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CTAB-Based RNA Extraction with Qiagen RNeasy Cleanup

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We use this protocol and it's working

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Abstract

This protocol details a modified RNA extraction method that combines **CTAB-based** purification with **Qiagen RNeasy column cleanup**, specifically tailored for complex plant tissues like cannabis, which harbor high levels of secondary metabolites and polysaccharides. The CTAB extraction step effectively eliminates contaminants that could impede downstream applications, ensuring high RNA integrity. The following Qiagen cleanup step further improves RNA purity, resulting in high-quality RNA suitable for RT-qPCR, RNA sequencing, and various molecular biology applications. This protocol especially benefits researchers working with medicinal plants, recalcitrant species, or tissues rich in **secondary metabolites**. The comparative table provided in this protocol showcases key optimizations, contrasts this method with previously published techniques, and offers suggested adjustments for further refinements.

	A	B	C	D
	Aspect	PureGene Protocol	Published Protocols	Recommended Adjustments
	Alternative Precipitation Method	Uses Sodium Acetate (NaOAc) precipitation	Some protocols suggest Lithium Chloride (LiCl) precipitation for polysaccharide-rich samples	Consider testing LiCl precipitation if polysaccharide contamination remains
	DNase Treatment for Cleaner RNA	On-Column DNase I treatment (15 min)	Kiss et al. (2024) includes DNase treatment to remove gDNA contamination	Keep DNase treatment (15 min is sufficient)
	RNA Integrity & Yield	Expected yield: 2-91 µg/µl; Air-drying step before dissolving RNA	Kiss et al. (2024) reports RIN of 7.1–8.1 with similar workflow	No major changes needed
	Polysaccharide & Polyphenol Removal	CTAB (2%), PVP-40 (2%), β-mercaptoethanol (2%)	Kiss et al. (2024) uses β-mercaptoethanol (10% v/v)	Increase β-mercaptoethanol to 10% v/v for better secondary metabolite removal
	Phase Separation & RNA Cleanup	Chloroform:Isoamyl Alcohol (24:1) + Qiagen RNeasy Cleanup	Wang & Stegemann (2010) also integrate CTAB with silica column purification	No major changes needed

This comparison reviews the CTAB + Qiagen RNA extraction protocol in relation to established plant RNA extraction methods. It assesses critical factors such as polysaccharide removal, phase separation, RNA integrity, precipitation

techniques, and DNase treatment. Based on insights from the literature, suggested modifications are offered to enhance RNA yield and purity.

Attachments

Kiss et al. - 2024 -... 1.2MB

Wang and Stegemann -... 1.3MB

Liyanage et al. - 20... 1.1MB

Guidelines

- This protocol combines CTAB-based RNA extraction for removing polysaccharides and secondary metabolites with Qiagen RNeasy column purification for high-purity RNA.
- Work quickly but carefully to prevent RNA degradation—keep samples frozen in LN_2 whenever possible.
- Use fresh reagents and pre-warm CTAB buffer to 65°C before starting.
- Maintain an RNase-free environment by cleaning surfaces with RNaseZap and using filtered RNase-free pipette tips.
- Do not overload columns—follow recommended sample volumes to avoid clogging.

Materials

1️⃣ Sample Collection & Preparation:

- Cannabis flowers & scissors
- Weigh boats & precision balance
- Liquid nitrogen (LN_2) & Dewar flask
- Ceramic mortar & pestle
- Cryogenic-safe plastic containers

2️⃣ Safety & Waste Management:

- Fume hood (for handling chloroform & β-mercaptoethanol)
- Chemical waste collection bottle *(HDPE or Glass, 1L or 2L, labeled: "Chloroform:Isoamyl Alcohol Waste – Hazardous Chemical Waste")*
- Biohazard waste bag (for contaminated disposables)
- Nitrile gloves & safety goggles (for working with hazardous chemicals)
- Absorbent spill pads (for chloroform & ethanol spills)
- Flammable Storage Cabinet (100% Ethanol & chloroform:isoamyl alcohol are highly flammable)

3️⃣ RNA Extraction & Phase Separation:

- RNA-Optimized CTAB Extraction Buffer *(see Appendix)*
- Water bath (65°C, with floating tube racks)
- Chloroform:Isoamyl Alcohol (24:1) – Must be used in fume hood
- Sodium acetate (3 M, pH 5.2 for RNA precipitation)
- Lithium chloride (LiCl, 8 M, for RNA precipitation)
- Ethanol 100% & 75% Ethanol

4️⃣ Qiagen RNeasy Cleanup Kit & Buffers:

