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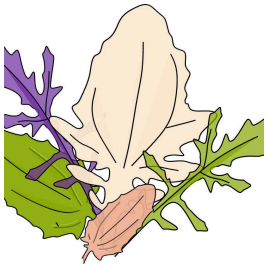
High-Molecular-Weight SPRI-aided DNA extraction from Mimulus (Phrymaceae) leaf tissue V.2



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We use this protocol and it's working

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Disclaimer

Abstract

This protocol is adapted from **Russo et al. 2023** methods for high-molecular weight DNA extraction. I modified the protocol to use reagents and incubation conditions used by the Ferris Lab at Tulane University to extract DNA from *Mimulus* spp. (monkeyflower) tissue (leaf and buds), see protocol. The original CTAB:Chloroform protocol used by the Ferris lab traces back to the Willis Lab at Duke University. The objective of these DNA extractions are to collect fungal DNA present in leaf tissue. I have **incorporated sample preparation procedures used by the Arnold Lab** at The University of Arizona to eliminate contamination of samples and optimize DNA extractions from preserved leaf tissue for future fungal ITS Illumina MiSeq sequencing. Solid phase reversible immobilization beads (SPRI) utilized are adapted from **Rohland and Reich 2012** and **Liu et al 2023**. All extractions were performed un a sterile biosafety cabinet to prevent sample contamination.

Prior to being placed in CTAB, photosynthetic tissues from plants were surface sterilized with sequential washes in 95% EtOH (10 s) , 0.5% sodium hypochlorite (1 min), and 70% EtOH (1min) and air dried under sterile conditions. Due to the small size of monkeyflower plants, the maximum amount of leaf tissue produced per host were placed in 750- 1000 µL of CTAB buffer and kept at RT until DNA extraction process.

*Several aspects of this protocol, mainly those with reagent amounts and molarities, have changed compared to the Russo et al 2023 protocol and the Willis Lab standard CTAB extraction protocol from ca. 2010.

Guidelines

General Notes

DNA Extractions for Illumina Seq. are the most sensitive. If you need high quality low contamination DNA that is high molecular weight, make sure to use the gentler methods of mixing. Use wide bore pipet tips to help reduce shearing of DNA. Do not vortex any samples to mix, just invert or gently tap/flick tubes. To reduce contamination, be sure to check that all samples come out of the first chloroform step with a clear aqueous layer, not cloudy. Sodium chloride (5M NaCl) helps reduce polysaccharide contamination (compared to the Isopropanol precipitation without the added NaCl). High salt concentrations also aid in DNA precipitation, so you should get more DNA precipited and cleaned with chloroform wash.

Sample Handling

1. Gloves must be worn at all times. Change gloves frequently and decontaminate often with DNA Away.
2. Pipetting must be done extremely carefully to minimize the risk of aerosols that can easily cause contamination between samples.
3. Use only unopened, sterile, aerosol-resistant pipette tips (filter tips) to minimize contamination of the pipette shaft and your samples. Clean the body of the pipettes regularly with DNA Away.
4. Discard pipette tips after each use to avoid cross-contamination. Eject pipette tips carefully to prevent aerosol formation or other splashing.
5. Remember to always clean the hood and any equipment with DNA Away and decontaminate where possible with UV for 30 minutes prior to use to avoid cross-contamination.
6. The pipettes used need to be calibrated annually.
7. Be careful not to touch the inside lid of tubes as it will cross-contaminate samples.

Materials

Equipment

- Geno Grinder (model 2010)
- TissueLyzer LT
- Centrifuge capable of 24 tubes, 10,000 rpm or 96 well-plate at 4,000 rpm
- Class II Biosafety Cabinet
- Stainless stee; beads 3.2mm **Next Advance Catalog #**SSB32
- Stainless stell bead blend 0.9-2mm **Next Advance Catalog #**SSB14B
- Ziconium oxide beads **Next Advance Catalog #**ZROB20-RNA
- Liquid Nitrogen
- Freeze Dryer/Lyophilizer (able to achieve -40°C)
- Water Bath (that can be set to 60°C)
- Benchtop shaking incubator (Labnet 222DS)
- Heating block (able to set at 50°C)
- Eppendorf or Rainin Pipettes: 1000 µL, 200 µL, and 20 µL
- 1.5/2ml tube magnetic rack.

