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Mimulus sp. CTAB Low Salt Nucleic Acid Prep

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Mimulus Protocol

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This protocol was used for Urquhart-Cronish et al. (2025) and Anstett et al. (2024-bioRxiv)

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Abstract

This protocol is an adaptation of Xin & Chen (2012) and Arseneau *et al.* (2017). From the high salt suspension step, users can follow Xin & Chen (2012) protocol to completion for quick clean-up in a 96-well format. If there is no access to or desire for bead-based cleanup, users can follow the protocol outlined by Arseneau *et al.* (2017) for the DNA pellet and washing steps.

It is suggested for use with less than ideal samples (mature fresh leaf with high secondary metabolites, field collection silica dried young leaves, oven-dried leaves). Young Mimulus leaf material generally passes in-house QC using a high salt + CTAB method. I recommend starting with Yuan & Sweigart (2019) or Fishman (2020) Mimulus protocols on Protocol.io and then trying this protocol for challenging samples.

This protocol is designed for individual tube format. Users can comfortably complete 96 samples in 3 days using a stacked method (Day 1 Extract, Day 2 Extract/Purify, Day 3 Purify/Quantify). 96 samples are possible in a 2-day format. The volumes are adapted to make this process efficient, utilizing individual tubes (the use of a combi tip during washes and a larger aqueous phase to draw from during organic phase separation).

Populations with higher trichome density or purple hue (pigmentation) in leaves were observed to be more challenging to extract cleanly using a standard CTAB protocol in my hands. This protocol was able to handle these samples, along with 2-year-old oven-dried mature leaf tissue.

Modifications:

- CTAB-DNA complex incubation time is increased to 2 hours, including a cool-down step meant to flocculate the complexes. This can be shortened to 1 hour, depending on your sample. Once the solution begins to precipitate CTAB-DNA complexes, it is fine to begin the spin. High-yielding samples are usually ready in 1 hour of incubation. If the sample fails to precipitate after 2 hours, spin anyway, as a small pellet will likely form. In this case, reduce the final elution volume to 35 uL.

- CTAB-DNA centrifugation is increased (3k rcf → 6k rcf) to stabilize the pellet during decanting (pour-off).

- DNA-CTAB precipitation centrifugation has increased (3k rcf → 10k rcf) before the high salt suspension for higher yields without sacrificing purity.

- Two rounds of desalting are used before the final elution. Using one wash yields 260/230 ~ 1.6-2.0, while two washes are generally >2.0.

Average nanodrop 260/280 1.9 $\pm$ 0.16 , 260/230 2.16 $\pm$ 0.3 across all 3 tissue types used n = 690.

Materials

Equipment

Fume hood

-20C freezer

Liquid nitrogen dewar
Liquid nitrogen 2L plastic dewar flask (Thermofisher Nalgene 4150-2000)
Metal Sample basket (for dewar flask)

Mix mill/bead beater
Waterbath or heat block
Eppendorf 5424 or any centrifuge capable of 16,000 rcf with MCT rotor
Shaker (with temp control is ideal)

p1000 pipette
p200 pipette
p2 pipette
Eppendorf Repeater M4 (optional)

Expendables

p1000 tips
p200 tips
p2 tips
25 mL Orange Combitip

2.0 mL MCT tubes (Fisherbrand 05-048-138)
1.5 mL MCT tubes (Fisherbrand 05-408-129)
3.2 mm chrome steel beads (Biospec, cat. 11079132c)

Qubit approved assay tube 0.5 mL (Invitrogen Q32856)

Eppendorf twin.tec, Full Skirt (Cat# 951020401)
VWR Aluminum Foil for PCR (Cat# 60941-074)

Chemicals & Reagents

Liquid Nitrogen 1-2 L

Enzymes

RNase A (10 mg/mL) **Thermo Fisher Scientific Catalog #**EN0531

CTAB

Hexadecyltrimethylammonium bromide (CTAB) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H9151