- Qiagen RNeasy Plant Mini Kit (for Cleanup Step)
- Buffer RLT *(with β-ME added: 10 µL per 1 mL RLT)*

5️⃣ Reagents for CTAB Buffer Preparation:

- CTAB (for RNA extraction buffer preparation)
- PVP-40 *(removes polysaccharides and phenolic compounds)*
- NaCl (2 M, for CTAB buffer)
- Tris-HCl (pH 8.0, 100 mM)
- EDTA (25 mM, for chelation of RNase cofactors)
- Spermidine (stabilizes RNA during extraction)
- β-mercaptoethanol (2%, prevents oxidation of RNA by phenolics)
- RNase-free water

6️⃣ Laboratory Equipment & Consumables:

- Heat block

- -80°C Freezer (For long-term storage of RNA samples)
- -80°C Freezer (For reagents and RNA samples)
- Water bath (65°C)
- Centrifuge *(capable of 12,000 x g, temperature-controlled)*
- Racks for Eppendorf 1.5mL tubes
- Pipettes (P1000, P200, P10)
- RNase-free pipette tips *(P1000, P200, P10, filtered tips)*
- RNaseZap (for surface decontamination of workspace)
- 2mL RNase-free tubes (for sample processing)
- 1.5mL RNase-free tubes (for final RNA storage)

7️⃣ RNA Quality Control & Storage:

- Qubit *(for RNA quantification)*
- *Tapestation (for RNA integrity check)*
- *-80*

Safety warnings

- Chloroform:Isoamyl Alcohol is hazardous—always work inside a fume hood and dispose of waste in a properly labeled chemical waste container.
- β-Mercaptoethanol is toxic and volatile—handle only in a fume hood and wear gloves.
- Ethanol and chloroform are flammable—keep away from open flames and use only in well-ventilated areas.
- Liquid nitrogen (LN_2) can cause severe burns—handle with cryogenic gloves and never seal containers with LN_2 inside.

Before start

Prepare all reagents and equipment:

- Pre-warm CTAB buffer to 65°C in a water bath.
- Set up the centrifuge at 4°C and ensure it can reach 12,000 x g.
- Chill spatulas in LN_2 before handling plant material.

Prepare working space:

- Clean the bench with RNaseZap.
- Set up a fume hood for handling chloroform:isoamyl alcohol and β-mercaptoethanol.
- Label all tubes before starting to avoid confusion.

Ensure correct PPE (Personal Protective Equipment):

- Wear gloves, safety goggles, and a face mask when handling hazardous chemicals.
- Use cryogenic gloves when working with liquid nitrogen (LN_2).

Plan your workflow:

- Keep samples frozen until the extraction step.
- Work quickly to minimize RNA degradation.
- Perform all centrifugation steps at the correct speed and temperature.

Preparation Before Going to the Greenhouse (GH)

1 Set up a portable **analytical balance** in the GH

2 Label **one tube** per cultivar. Use 50 mL Falcon tubes

3 Bring **RNase-free** gloves and sterile tools (scissors, tweezers).

Go to the greenhouse and collect fresh cannabis flowers

4 Cut fresh flowers using **sterile scissors** (one sample at a time)

5 Place the **weighed ~100 mg** sample into a labeled 50 mL Falcon tube. Poke some holes in lid of Falcon tube.

6 **Flash-freeze** the plant material (in 50 mL Falcon tube) immediately in liquid nitrogen.

Tissue Collection & Homogenization

7 Prepare f**resh CTAB** (see Appendix) before harvesting and grinding, keeping it at 65°C. Then, start the centrifuge (4°C).

8 **Pre-chill** spatulas in cryogenic-safe plastic containers with LN_2. Tare the calibrated precision balance before weighing samples.

9 Immediately flash-freeze in liquid nitrogen (LN2). **Using a mortar and pestle**, grind to a fine powder in LN2, **ensuring the sample remains fully frozen** during grinding!

