

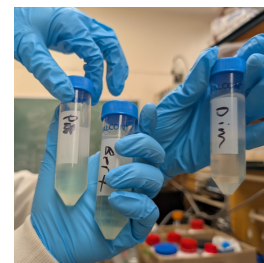
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Version 1

High Molecular Weight DNA Extraction of Pomegranate V.1

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We use this protocol and it's working

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California Dept. of Food and Agriculture -University of Florida: Developing Strategies for Increasing Marketable Yield in California and Florida Pomegranate Orchards
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Abstract

The purpose of this protocol is to share a modified CTAB DNA extraction method that can be generally applied to all plants, but is especially effective in extracting High Molecular Weight (HMW) DNA from plants with high concentrations of secondary plant metabolites. Pomegranates and other plants rich in polyphenolic compounds and polysaccharides, which may have difficult-to-penetrated cell walls, make DNA extraction difficult when the extraction products are meant for long-read sequencing. The most unfortunate scenario resulting from sequencing a plant with high levels of polyphenolics is a partial sequencing run that can be a waste of thousands of dollars. This protocol requires approximately 10 grams of tissue to yield adequate results for HMW extraction.

This protocol typically yields approximately 20-40 µg of DNA at a concentration of at least 100ng to an upwards of 1000 ng/µL with a DIN of at least 6.7.

Attachments



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Guidelines

When grinding the leaves with the mortar and pestle, ensure that you consistently add small amounts of LN2 to the mortar to maintain a cold temperature until the leaf tissue is reduced to a fine powder.

When performing the sorbitol washes, use your judgment to qualify the quantity and quality of your DNA pellet. Some plant species require more washes than others.

Sand in this protocol is used to break rigid cell walls.

DNA is most fragile after cell lysis - if your goal is to keep DNA intact be gentle when handling/agitating the pellet.



Materials

Abbreviations:

NIB: Nuclei Isolation Buffer

SWB: Sorbitol Wash Buffer

CLB: Carlson Lysis Buffer

LN2: Liquid Nitrogen

BME: Beta-mercaptoethanol

PVP: polyvinylpyrrolidone mw 360,000

PCI: 25 parts Phenol: 24 parts Chloroform: 1 part Isoamyl Alcohol

Tissue:

10 g frozen leaves (-80°C) harvested during bud break before sunrise

Buffers:

NIB (Nuclei Isolation Buffer): 200 mL (10 mM Tris pH 9.5, 10 mM EDTA, 100 mM KCl, 500 mM sucrose, 0.0278 mM PVP-360, 4 mM spermidine, 1 mM spermine, 1% BME fresh)

SWB (Sorbitol Wash Buffer): 250 mL (NIB without BME + 0.35 M sorbitol, 1% PVP-360, 1% BME fresh)

CLB (Carlson Lysis Buffer): 200 mL (100 mM Tris pH 9.5, 2% CTAB, 1.4 M NaCl, 1% PEG (Polyethylene glycol) 8000, 20 mM EDTA, 0.25% BME fresh)

TE Buffer: 400 µL (10 mM Tris pH 8.0, 1 mM EDTA)

Reagents:

Liquid Nitrogen (LN2)

BME (Beta-mercaptoethanol): 2 mL (NIB), 2.5 mL (SWB), 500 µL (Carlson)

RNase A: 400 µL (10 mg/mL)

PCI (Phenol:Chloroform:Isoamyl Alcohol at ratio 25:24:1, v/v)

Isopropanol: ~140 mL (Ice cold)

70% Ethanol: 400 mL (Ice cold)

3M Sodium Acetate (NaOAc)

Equipment:

- 1-4 mortars/pestles (autoclaved and pre-cooled in -80)
- 4-8 × 50 mL Falcon tubes
- Refrigerated centrifuge (50 mL capacity)
- Water bath (65°C)
- Dry ice
- Ice bath
- Autoclaved Kimwipes
- Autoclaved Glass rods or metal spatulas
- 50 mL tube rack
- P1000 / p5000 (or equivalent to transfer supernatant - see attached photos) / p20 / p10 (for ethanol cleanup and Nanodrop)