Reagents and consumables

- 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250
- Polyvinylpyrrolidone (PVP-40) **VWR International (Avantor) Catalog #**0507
- 5M NaCl solution (Make ahead and autoclave if there isn't any in the cabinet)
- 10 mM Tris-HCL pH 8.0 or

1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA)

- 95% Ethanol
- 80% Ethanol (frozen at -20°C)
- DNase Away
- 10 % sodium hypochlorite solution (Bleach)
- CTAB buffer **OPS Diagnostics Catalog #**CEB 500-02
- Pipette tips: 1000 µL (×4), 200 µL (×2), and 20 µL (×1)
- SPRI-beads (homemade, see protocol).
- Molecular grade water (100 mL)
- Screw cap micro tubes 2 mL **Thermo Scientific Catalog #**3469NK
- Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #**022431048

Protocol materials

- Polyvinylpyrrolidone (PVP-40) **VWR International (Avantor) Catalog #**0507
- 1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA)
- CTAB buffer **OPS Diagnostics Catalog #**CEB 500-02
- Screw cap micro tubes 2 mL **Thermo Scientific Catalog #**3469NK
- Stainless stell bead blend 0.9-2mm **Next Advance Catalog #**SSB14B
- 10 mM Tris-HCL pH 8.0
- 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250
- Polyvinylpyrrolidone (PVP-40) **VWR International (Avantor) Catalog #**0507
- Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #**022431048
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- Polyvinylpyrrolidone (PVP-40) **VWR International (Avantor) Catalog #**0507
- 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250
- CTAB buffer **OPS Diagnostics Catalog #**CEB 500-02
- RNase A 10mg/ml, DNase and Protease-free **Thermo Scientific Catalog #**EN0531
- Sodium Chloride **Fisher Scientific Catalog #**S271
- Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #**022431048
- Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #**022431048
- 1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA)
- 10 mM Tris-HCL pH 8.0
- SeraMag SpeedBeads Carboxyl-Magnet-Beads hydrophob **Fisher Scientific Catalog #**GE Healthcare 65152105050250
- Polyethylene glycol (PEG-8000) **Fisher Scientific Catalog #**FLBP2331
- EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E9884
- Polysorbate 20 (Tween-20) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416

Fresh 80% Ethanol

1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA)

10 mM Tris-HCL pH 8.0

Troubleshooting

Safety warnings

Careful handling all reagents, specially CTAB and Chlorofom:IAA

Before start

Make sure to...

- Turn on the water bath to 60°C and heating block to 50°C.
- Get liquid nitrogen.
- Get sample tubes/plates from the -80°C or 20°C freezer.
- Keep samples frozen! (On dry ice).

If and only if...

You have a fresh homemade CTAB buffer that **does not** have **polyvinylpyrrolidone**and **β-mercaptoethanol** will you add
these reagents to stock buffer.

Instructions

- Right before use of CTAB buffer; prior to starting extraction, add polyvinylpyrrolidone and β-mercaptoethanol.
- Dissolve 1.1 – 2.2 grams of Polyvinyl-Pyrrolidone (PVP) **per each 50-100ml CTAB** (1.1 – 2.2% of the total buffer vol.).

Once these have been added the shelf life of the buffer is only 2-3 days.

- If tissue is in homemade CTAB buffer and no freeze drying/lyophilization was performed (jumped directly to DNA extraction) you can add 2.25 µL of β-mercaptoethanol per 700 µL L CTAB, as well as 8 -16 µL of Polyvinylpyrrolidone (PVP-40) **VWR International (Avantor) Catalog #**0507 (1.1-2.2 % solution) per 700 µL CTAB.

DO EXTRACTIONS IN THE BIOSAFETY CABINET (except for grinding, and spinning steps).

Sample preparation

1 Clean the biosafety cabinet, pipettes, tip boxes and any other instrument to be used with 70% EtOH, 10 % Bleach and 95 % EtOH. Surface sterilize all instruments in biosafety cabinet with UV light for 00:30:00 . 30m

2 Transfer 0.15-0.3 grams of leaf tissue to new 2 mL tube. Leaving remaining leaf tissue in original 15 mL vial tube.

Note

This step depends on how easy to handle your plant tissue is. Fresh tissue is easier to handle. Mimulus spp. tissue preserved in CTAB turns "mushy" and it is difficult to handle and transfer.