10 Use a **chilled spatula** to transfer the powdered tissue into a **2mL** RNase-free tube (ensure to use a 2mL tube).

RNA Extraction with CTAB

11 Add 1 mL (use the P1000 pipette) of pre-heated CTAB buffer to the 2 mL RNase-free tube containing plant powder.

12 Vortex thoroughly for 1 minute

13 Incubate in a **water bath** at **65°C** for 10 minutes, mixing occasionally (invert the tubes).

14 Add an equal volume (**1mL**) of chloroform:isoamyl alcohol

15 Vortex for **30 seconds**.

16 Incubate for **15 minutes** at room temperature for optimal phase separation

17 Centrifuge at **12,000 x g** for **10** minutes at **4°C**

18 Transfer ~ **500 µL** of the upper aqueous phase (RNA) to a fresh **1.5 mL RNase-free tube** (**do not disturb the interface** between the aqueous vs. organic phase). Use the P200 pipette. The aqueous phase should be clear and colorless to pale yellow

RNA Precipitation: Sodium Acetate (NaOAc) + Ethanol (Standard Method)

19 Add **50 µL** of 3M NaOAc and **1250 µL** of 100% ethanol. **Invert** the tube 5–6 times to mix

20 Incubate at –20°C for 1 hour

21 Centrifuge at 12,000 x g for 20 min at 4°C. The RNA will be visible as a **white pellet** (bottom of the tube)

22 Discard the supernatant by **removing** the liquid slowly to discard the supernatant. Be careful not to touch the pellet with the pipette tip.

23 Wash the pellet with **1 mL** of **75% ethanol**. Invert the tube 5–6 times. The goal is to ensure the **RNA pellet** is well-washed without **disrupting it**. DO NOT VORTEX

24 Centrifuge at 12,000 x g for 5 minutes at 4°C**. Pipette the ethanol out** with a **P200**

25 Repeat Steps 18-19

26 Air-dry the RNA pellet for **10 minutes at RT** to evaporate the residual ethanol

RNA Cleanup with Qiagen RNeasy Plant Mini Kit

27 Pre-warm **400 µL Buffer RLT** (with β-ME **added**) to 37°C in heat block

28 Dissolve RNA pellet in **400 µL** Buffer RLT (with β-ME **added).** Pipette **up & down** gently until fully dissolved (If RNA does not fully dissolve, incubate at **37°C for 5 min** and vortex briefly)

29 Pass the sample through a QIAshredder spin column (lilac) placed in a 2 mL collection tube. Centrifuge at ≥8000 x g for 2 min.

30 Carefully transfer the **cleared lysate (~400 µL flow-through)** to a **new tube**, avoiding any visible pellet at the bottom. **Discard the QIAshredder** column.

31 **Add 200 µL of ethanol (0.5x volume, 96–100%)** to the flow-through. Mix gently by pipetting up and down

32 **Transfer the sample onto a pink RNeasy spin column** (in a new 2 mL collection tube).

33 Centrifuge at **≥8000 x g for 15 sec**. Discard the flow-through.

34 **Add 700 µL Buffer RW1** to the spin column. Centrifuge at **≥8000 x g for 15 sec**. Discard flow-through

35 Prepare **DNase** incubation mix: **10 µL DNase I** *(RNase-free)*+ **70 µL Buffer RDD** *(from Qiagen RNase-Free DNase Set)* + **Mix** gently (pipetting up and down)

36 Apply the **DNase mix (80 µL) directly** onto the membrane of the RNeasy spin column

37 Incubate at room temperature for **15 minutes** to "kill" DNA

38 Add **350 µL Buffer RW1** to the spin column to wash out DNase.

39 Centrifuge at ≥8000 x g for 15 sec. Discard flow-through.

40 **Add 500 µL Buffer RPE.** Centrifuge at **≥8000 x g for 15 sec**. Discard flow-through

41 **Add 500 µL Buffer RPE again.** Centrifuge at **≥8000 x g for 2 min***(removes residual ethanol).*

42 Place the RNeasy spin column in a **new 2 mL collection tube**. Centrifuge at **full speed (≥10,000 rpm) for 1 min** *(ensures ethanol removal).*

43 Transfer the RNeasy spin column to a **new 1.5 mL RNase-free tube**.

44 Add **30 µL o**f pre-warmed RNase-free water **(37°C)** directly onto the membrane. Let it sit for 1 min

45 Centrifuge **at ≥8000 x g for 1 min** to elute RNA.

Protocol references

A Modified CTAB Method for the Extraction of High-Quality RNA from Various Plant Species
* **Summary**: Optimized CTAB protocol for extracting RNA from woody and herbaceous plants.
* **Link**: https://plantmethods.biomedcentral.com/articles/10.1186/s13007-024-01198-z

Extraction of High-Quality RNA from Polysaccharide Matrices Using CTAB
* **Summary**: Explains how CTAB efficiently isolates RNA from polysaccharide-rich plant tissues.
* **Link**: **https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2813910/**

A CTAB Protocol for Obtaining High-Quality Total RNA from Cinnamon
* **Summary**: Optimized CTAB method for RNA extraction from different tissues of cinnamon plants.
* **Link**: **https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8007690/**

NOTE: these 3 PDFs are added in the "Description" section