- Autoclaved sand in glass petri dish covered in foil

Safety warnings

- ! **Tris(hydroxymethyl)aminomethane hydrochloride:** This chemical is not considered hazardous
- Ethylenediaminetetraacetic Acid (EDTA):** Causes serious eye irritation, harmful if inhaled, may cause damage to organs through prolonged or repeated exposure
- Potassium Chloride (KCl):** May be harmful if swallowed, causes serious eye irritation, causes skin irritation, toxic to terrestrial vertebrates; considered hazardous.
- Sucrose:** This chemical is not considered hazardous
- Polyvinylpyrrolidone (PVP-360):** May be harmful by inhalation, ingestion, or skin absorption, may cause eye irritation, may be irritating to mucous membranes and upper respiratory tract
- Spermidine:** Causes severe skin burns and eye damage
- Spermine:** Causes severe skin burns and eye damage, may cause respiratory irritation
- Beta-mercaptoethanol (BME):** Combustible liquid, fatal in contact with skin, causes skin irritation, may cause an allergic skin reaction, causes severe eye damage, suspected of damaging fertility, may cause damage to organs through prolonged or repeated exposure, toxic if swallowed or inhaled
- Sorbitol:** This chemical is not considered hazardous
- Cetyltrimethylammonium Bromide (CTAB):** Harmful if swallowed, causes skin irritation, causes severe eye damage, may cause respiratory irritation, very toxic to aquatic life
- Sodium Chloride (NaCl):** This chemical is not considered hazardous
- PEG (Polyethylene glycol) 8000:** This chemical is not regarded as hazardous
- Liquid Nitrogen (LN2):** May cause cryogenic burns or injury
- Chloroform/isoamyl alcohol 24:1:** Harmful if swallowed, causes skin irritation, causes severe eye irritation, toxic if inhaled, may cause respiratory irritation, may cause drowsiness or dizziness, may cause cancer, suspected of damaging the unborn child, causes damage to organs through prolonged exposure
- RNase A:** May cause allergy or asthma symptoms or breathing difficulties if inhaled
- Isopropanol:** highly flammable liquid and vapor, causes severe eye irritation, may cause drowsiness or dizziness
- 70% Ethanol:** Highly flammable liquid and vapor, toxic if swallowed, may cause drowsiness or dizziness, may damage fertility, may cause damage to organs through prolonged or repeated exposure
- Hydrochloric acid (HCl):** corrosive to metals, skin corrosion/irritation, severe eye damage/eye irritation, specific target organ toxicity



Before start

Before starting the protocol, autoclave all equipment and reagents for buffers as mentioned (AUTOCLAVE). Liquids are to be thoroughly sanitized at a liquid phase for 20 minutes in an autoclave (L20). Additionally, have excess dry ice (0.5 lbs/prep) and approximately 4L of liquid nitrogen (a full medium dewar) readily available on the day of DNA processing. Wear personal protective equipment correctly, including a lab coat, properly sized gloves, and safety goggles. When obtaining liquid nitrogen, a face shield is necessary to dispense. Prepare the buffers a day in advance of your protocol, as compounds in the buffers require time to homogenize and need to be stirred overnight before use. Prepare autoclaved material a day or two in advance to properly sterilize and cool for the protocol. Make sure to cool the mortars and pestles - tissue and buffer will stick to components otherwise.

Buffer Preparation

1 **Nuclei Isolation Buffer (NIB) - 200 mL**

Limits chemical interactions

- 1.1 Begin by autoclaving all heat-resistant liquid reagents at a liquid phase for 20 minutes (L20)
- 1.2 The base is created by adding 2 mL of 1 M Tris pH 9.5, 4 mL of 0.5 M EDTA, and 20 mL of 1 M KCl. Then, add water to the 200 mL mark. If particulate matter is present (dust, microplastics) filter the solution. Add a magnetic stir bar.
- 1.3 Let the solution mix overnight on an automatic stirrer
- 1.4 Autoclave, and then after autoclaving, add 2 grams of PVP-360, 0.116 g. of spermidine, 0.040 g. of spermine, and 34.23 g. of sucrose.
- 1.5 On the day of use, add 2 mL of BME.