Screw cap micro tubes 2 mL **Thermo Scientific Catalog #**3469NK

2.1 Weigh empty 2 mL tube to determine how much tissue you need to place in tube.

Screw cap micro tubes 2 mL **Thermo Scientific Catalog #**3469NK

2.2 Weigh 2 mL tube with leaf tissue to determine if it has the correct amount.

Note

Samples with more tissue (> 0.3 <0.6) can be split into two tubes.

3 Quickly after placing tissue in tubes, place tube cap and close tightly.

Note

Don’t let samples sit at room temperature for very long because the DNA will degrade.

4 Place tubes in the -20 °C or -80 °C freezer. Until ready for freeze drying/lyophilization step.

Freeze drying/Lyophilization 2d 1h

5 Once all your samples are cold, turn on the refrigerator for the lyophilizer and place the glass plate on the front.

Note

Make sure there is no water in the chamber or drain tube.

6 Once the lyophilizer temperature is at -40 °C, turn on the vacuum pump. 1h

Note

Vacuum pressure must be below 150 milliTorr. If pressure doesn't drop within the hour do not use machine.

7 When the pressure is at the appropriate level, release the pressure by turning the white port 180 degrees.

Note

Make sure there is a vacuum seal, and the pressure returns to <150 milliTorr.

7.1 Quickly take frozen tubes out of the freezer and place inside the lyophilizer chamber. Close the lid and close the port.

8 Lyophilize samples for a minimum of 48:00:00. 2d

Note

To remove the samples, first turn the white port on the lyophilizer 180 degrees to release the pressure in the chamber. Once the pressure is released and the gauge reads 0, turn off the vacuum pump and then the refrigerator. Remove the glass plate from the front and wipe out the water after it has melted (also shake out the drain tube).

Pulverization

9 In a sterile laminar flow hood, remove the parafilm or lid from the tubes (being careful not to touch the inside lip of the cap) and add beads for homogenization. For all plant samples except mosses, add the following:

Note

The pulverization/homogenization of tissue can be performed beforehand and samples stored away until DNA extraction. I prefer to pulverize all my samples, store them and work my way through the DNA extraction.

9.1 Add 100 µL (2 beads) of the autoclaved 3.2 mm stainless steel beads.
Stainless stee; beads 3.2mm **Next Advance Catalog #**SSB32

Note

Beads can also be added at the same time sample tissue is weighed and transffered to 2 mL tubes. Organize your workflow as you see fit.

9.2 Add 2-3 of the autoclaved 2 mm zirconium oxide beads to each tube with a sterile scoop. Stainless stell bead blend 0.9-2mm **Next Advance Catalog #**SSB14B

9.3 Add 100 µL of the autoclaved stainless steel bead blend, (0.9-2.0mm) using a sterile scoop. **Close the lids securely.**
Ziconium oxide beads **Next Advance Catalog #**ZROB20-RNA

10 Tubes with beads can be placed in a -20 °C freezer until ready to extract DNA.

11 11. Dip tubes/96-well plates in liquid nitrogen.

Note

a) Make sure there are no cracks in the plastic and that the plate has its bottom panel to keep the tubes and racks from shattering.
b) Clamp plates into shaker rack in Geno Grinder 2010 or TissueLyser LT. Always clamp plates into both shaker racks and make sure tubes are evenly distributed across plates.

12 12. Shake at **1500 strokes/minute** for 3 minutes in GenoGrinder 2010 or **25-30 Hz** for 3 minutes if using TissueLyzer LT.

Equipment

GenoGrinder 2010

NAME

SPEX-SamplePrep

BRAND

SP 2010-115

SKU

https://www.spexsampleprep.com/2010genogrinder

LINK

High-throughput plant & animal tissue homogenizer and cell lyser.

SPECIFICATIONS

Equipment

Tissuelyser LT

NAME

Qiagen

BRAND

85600

SKU

https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/instruments-equipment/tissuelyser-lt?catno=85600

LINK

Tissue and cell lyser

SPECIFICATIONS

Note

a) **Decrease or increase time as necessary.**
b) Check periodically and do not grind for longer than necessary. Do not let tissue thaw, if you notice melting, re-dip in liquid N2.
c) When tissue is ground to a fine powder, dip briefly into liquid nitrogen again to keep frozen.
d) Examine tubes carefully to ensure proper pulverization. **Repeat if necessary.**

13 Centrifuge plate fast and briefly at 4000 rpm when using 96-well plate centrifuge or 10.000 rpm when using Corning LSE centrifuge to get powder off lids.