1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific Catalog #**15568025

UltraPure 0.5M EDTA, pH 8.0 **Thermo Fisher Scientific Catalog #**15575-038

NaCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**53014

b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M3148-25ML

Ambion Proteinase K **Thermofisher Catalog #**AM2546

Organic Extraction

Chloroform **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**366919-1L

isoamyl alcohol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**W205702

Wash Buffer / Precipitation

Ethanol

Quantification

Qubit™ dsDNA BR Assay Kit **Thermo Fisher Catalog #**Q32850

Use at minimum distilled water for stock prep, ultrapure recommended for TE elution buffer

UltraPure Distilled Water **Invitrogen - Thermo Fisher Catalog #**10977-015

Stocks

5M NaCl

	A	B
	75 mL	Distilled Water
	58.44 g	NaCl
	200 mL	**Final Volume**

Add salt slowly (3-5g) while mixing thoroughly between additions. Add water as the solution becomes saturated. Complete volume in a volumetric flask. Autoclave 20 min hold liquid cycle.

Te Buffer (Genome Quebec)

	A	B	C
	490 mL		Distilled Water (Ultrapure/Milli-Q preferred)

	A	B	C
	5 mL	10 mM	1M TRIS pH 8.0
	100 uL	0.1 mM	0.5M EDTA pH 8.0
	500 mL		**Final Volume**

Add in order, finalize volume in a volumetric flask

Te High Salt (Xin & Chen, 2012)

	A	B	C
	50 mL		Distilled Water (Ultrapure/Milli-Q preferred)
	1 mL	10 mM	1M TRIS pH 8.0
	200 uL	1.0 mM	0.5M EDTA pH 8.0
	20 mL	1.0 M	5M NaCl
	100 mL		**Final Volume**

Add in order, mixing between additions. Finalize volume in a volumetric flask.

Wash Buffer (Xin & Chen, 2012)

	A	B
	70 mL	Ethanol, molecular biology grade
	vol to	TE Buffer
	100 mL	**Final Volume**

Mix day of extraction, discard after 2 week

CTAB Extraction & Dilution Buffer (Xin & Chen, 2012)

	A	B	C
	50 mL		Distilled Water (start with 75 mL for Dilution Buffer)
	10 mL	100 mM	1M TRIS pH 8.0
	4 mL	20 mM	0.5M EDTA pH 8.0
	24 mL	1.2 M	5M NaCl (omit for Dilution Buffer)
	2 g	2% w/v	CTAB
	100 mL		**Final Volume**

Add in order.
Once CTAB is added, mix gently on a stir plate for 5 min 200-300 rpm (too high will cause foam) until any chunks break apart
Heat in a 60-65C water bath for 5 min, lightly swirling to fully dissolve CTAB.
Complete volume in a volumetric flask (pour slowly down the sidewall!!).
Return to stir plate for 5 minutes to ensure well mixed.
Optional: Autoclave liquid cycle 20 min - I do not because all liquid stocks are already sterilized/autoclaved and CTAB grade is purified for molecular biology

Ideally use within 14 days (stable for months though)

Protocol materials

- NaCl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**53014
- b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M3148-25ML
- Chloroform **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**366919-1L
- RNase A (10 mg/mL) **Thermo Fisher Scientific** **Catalog #**EN0531
- UltraPure 0.5M EDTA, pH 8.0 **Thermo Fisher Scientific** **Catalog #**15575-038
- Hexadecyltrimethylammonium bromide (CTAB) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H9151
- Qubit™ dsDNA BR Assay Kit **Thermo Fisher** **Catalog #**Q32850
- 1M Tris-HCl (pH 8.0) **Thermo Fisher Scientific** **Catalog #**15568025
- Ambion Proteinase K **Thermofisher** **Catalog #**AM2546
- isoamyl alcohol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**W205702
- UltraPure Distilled Water **Invitrogen - Thermo Fisher** **Catalog #**10977-015
- Ethanol
- b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M3148-25ML
- Ambion Proteinase K **Thermofisher** **Catalog #**AM2546

Set-up

1 For each sample label:
1× 2.0 mL tube with 2× 3.2 mm chrome steel beads (Biospec, cat. 11079132c)
1× 1.5 mL tube

Tissue Prep

1h

2 Load tissue loosely into a 2.0mL tube with 2× 3.2 mm chrome steel beads (Biospec, cat. 11079132c)

50 mg dried tissue
or
100 mg fresh leaf

3 Flash freeze in liquid nitrogen and load into tube mix mill grind plates chilled to -20 °C

Equipment

Mix Mill (MM 300)	NAME
Mix Mill	TYPE
Retsch	BRAND
23392	SKU
https://americanlaboratorytrading.com/lab-equipment-products/qiagen-retsch-mm300-tissuelyser-lab-vibration-mill-mixer_11444	LINK

4 Grind 00:00:45 30/sec and gently knock tissue until free in the tube. Check how well the tissue is ground (should still be frozen).