2 **Sorbitol Wash Buffer (SWB) - 250 mL**

Efficiently washes leaf tissue of extreme impurities.

- 2.1 Begin by autoclaving all heat-resistant liquid reagents in a liquid phase for 20 minutes (L20)
- 2.2 The base is created by adding 2.5 mL of 1 M Tris pH 9.5, 5 mL of 0.5 M EDTA, and 25 mL of 1 M KCl. Then, add water to the 250 mL mark. (Note this is similar to NIB) If particulate matter is present filter the solution. Add a magnetic stir bar.
- 2.3 Autoclave, and then after autoclaving, add 16 g. of sorbitol, 2.5 g. of PVP-360, and 42.79 g. of sucrose.
- 3 Let the solution mix overnight on an automatic stirrer
- 3.1 On day of use, add 2.5 mL of BME.

4 **Carlson Lysis Buffer - 200 mL**



Breaks open cellular membranes to release DNA.

- 4.1 Begin by autoclaving all heat-resistant liquid reagents in a liquid phase for 20 minutes (L20)
- 4.2 Create the base by adding 20 mL of 1 M Tris pH 9.5, 4 mL of 0.5 M EDTA, 56 mL of 5 M NaCl, 4 g. of CTAB, and water to the 200 mL mark. Add a magnetic stirrer. Autoclave at L20, filter if needed, and then add 2 g. of PEG 8000.
- 5 Let the solution mix overnight on an automatic stirrer.
- 5.1 On the day of use, combine with 500 μ L of BME and add 12 μ L of RNase A diluted to 100 μ g/mL in resuspension buffer (50 mM Tris·Cl, pH 8.0; 10 mM EDTA)
- 6 **TE/EB Buffer - 100 mL**
Final solution to store DNA
- 6.1 Begin by autoclaving all heat-resistant liquid reagents in a liquid phase for 20 minutes (L20)
- 6.2 [TE] Create the base by adding 1 mL of 1 M Tris pH 9.5, 0.2 mL of 0.5 M EDTA, water to the 100 mL mark, and pH to 8.0 with HCl.
- 7 [EB] 10 mM TRIS pH 8.5

Tool Preparation

1h 30m

- 8 Autoclave mortars, pestles, kimwipes in a foil packet, sand, glass rods or metal spatulas, autoclave 5 mL and 1 mL tips if needed all at a gaseous phase G30 on the autoclave.
- 9 Place 4 autoclaved mortars/pestles at room temp and then at -80°C (for at least 30 min; overnight preferred).

Sample Preparation

- 10 Extraction can be completed within a day, typically in six hours. Budget for this as you start this section.



- 11 Split 10 g into approximate 4×2.5 g batches or just grind half a 50 mL tube in a larger pestle with about 1 gram of sand for each prep.
- 12 For each mortar: Add ~2.5 g of flash frozen tissue in liquid nitrogen (LN2)
- 13 Add 1 g of sand to each mortar
- 14 Grind tissue, sand, and LN2 until a light green powder develops
- 15 Once the LN2 begins to dissipate add a small amount of NIB ~10 mL straight into the mortar and crush the NIB and tissue amalgamation into a fine powder once again. These cold conditions arrest redox reactions in this process.
- 16 Pour light green powder into 50 mL tubes with an autoclaved spatula or sterile tool.

Nuclei Isolation and Wash

2h

- 17 Add enough NIB (with BME) to even out each tube to the same mL level, swirl to homogenize (can be 15 min total); this helps to melt any remaining ice crystals. (You may have between 20-25 mL at this step)
- 18 Centrifuge the tubes at a rate of 4000 g, at 4°C, and for 10 minutes. Remove supernatant
- 19 Add about 10 mL of SWB to each tube and invert the tubes to mix pellet and SWB.
- 20 Centrifuge the tubes at a rate of 4000 g, at 4°C, and for 10 minutes. Remove supernatant
- 21 Repeat the SWB step (17) to further remove tissue and remove supernatant.
- 22 If tissue (and DNA) is limiting, discard the supernatants into a collection tube.