Note

Anywhere from 1-3 minutes depending on how much the sample adheres to the lid of the tube.

14 Store in -20 °C or -80 °C until DNA extraction steps.

DNA Extraction

30m

15 Surface sterilize all workbench surfaces and instruments with a 10% solution of 0.5% sodium hypochlorite, 70% EtOH and 95% EtOH prior to placement in biosafety cabinet. Surface sterilize all instruments in biosafety cabinet with UV light for 00:30:00 .

30m

16 **Make Extraction Master Mix** (Pre-warm to 60**°**C):
Measure out 700 µL of CTAB extraction buffer per sample into 15-50 mL conical centrifuge tube. Add 2.25 µL β-mercaptoethanol per 700 µL of CTAB extraction buffer to the same conical tube (0.3% of the total extraction buffer volume).

Basic recipe:
[(# of samples x 1.1) x **700** µL of CTAB] + [(# of samples + 1) x **2.25** µL β-mercaptoethanol]

Note

See **Guidelines and Warning** for more information.

2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250
CTAB buffer **OPS Diagnostics Catalog #**CEB 500-02
Polyvinylpyrrolidone (PVP-40) **VWR International (Avantor) Catalog #**0507

17 Add 700 µL of preheated Extraction Master Mix to each tube, replace the lids (if using strip tubes).

Note

a) Shake tubes by hand vigorously until tissue is thawed and fully suspended in the buffer. Dip plate or tubes in hot water bath to assist the thaw if needed.
b) If fine tissue powder is collected at the bottom and it does not mix well with CTAB buffer, then place in Vortex 5-30 seconds (depending on tissue) or TissueLyser LT for 1 minute at 15 Hz.
Beware of DNA shearing.

Note

Always make sure to include an extraction blank (negative control).

18 Incubate tubes for 00:20:00 in a 60 °C water bath. 20m

Note

Important: Invert tubes/plates gently about 10 times every ~10 minutes during incubation.
If using strip tubes: Keep costar plate lid off during water bath and grinding step (prior) and refasten strip tube caps before inverting – they will pop off! Keep fingers firmly on strip tube lids while inverting.

19 **After 20 minutes** incubation in step 18, **add** 4 µL **of RNase A**.

RNase A 10mg/ml, DNase and Protease-free **Thermo Scientific Catalog #**EN0531

20 Incubate at 60 °C for 00:10:00 . 10m

Note

Invert 10 times once during incubation.

21 All 200 µL of 5M NaCl (or 5M Sodium/Potassium acetate). To fully precipitate proteins and polysaccharides in extraction buffer.

Note

Slowly pipette up and down or invert 25 times.

Sodium Chloride **Fisher Scientific** **Catalog** #S271

22 Add 900 µL of chloroform/isoamyl (24:1) (1 volume) to each tube.

23 Place tubes in a nutating mixer for 00:10:00 at 24 rpm.

10m

Note

a) If possible, use instruments that can produce 10-20 rpms. **Less shaking = less shearing.**
b) Can invert by hand 20 - 40 times, instead of shaking, to further decrease shear. *Wear extra thick nitrile gloves to prevent chloroform exposure to skin. If you get chloroform on your gloves, remove them as soon as possible and discard them in the solid hazardous waste bag in the hood!*

Equipment

S0500 Mini Nutating Mixer	NAME
VWR	BRAND
82007-202	SKU
https://us.vwr.com/store/product/4787436/null	LINK
Mixer	SPECIFICATIONS

24 Centrifuge for 00:10:00 at 4000rpm when 96-well plate centrifuge or 00:10:00 at 10.000 rpm when using Corning LSE centrifuge. 20m

Note

A band of tissue debris will separate the aqueous (upper) and chloroform/organic (lower) layers. The top aqueous layer should be clear, not cloudy. If cloudy, go back and re-do steps 23 and 24.

25 **Carefully pipette off ~** 800 µL **-** 900 µL **of the top aqueous layer** using filter pipet tips and
transfer to sterile new tubes or 96-well plate.

Note

Avoid drawing up debris or chloroform from the middle and lower layers (if so, re-centrifuge briefly and transfer again).

Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #**022431048

26 Repeat steps 22-24 go to step #22 .

Note

This will remove any leftover RNase and contaminants.

