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Protocol status: Working
We use this protocol and it's working

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Annonaceae DNA extraction protocol from silicagel dried and herbarium preserved leaves

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DISCLAIMER

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ABSTRACT

This protocol is used for DNA extraction of samples from the tropical plant family Annonaceae for leaves dried using silicagel or sampled from herbarium sheets.
This protocol is made for generation of small to long fragments (depending quality of sample) to prepare NGS libraries.
This protocol is designed to extract DNA in batches of 48 samples, but this can also be undertaken in 2 times 48 (96) samples.

IMAGE ATTRIBUTION

Art work by Vincent Soulé

GUIDELINES

Work in a clean environment to avoid contamination, use as much as possible filter tips, wear gloves and lab coat. Wash work space and pipettes before and after use, with DNAaway and DNAse away.
Manipulate with extreme caution rare or old samples.

MATERIALS

2mL Screw tube
2mL secure lock ependorf tube

REF MATAB

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PROTOCOL integer ID: 75464

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PROTOCOL MATERIALS

- 70% alcohol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #793213** — Step 4.2
- Gel Red Nucleic Acid Gel Stain **Biotium Catalog ##41003** — Step 7
- Na acetate 3M pH 4.8 or 5.2 — Step 4
- Proteinase K **Life Technologies Catalog #17916** — In 2 steps
- RNase A Solution, 4mg/ml **Promega Catalog #A7973** — Step 3.3
- 1M TE buffer (1M Tris-HCl, 0.1M EDTA, pH 8.0) — Step 5
- Agarose **Catalog #A5304** — Step 7
- Isopropanol — Step 4
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632** — Step 2.1

SAFETY WARNINGS

Always work under an extractor hood when manipulating of Chloroform, DTT or Isopropanol

Leaf grinding

1 Prepare 48 2 mL Screw-Top tubes in rows of 8 in a 96 well rack. Add one 1/4" ceramic beads (MP Biomedical REF 116540422).

1.1 Add leaf sample inside the tube using clean tweezers. The leaf samples can be between 1x1 cm and 3x3 cm in size. Closes the tubes.

1.2 Grind samples using a MP FastPrep grinder, twice for 00:00:40 at 4m/second speed with a 2 4m minute pause in between each grind as not to over heat the samples.

Equipment

FastPrep-24™ 5G	NAME
grinder	TYPE
MP Biomedicals™	BRAND
116005500	SKU
https://www.fishersci.fr/shop/products/mp-biomedicals-fastprep-24-5g-instrument/15260488	LINK
24 samples	SPECIFICATIONS

file

Lysis buffer preparation and lysis

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Lysis buffer **LB** needs to be freshly made the day of the extraction using a previously made **LBmix** + MATAB, Proteinase K Life Technologies Catalog #17916 and DTT (DL-Dithiothreitol) (final concentration 1mM).

Preparation of **LBmix** (1000 mL) for 1000 samples:

1000 mL	miliQ water	Final concentration
9.31 g	EDTA	[M] 25 millimolar (mM)
15.76 g	Tris-HCL	[M] 100 millimolar (mM)
81.82 g	NACl	[M] 1.4 Molarity (M)

2.1

Preparation of **LB** for 48 samples:
In a 50mL tube add 2 g of MATAB + 20 ml of **LBmix** Final concentration [M] 4 Mass / % volume
Disolve MATAB in 65 °C water bath and vortex (aprox 10 min).
Add 250 µL of Proteinase K Life Technologies Catalog #17916 at [M] 1 mg/mL then vortex.

Add 50 µL of DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632** then vortex.

Add **LBmix** to adjust volume to 50 mL and put solution at 65 °C

Final volume 50 mL

2.2 Using a p1000, add to each grinded sample 1 mL of still hot **LB;** close the tube then vortex e 17h

sample until the leaf powder is totally disolved.

Place the rack at 65 °C for a minimum of 03:00:00 for silicagel preserved samples; for herbarium sampled leaves place at 65 °C for a recommended 06:00:00 to 08:00:00.

Lysis can be done over night.

Shake the rack/tubes every 30 minutes during lysis.

2.3 If needed, the process can be stopped after lysis, and the tubes can be conserved up to 1d

24:00:00 at -20 °C

Chloroform DNA Isolation

3 For herbarium samples, limit to one round of chloroform isolation. 12m

After the lysis step, let the samples get to room temperature.