Lysis

1h 30m

- 23 Add 20 mL Carlson lysis buffer (with BME) to each pellet; invert to homogenize.
- 24 Incubate 55-65°C, 1 hr, invert GENTLY after 30 min and add 12 µL of RNase A per tube if you have between 20-25 mL of CLB in the tube - if you have more adjust to the ratio. The DNA is at its most fragile state at this step.

Phase Separation

1h

- 25 Cool to room temp (~10 min). This is required! Do not skip this step.
- 26 Add 20 mL of Phenol:Chloroform:Isoamyl (PCI) , shake gently to incorporate - 20x inversions ok, centrifuge 4000g, 4°C, 15 min.
- 27 Pool upper aqueous layers **of the same variety** into new tubes while avoiding the interphase layer of the PCI.
- 28 Repeat the PCI cleanup if any of the product thus far prepared is brown (oxidized)

DNA Precipitation

1h

- 29 Add ~14-20 mL of freshly prepared and ice cold isopropanol (0.7x volume) to ~15-20mL of product, invert 10x.
- 30 Add 1 mL of 3M NaOAc and incubate in -20°C for 1 hour.
- 31 Centrifuge at a rate of 10000g, at 4°C, and for 45 minutes.

Pellet Wash

1h

- 32 Discard supernatant, add 20 mL of freshly prepared and ice-cold 80% ethanol, invert 10x, centrifuge at a rate of 5000 g, at 4°C, and for 15 minutes.
- 33 Repeat ethanol wash.



- 34 Air dry pellet(s) on an autoclaved kimwipe for 5-10 minutes.

Final Resuspension

7h

- 35 Resuspend in 200 μ L of TE or EB (overnight preferred) and then perform quality control.

30m

Quality Control

2h

- 36 Nanodrop to measure 260/280 and 260/230 ratio
- 260/280 ratio around 1.8-2.0
 - 260/230 ratio between 2.0-2.2
- 37 Qubit 4 Fluorometer to measure DNA concentration:
- 38 Add 199 μ L of Qubit Kit Buffer to two 0.5 mL tubes and an additional tube for each DNA sample, which will serve as the working solution tubes.
- 39 Add 1 μ L ds DNA HS to all tubes, then vortex mix tubes for 2-3 seconds.
- 40 Transfer 190 μ L from each of the two working solution tubes to two new 0.5 mL tubes, which will serve as the standard solutions.
- 41 Add 10 μ L of Standard 1 into one of the standard solution tubes, now labeled S1, and 10 μ L of Standard 2 into the other standard solution tube, now labeled S2, then vortex mix 2-3 seconds.
- 42 Add 199 μ L from each of the other working solutions, designated for the DNA samples, to new tubes.
- 43 Add 1 μ L of the corresponding DNA sample to its designated tube, then vortex mix 2-3 seconds.
- 44 Incubate all standard and DNA sample tubes for 2 minutes at room temperature.



- 45 Calibrate Qubit 4 Fluorometer by selecting ds DNA HS and inserting Standard 1, then Standard 2. (There should be a graph with an increasing line)
- 46 After calibration, insert a DNA sample and press "Read tube", then record the concentration in ng/μL.
- 47 Repeat for each DNA sample.

Expected Outcome

- 48
 - **Yield:** ~20 μg
 - **Volume:** 200 μL
 - **Concentration:** 100 ng/μL (>100 ng/μL target)

Notes

- 49
 - **Check:** Measure concentration (Qubit/Nanodrop) post-resuspension.
 - Measure QC ratios with Nanodrop first
 - Once Nanodrop QC is adequate measure concentration with Qubit or send through tapestation

Protocol references

Publications and links that inspired the protocol herein

Cheng, YJ., Guo, WW., Yi, HL. et al. An efficient protocol for genomic DNA extraction from Citrus species. Plant Mol Biol Rep 21, 177–178 (2003). <https://doi.org/10.1007/BF02774246>

High molecular weight DNA isolation method from diverse plant species for use with Oxford Nanopore sequencing. Brieanne Vaillancourt, C. Robin Buell bioRxiv 783159; doi: <https://doi.org/10.1101/783159>

<https://nanoporetech.com/document/extraction-method/fever-tree-gdna>

<https://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Guidelines-for-Using-a-Salt-Chloroform-Wash-to-Clean-Up-gDNA.pdf>

Byeong-Ju Lee, Sohyeon Kim, Jei-Wan Lee, Hyun-Mi Lee, Soo Hyung Eo, Technical note: Polyvinylpyrrolidone (PVP) and proteinase-K improve the efficiency of DNA extraction from Japanese larch wood and PCR success rate, Forensic Science International, Volume 328, 2021, 111005, ISSN 0379-0738, <https://doi.org/10.1016/j.forsciint.2021.111005>.