27 **After repeating steps 22-24.** Carefully pipette ~ 700 µL - 800 µL of the top aqueous layer using *wide-bore* filter pipet tips and transfer to sterile new tubes or 96-well plate.

Note

a) Avoid drawing up debris or chloroform from the middle and lower layers (if so, re-centrifuge briefly and transfer again).
b) You can cut 3-4 mm of P1000 tips to make yourself "wide-bore" tips. Do this beforehand in a sterile environment or autoclave tips afterwards.

Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #**022431048

Note

Feel free to pause and get yourself ready for DNA purification. DNA is stable at this stage and can be left at room temperature while you prepare.

SPRI aided isolation and purification of DNA

45m

28 Turn ON heating block to 50 °C and place 10 mM Tris-HCl or 1X TE buffer to pre-heat.

1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA) 10 mM Tris-HCL pH 8.0

29 Add SPRI bead mixture according to the following:
Volume of SPRI beads to add = 1.2 × DNA volume (from step 27)

Note

Adjust the ratio of SPRI beads as you see fit. **Russo et al 2023** use 0.7x. See **Rohland and Reich 2013** and **Liu et a 2023** for SPRI protocol and

	A	B	C	D
	Final concentration for a 1.25% SPRI bead solution for use in DNA extraction process.			
	Reagent	10 mL	50 mL	Final Concentration
	Sera-Mag Speed Beads	125 µL	625 µL	1.25%

	A	B	C	D
	PEG-8000	2.5 g	12.5 g	25%
	5 M NaCl	1000 µL	25 mL	2.5 M
	1 M Tris-HCl (pH 8.00	100 µL	500 µL	10 mM
	0.5 M EDTA (pH 8.00	20 µL	100 µL	1 mM
	10% Polysorbate 20 (Tween 20)	5.5 mL	275 mL	10 %
	Nuclease free H2O	fill to 10 mL	fill to 50 mL	n/a

SeraMag SpeedBeads Carboxyl-Magnet-Beads hydrophob **Fisher Scientific Catalog #**GE Healthcare 65152105050250

Polyethylene glycol (PEG-8000) **Fisher Scientific Catalog #**FLBP2331

EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E9884

Polysorbate 20 (Tween-20) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416

30 Incubate for 00:30:00 in a nutating mixer at 24 rpms (default). 30m

31 Spin down for 00:00:05 on centrifuge. 5s

32 Place the tubes on a magnetic rack for 00:05:00 or wait until the solution is clear. 5m

Note

This can take several minutes depending on the viscosity and type of sample. Wait until the solution is clear before proceeding to the next step.

33 Aspirate the cleared solution from the tubes, inspect pipette tips for bead residue, and discard.

Note

This step must me performed while the tubes are placed on the magnetic rack. Avoid disturbing the settle magnetic beads. If beads are drawn into tips, leave behind a few microliters of solution..

34 Dispense 1 mL of **fresh 80% ethanol** to each tube and invert 25 times to resuspend bead pellet.

Note

Flick tubes gently to help separate the beads from the tube wall. Avoid prolonged incubation in EtOH.

Fresh 80% Ethanol

35 Spin down for **1-2 s** on centrifuge. 2s

36 Place tubes on **magnetic rack**.

37 Aspirate out the ethanol, inspect pipette tips for bead residue, and discard.

38 Repeat for a total of **three** washes. go to step #33

39 Let beads air-dry for 00:01:00 after final wash. 1m

40 Take tubes out of the magnetic rack, add 50 µL of elution buffer (10 mM TRIS-HCl pH 8.0,
or 1X TE buffer) pre-heated to 50 °C to each tube and pipette mix 5 times.

1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA) | 10 mM Tris-HCL pH 8.0

41 Spin down for **1-2 s** on centrifuge. 2s

42 Incubate tubes at 37 °C **for** 00:15:00 in benchtop shaking incubator at **20 rpm.** 15m

Note

If possible, use instrument that can produce 10-20 rpms.

43 Place tubes in magnetic rack and let sit for 00:15:00 - 00:30:00 . 45m

Note

I've had best results with 30 minutes.

44 Collect ~ 50 µL of supernatant and transfer to a **new tube.**

45 Place in tube stand and let sit at **RT overnight**. 12h

Note

This will allow the DNA to homogenize and relax. You can relax too!

46 Store DNA at -20 °C or -80 °C until ready for quantification with **Qubit** or **PicoGreen** assay and downdtreams analyses.

Protocol references

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