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# High-Molecular-Weight SPRI-aided DNA extraction from Mimulus (Phrymaceae) leaf tissue V.1

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**We use this protocol and it's working**

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## 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 in a sterile biosafety cabinet to prevent sample contamination.

Prior to being placed in CTAB, photosynthetic tissue from plants was 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  $\mu$ L 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 precipitated 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 steel; beads 3.2mm **Next Advance Catalog #SSB32**
- Stainless steel bead blend 0.9-2mm **Next Advance Catalog #SSB14B**
- Zirconium 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**
- ✕ Stainless steel; beads 3.2mm **Next Advance Catalog #SSB32**
- ✕ Eppendorf DNA low-bind microtube 2 mL **Fisher Scientific Catalog #022431048**
- ✕ Stainless steel bead blend 0.9-2mm **Next Advance Catalog #SSB14B**
- ✕ Zirconium oxide beads **Next Advance Catalog #ZROB20-RNA**
- ✕ CTAB buffer **OPS Diagnostics Catalog #CEB 500-02**
- ✕ 1X TE buffer (10 mM Tris-HCl pH 8.0 1 mM EDTA)
- ✕ Screw cap micro tubes 2 mL **Thermo Scientific Catalog #3469NK**
- ✕ 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M6250**
- ✕ 10 mM Tris-HCl pH 8.0
- ✕ Screw cap micro tubes 2 mL **Thermo Scientific Catalog #3469NK**
- ✕ 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**
- ✕ EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E9884**
- ✕ Polysorbate 20 (Tween-20) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P9416**
- ✕ SeraMag SpeedBeads Carboxyl-Magnet-Beads hydrophob **Fisher Scientific Catalog #GE Healthcare 65152105050250**
- ✕ Polyethylene glycol (PEG-8000) **Fisher Scientific Catalog #FLBP2331**
- ✕ Fresh 80% Ethanol

## Safety warnings

- ! Careful handling all reagents, specially CTAB and Chloroform: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 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

30m

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 .

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.

II

## 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 Bar . 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 Bar .

- 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  $\mu$ L (2 beads) of the autoclaved 3.2 mm stainless steel beads.

 Stainless steel; beads 3.2mm **Next Advance Catalog #SSB32**

**Note**

Beads can also be added at the same time sample tissue is weighed and transferred 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 steel bead blend 0.9-2mm **Next Advance Catalog #SSB14B**

- 9.3 Add 100  $\mu$ L of the autoclaved stainless steel bead blend, (0.9-2.0mm) using a sterile scoop. **Close the lids securely.**

 Zirconium oxide beads **Next Advance Catalog #ZROB20-RNA**

- 10 Tubes with beads can be placed in a  -20 °C freezer until ready to finish pulverization steps.

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





### 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 N<sub>2</sub>.

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  $\mu\text{L}$  of CTAB extraction buffer per sample into 15-50 mL conical centrifuge tube. Add 2.25  $\mu\text{L}$   $\beta$ -mercaptoethanol per 700  $\mu\text{L}$  of CTAB extraction buffer to the same conical tube (0.3% of the total extraction buffer volume).

### Basic recipe:

$[(\# \text{ of samples} \times 1.1) \times 700 \mu\text{L of CTAB}] + [(\# \text{ of samples} + 1) \times 2.25 \mu\text{L } \beta\text{-mercaptoethanol}]$

### 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  $\mu\text{L}$  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  $\mu$ L **-**  900  $\mu$ L **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  .

#### Note

This will remove any leftover RNase and contaminants.

27 **After repeating steps 22-24.** Carefully pipette ~  700  $\mu\text{L}$  -  800  $\mu\text{L}$  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                   |
|----------------------------------------------------------------------------------------------|---------------|---------------|---------------------|
| <b>Final concentration for a 1.25% SPRI bead solution for use in DNA extraction process.</b> |               |               |                     |
| Reagent                                                                                      | 10 mL         | 50 mL         | Final Concentration |
| Sera-Mag Speed Beads                                                                         | 125 µL        | 625 µL        | 1.25%               |
| 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 be performed while the tubes are placed on the magnetic rack. Avoid disturbing the settled 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.

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**.

Note

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

12h



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

## Protocol references

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