Links to Chemicals Used:

- Tris <https://www.fishersci.com/store/msds?partNumber=BP1531&productDescription=TRIS+HYDROCHLORIDE+1KG&vendorId=VN00033897&countryCode=US&language=en>
- KCl https://www.fishersci.ca/content/dam/fishersci/en_US/documents/programs/education/regulatory-documents/sds/chemicals/chemicals-p/S25484A.pdf
<https://coopersurgical.marketport.net/MarketingZone/MZDirect/Source/e68b4128-798e-48f7-902b-ab45852ff1ac>
- PVP-360 https://www.fishersci.ca/content/dam/fishersci/en_US/documents/programs/education/regulatory-documents/sds/chemicals/chemicals-p/S25484A.pdf
- BME <https://www.fishersci.com/store/msds?partNumber=O34461100&productDescription=2-MERCAPTOETHANOL+R+100ML+IND&vendorId=VN00033897&countryCode=US&language=en>
- NaCl <https://www.fishersci.com/store/msds?partNumber=S640500&productDescription=SODIUM+CHLORIDE+USP%2FFCC+500G&vendorId=VN00033897&countryCode=US&language=en>
- CTAB [https://www.columbuschemical.com/MSDS/SDS/Cetyltrimethylammonium%20Bromide%20\(CTAB\)%201320.pdf](https://www.columbuschemical.com/MSDS/SDS/Cetyltrimethylammonium%20Bromide%20(CTAB)%201320.pdf)
- PEG <https://www.fishersci.com/store/msds?partNumber=BP233100&productDescription=PEG+8000+100G&vendorId=VN00033897&countryCode=US&language=en>
- LN2 https://static.prd.echannel.linde.com/wcsstore/HK_RGC_Industrial_Ntl_Store/pdf/product/en_HK/Nitrogen_liquid.pdf

- RNase <https://www.qiagen.com/binary/resource/sds/0011/800000000011-en-GB-KR--1.pdf>
- 25:24:1 Phenol:Chloroform:Isoamyl Alcohol https://www.thermofisher.com/order/catalog/product/15593031?ef_id=CjwKCAjwlaTGBhANEiwAoRgXBSwYOLM8ohcInni5EW_z3q1Qf7kkvaBPiszffDkeE2u7AIFf0QvWohoC5fMQAvD_BwE:G:s&s_kwcid=AL!3652!3!606515189508!!!g!!!16893189581!143415109412&cid=bid_sap_rst_r01_co_cp1362_pjt0000_bid00000_0se_gaw_dy_awa_con&gad_source=1&gad_campaignid=16893189581&gbraid=0AAAAADxi_GSm11NPKMk0Yw7UTusz2ZYBd&gclid=CjwKCAjwlaTGBhANEiwAoRgXBSwYOLM8ohcInni5EW_z3q1Qf7kkvaBPiszffDkeE2u7AIFf0QvWohoC5fMQAvD_BwE
- Isopropanol https://www.fishersci.com/content/dam/fishersci/en_US/documents/programs/education/regulatory-documents/sds/chemicals/chemicals-i/S25372A.pdf
- Spermine <https://www.fishersci.com/store/msds?partNumber=AAL1956203&productDescription=SPERMINE+97%25+1G&vendorId=VN00024248&countryCode=US&language=en>
- HCl <https://www.fishersci.com/store/msds?partNumber=A481212&productDescription=HYDROCHLORIC+ACID+NF%2FFCC+21%2F2L&vendorId=VN00033897&countryCode=US&language=en>
- Spermidine <https://www.fishersci.com/store/msds?partNumber=AC132740250&countryCode=US&language=en>

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