Under an extractor hood, add 700 µL of 24:1 Chloroform : Isoamyl alcohol. Close the tube and shake vigorously by inversion the rack for 00:02:00 to 00:10:00

3.1 Centrifuge 4000 rpm, Room temperature, 00:10:00 10m

Prepare in a new rack of 48 2mL tubes.

3.2 Under an extractor hood, transfer 8 by 8, using a multichannel pipette, 800 µL of the aqueous 10m supernatant phase into the new 2mL tubes. Avoid pipetting the interphase pellet.

3.3 Add 10 µL of RNase A Solution, 4mg/ml **Promega Catalog #A7973** at [M] 0.5 mg/mL 30m

Place the rack with the samples at 37 °C for 00:30:00 .

3.4 Under an extractor hood, proceed with the second chloroform cleaning step. Add 700 µL of 2 10m

Chloroform Isoamyl alcohol; close tubes and shake the rack of tubes.

3.5 Centrifuge 4000 rpm, Room temperature, 00:10:00 10m

During centrifugation, prepare and name new 2ml eppendorf safe lock tubes.

3.6 Under an extractor hood, transfer 8 by 8 using a multichannel pipette, 600-800 µL of the aque 5m

supernatant phase into the new 2mL tubes. Avoid pipetting the interphase pellet.

DNA precipitation 2h

4 Add 360 µL of Isopropanol at -20 °C 2h

Add 60 µL of Na acetate 3M pH 4.8 or 5.2 pH=5 at 4 °C

Slowly shake the tubes and then place the rack at -20 °C for 02:00:00 to Overnight

4.1 Centrifuge 14000 rpm, -20°C, 00:10:00 10m

Slowly remove liquid phase using a pipette with P1000, be aware to not pipette DNA pellet.

Washing DNA 25m

4.2 Add 700 µL of 70% alcohol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #793213** 10m in

each tube to wash pellet.

4.3 Centrifuge 14000 rpm, -20°C, 00:10:00 25m

Remove liquid phase with P1000, be aware to not pipette DNA pellet.

Let dry for 00:15:00 at Room temperature .

DNA elution

5 Depending on DNA pellet size, add 60-100 µL of 1M TE buffer (1M Tris-HCl, 0.1M EDTA, pH 8.0), shake tubes. 20m

10 rpm, Room temperature

Eluate Overnight at 4 °C

5.1 Tubes can be stored for 48:00:00 at 4 °C before quality check and quantification. 2d

DNA quantification

55m

6 Transfer 6 µL of each samples to a 96 well PCR plate.

Use 2 µL for DNA quantification using TECAN spark or nanoquant with nanoquant 16 holes plate; or quantify one by one using Nanodrop.

Equipment	
SPARK	NAME
Microwell plate reader	TYPE
TECAN	BRAND
SPARK	SKU
https://www.tecan.com/blog/spark-multimode-microplate-reader-for-high-performance-cell-based-fluorescence-assays	LINK

DNA quality check on gel

55m

7 To the 4 µL left add 6 µL 1.5X red blue yellow loading buffer. 55m

Prepare 1% of Agarose **Catalog #A5304** gel with TAE 1X.
Add your samples and:
+ promega 100pb dna ladder
+ promega 2.5kb lambda eco R1 hind3 dna ladder on gel well
Then proceed with gel electrophoresis at 135V 00:40:00 on TAE 0.5X.

Put gel 00:15:00 in 1X Gel Red Nucleic Acid Gel Stain **Biotium Catalog ##41003**
Place gel in imaging machine. Turn on UV light, take a picture.

DNA conservation

55m

8 Extracted DNA needs to be stored at -20 °C .

For long term conservation, transfert to barcoded screw-top tube on 96 rack.
For the GLOBAL projet we used **Thermo Scientific™ Matrix™ 0.5mL 2d barcoded.**

Expected result

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Expected result

Within the GLOBAL project, around 3600 DNA extractions were undertaken, some on silicagel leaves others on herbarium preserved leaves.
For silicagel dried samples we extracted 1050 specimens, with a max concentration of 979 ng/ul and a minimum of 0.9 ng/ul for a total elution volume of 100 ul. On average we had 213 ng/ul (Standard error: 205.8).
For herbarium preserved leaves 2600 samples were extracted: the highest concentration was 1088ng/ul and the lowest was 0,1 ng/ul for a total elution volume of 60 ul. On average we had 230 ng/ul per extraction (Standard error: 221.8).
Concentrations below 10 ng/ul were rarely used to sequenced or frequently failed.
DNA size ranged between 100pb-2.5kb, but longer fragments were also possible.