45s

Ground & collected Mimulus leaf

4.1 If needed, flash freeze again and flip the orientation of grind plate (tubes closest to the machine to the further position away from the matching), repeat grind until well milled

4.2 Store at -20 °C or proceed directly to extraction. I prefer to load & grind the samples the day before extraction.

5 When ready to extract, spin on a short cycle [2-3 sec to ~ 2500 rcf (g)] to collect tissue from the cap.

Tissue Extraction

1h 30m

6 For 48 samples, prepare 40 mL CTAB Extraction Buffer + 40 µL Beta-mercaptoethanol (0.1%) + 100 µL Proteinase K (0.1mg/mL) under the fume hood and warm to 60 °C in a waterbath.

b-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M3148-25ML

Ambion Proteinase K **Thermofisher Catalog #**AM2546

Note

7 Under the fume hood, dispense 750 µL warmed CTAB buffer solution (w/ B-mercap) per sample using a p1000 pipette, changing the tip every 6-8 samples or if bubble forms in the tip

Note

Optional: Combitip 25mL step level 1.5 Eppendorf Repeater M4

When dispensing CTAB there are few things to note.
1. I like to 'prime' the pipette by drawing the solution up and down once using the first stop of the pipette, then drawing up the volume a second time. This is only the first time you use a fresh tip.

2. Do not go to the second stop on the pipette (air expel) if you are planning to reuse the tip. Doing so will create bubbles that can be sucked into the pipette body. For inexperienced users, consider starting with filter tips to protect the pipette until comfortable.

3. Warmed solutions can often be overdrawn (think of the effect of temperature on meniscus shape and height-of-rise). Pay attention to make sure the volume is not 'creeping' up the pipette if reusing the tip (generally I found every 6-8 samples needed a new tip for accuracy).

.

8 Vortex samples under the fume hood for 30-60 seconds or until the tissue is dispersed in the buffer.

9 Incubate in a water bath 60 °C 60 min , inverting several times gently every 20 minutes to ensure good contact between buffer and tissue. Let tubes cool in the fume hood for 5 minutes

Organic Phase Seperation 45m

10 Add equal volume 750 µL Chloroform: Isoamyl alcohol 24:1 to 24 samples. The other 24 samples can remain at room temperature in the fume hood until ready.

10.1 Close tubes, place a paper towel, and then a tube block on top. Shake samples by hand (5-7/sec) between the two blocks for 00:00:20 under the fume hood. 20s

Note

Preferred for speed of loading to centrifuge and keeping chloroform under the hood.

10.2 Alternatively, return samples to mix mill 00:00:20 at 7/s 20s

11 Immediately load and spin samples 3000 rcf, 00:15:00

11.1 In the first 10 minutes, dispense 1.5 mL tubes with 1000 µL CTAB Dilution Buffer

Note

NaCl molarity of CTAB extraction buffer is 1.2M, diluting reduces molarity to < 0.5M and precipitates DNA-CTAB complexes leaving polysaccharides in solution (Murray & Thompson 1980; Xin & Chen 2012)

12 Transfer 500 µL into the 1.5 mL tube containing CTAB Dilution Buffer using a p1000 pipette. Mix 1 -2 times by pipette. If interphase is disturbed, recentrifuge sample 3000 rcf, 00:03:00 .

2 mL tube with three phases separated out after centrifuging and before transferring 500 ul of the supernatant (top aqueous layer) to the 1.5 mL tube containing CTAB dilution buffer. This sample is from desiccant dried young leaf tissue.

