-ATAAAGTATGGCTGATGCTGAGG-3 and reverse: 5'-CAACAATGCTTGGGAACACA-3) was used as reference gene. The designed primers were intron-spanning to avoid amplification of genomic DNA.

The results showed that the modified CTAB RNA isolation method produced high-quality and quantity of RNA from different oil palm tissues, which may serve as a promising method of RNA isolation from palm. Even though MRIP performed well in case of palm leaves, it was inefficient in extracting quality RNA from other tissues of palm. The modified RNA isolation protocol discussed here compared well with other protocols such as MRIP¹⁹ and TRIzol (standard method), likely that best method to extract RNA from several tissues of oil palm

(Figure 1). The quantity and purity of the isolated RNA were determined using a Nanodrop-2000 spectrophotometer. The absorbance ratios $A_{260/280}$ and $A_{260/230}$ of the isolated RNA (extracted using the modified CTAB method) ranged from 1.95 to 2.02 and 1.81 to 2.02 respectively (Table 1), suggesting that the resulting RNA was of high quality and purity^{20,21}.

In order to examine the quality of extracted RNA, about 2 µg of total RNA was separated on 1.2% agarose-ethidium bromide gel (Figure 1). All RNA samples exhibited bands of 28S rRNA and 18S rRNA, indicating acceptable quality and purity of RNA (Figure 1a). Other methods (MRIP buffer and TRIzol) were unable to extract RNA from various reproductive tissues (Figure 1c and d). Actin was used as control (Figure 1b). The resulting RNA was used in real-time RT-PCR assays to test if the quality was acceptable for downstream applications. We chose WR11 as candidate to assess its expression in different tissues. Semi-quantitative RT-PCR was performed after extraction of RNA from various tissues. EgWR11 transcripts were detected in mesocarp (young and mature), endosperm and leaf (Figure 2).

Multiple methods have been used to extract RNA from the oil palm tissues, but without any success. The method described here is relatively easy, cheap and capable of isolating RNA efficiently from various oil palm tissues, rich in polyphenols and polysaccharides.

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