Note

Place the tip just below the surface of the top aqueous phase, slowly moving down the tube while drawing up the solution. There is enough aqueous phase (~650 - 750ul) that no interphase should be disturbed when using a single draw. If air bubbles occur, dispense the volume into the 1.5 ml tube, and then draw up another small portion to complete the volume to the 1.5 ml mark.

Top phase added to CTAB Dilution Buffer. Left: oven-dried young leaf, center: fresh mature leaf, right: fresh young leaf. All three resulted in clean DNA with sufficient yield.

13 With the next 24 samples go to step #10 and repeat.

Note

OPTIONAL: I like to begin the next 24 samples at the 2 minutes left mark on the spin cycle. The centrifuge is done by the time I finish dispensing chloroform. I unload the 1st batch, then shake and load the 2nd batch. During the 2nd batch spin, I transfer the top phase of the 1st batch.

DNA-CTAB Precipitation

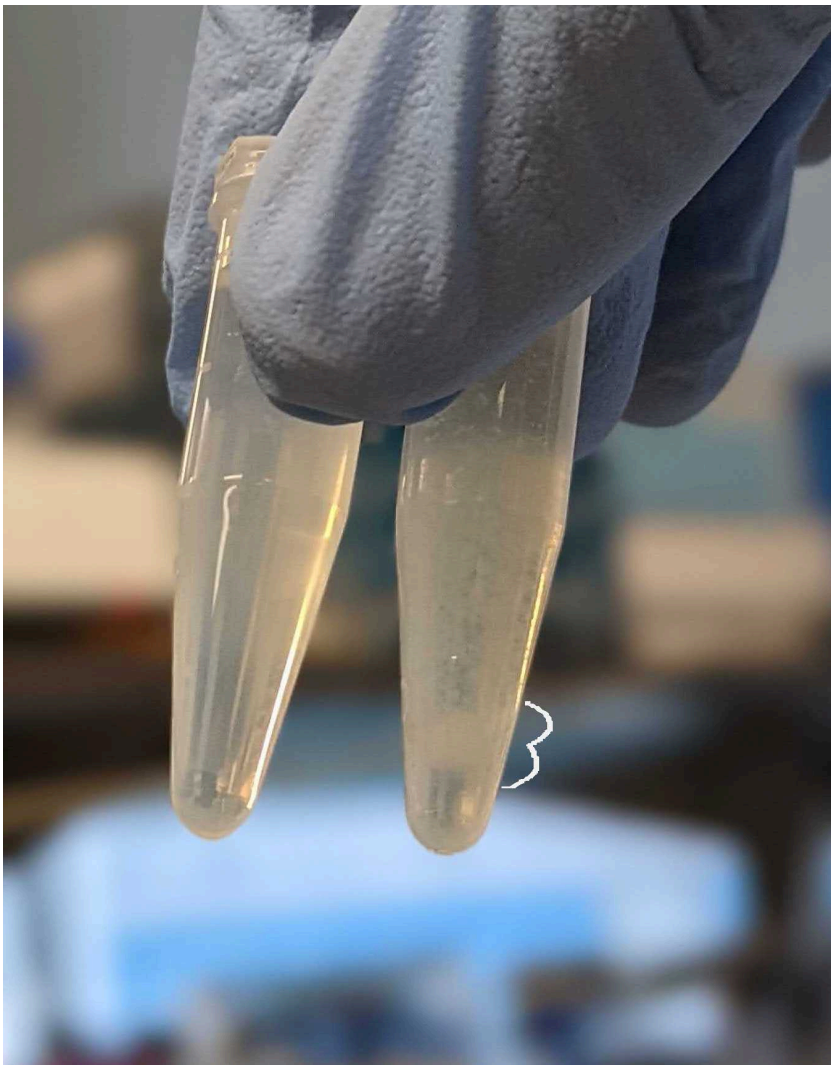
2h 30m

14 Incubate samples in a water bath or heat block 65 °C 60 min

15 Place samples on shaker 150 rpm, Room temperature , 01:00:00 or until the solution looks saturated in small 'crystals'.

Note

For most extractions, the solution will look saturated. If DNA concentrations are high enough, these 'crystals' will flocculate into long strands and drop out leaving the solution clear with DNA-CTAB 'clumps' at the bottom. These yields tend to be >2.5-3 ug. This solution has yielded up to 7.5 ug.



CTAB Precipitation Post Shaking. Left: "crystal" saturated sample (average appearance is like a hazy IPA) Right: formation of flocculate "strands" forming, note larger 'crystal' size above the aggregate.

16 Spin samples 6000 rcf, Room temperature, 00:15:00

17 Pour off supernatant gently and completely. If pellet detaches, stop pouring and respin for 5 min before finishing pour.

CTAB-DNA pellets. Left: oven-dried young leaf, Right: fresh mature leaf

DNA-CTAB Purification

1h 30m

18 Add 500 µL Wash Buffer and invert several times until pellet detaches from the wall and begins to dissolve.

Note

For faster dispensing, a 25 mL Combitip with step level 1 (500 uL) will fill 48x samples in under 3 minutes

This step cleans up contamination from the DNA-CTAB complexes

19 Put on shaker 150 rpm, Room temperature , 00:30:00

20 Spin tubes 12000 rcf, Room temperature, 00:15:00

Washed CTAB-DNA pellet. Left: dried leaf, Center: fresh mature leaf, Right : fresh young leaf

21 Pour off supernatant gently and completely. Dab tubes on an autoclaved paper towel to remove trace liquid

Note

If time-constrained, pipette off remaining traces of wash buffer using p200 pipette

22 Dry tubes open 37 °C 15-20 min or until tubes are just dry.

Note

Do not over-dry tubes, it will become difficult to resuspend the pellet and can shear DNA.

DNA High Salt RNASE Treatment

23 Gently suspend pellet in 100 µL High Salt TE (1M) + 50 ug/mL final concentration RNASE (0.25 uL/100 uL)

Note

Pellet can be suspended gently by pipette, or on shaker RT, 150 RPM, 30 minutes.

This step disassociates CTAB from DNA by introducing NaCl > 0.5M, along with degrading RNA.

For 48 samples, prepare 5mL TE Buffer 1M NaCl + 12.5 uL Purelink RNASE

24 Incubate either 37 °C 60 min and store 4 °C overnight OR Room temperature overnight

Note

This is usually my stopping point for the day. Another user reported good success with 37C for 1 hour and longer-term storage at -20C.

From this point, can either follow either alcohol precipitation and desalting (Arseneau), or use of paramagnetic beads (Xin and Chen). Personal preference is to follow Xin and Chen if Suspension G beads are available.

STEP CASE

Arseneau (2017) 15 steps

DNA Precipitation 1h 45m

25 Add 500 uL 90% ETOH (final concentration of 75%)

Note

For faster dispensing, a 25 mL Combitip with step level 1 (500 uL) will fill 48x samples in under 3 minutes

26 Invert several times and incubate Room temperature 60 min

27 Spin 16000 rcf, Room temperature, 00:20:00

28 Pour off supernatant slowly and completely.

DNA Purification 45m

29 Desalt the pellet using 500 uL Wash Buffer, flick tube to suspend pellet briefly

30 Spin 16000 rcf, Room temperature, 00:05:00

31 Pour off supernatant slowly and completely.

32 If DNA 260/230 of 1.6-1.8 with slightly higher yield is acceptable continue to step 33, else go to step #29 and repeat once for DNA 260/230 > 2.0 (Genome BC/Quebec QC standard).

33 Dry tubes open at 37 °C 15-20 min

DNA Elution and Quantification

34 Add 55 uL low TE (Genome Quebec) or 10mM Tris-Hcl pH 8.0

Note

Genome Quebec TE: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA
Standard TE (Cold Harbour Springs): 10 mM Tris-HCL pH 8.0, 1 mM EDTA

35 Incubate 150 rpm, 37°C, 00:30:00

36 Mix samples by pipette (gently!) and transfer 54 µL to a PCR plate

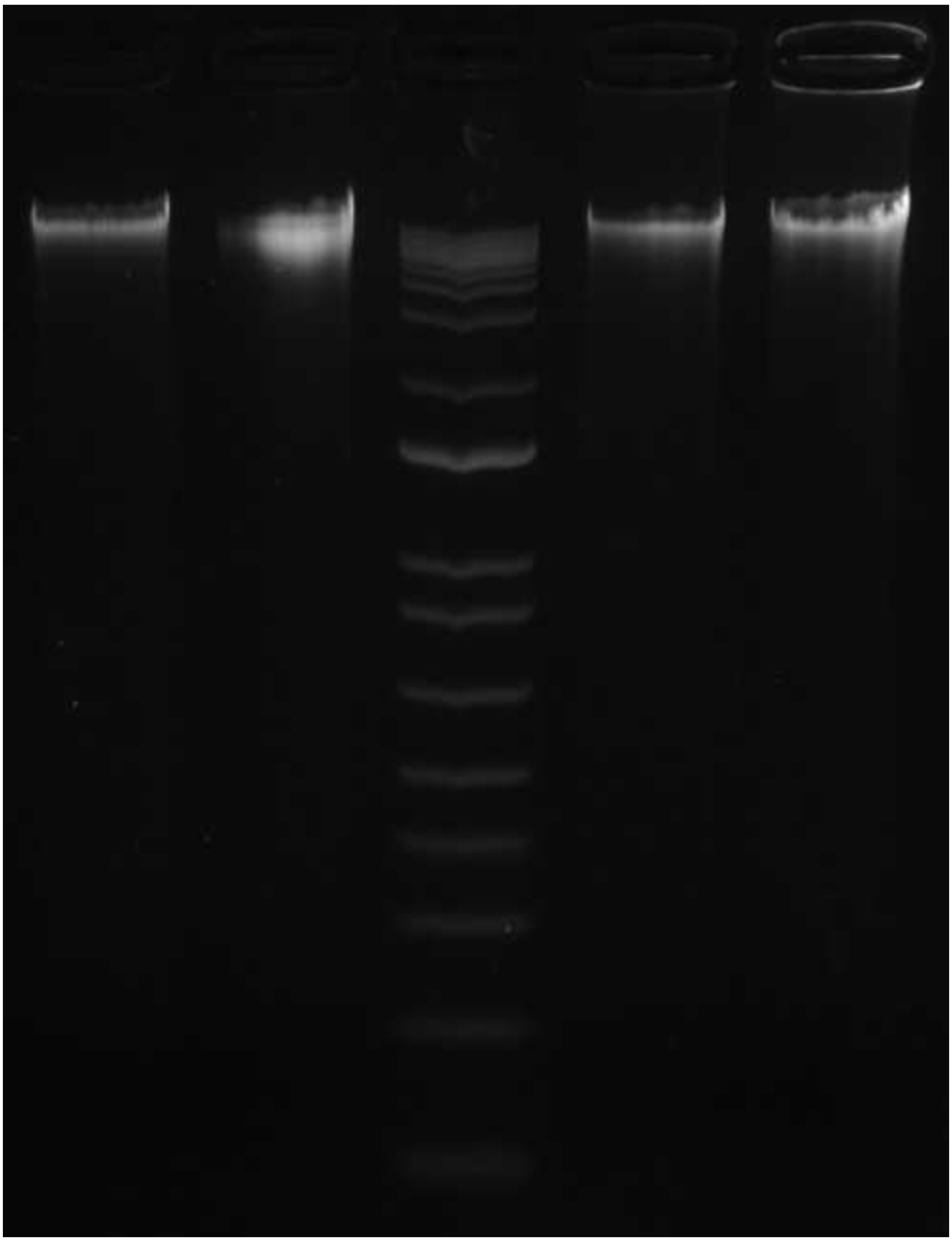
37 Access purity of the sample using 2 µL on a Nanodrop1000/2000

38 Quantify 2 µL sample using broad spectrum Qubit kit

Note

ug/mL can be converted to ng/uL by multiplying *10 for 2 uL volume, (i.e 0.280 ug/ml = 28.0 ng/uL and 1.19 ug/mL = 119 ng/uL) This saves clicking calculate stock concentration everytime between reading samples.

39 Optional: run 3-5 uL sample on 1.0% agarose gel in TAE buffer, 100V 30 min to check for average molecular weight, and sample integrity.



1% agarose TAE buffer electrophoresis gel. 100V 30 min. Left 2 lanes: fresh mature leaf, Center: Generuler 1kb plus, Right: 2 year old oven dried leaf

Protocol references

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