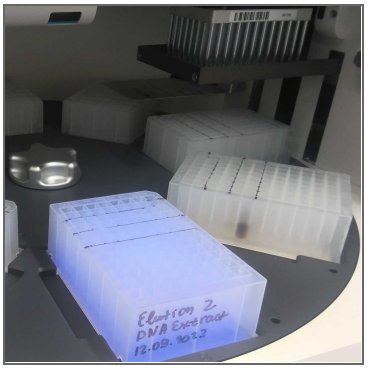

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Protocol status: Working
We use this protocol and it's working

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Sanger Tree of Life HMW DNA Extraction: Automated Plant MagAttract v.4

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ABSTRACT

This protocol describes the automated extraction and SPRI of HMW DNA from cryogenically homogenised or bead-beaten tissue samples from plants and fungi intended for long-read sequencing. It employs the Qiagen MagAttract HMW DNA extraction kit and the Thermo Fisher KingFisher™ Apex. This process is effective for a wide variety of plant species covered by the Tree of Life Programme. The output of this protocol is HMW DNA, which depending upon yield and genome size of the species, can be directed towards either HMW DNA Pooling, HMW DNA Fragmentation: Diagenode Megaruptor®3 for LI PacBio or HMW DNA Fragmentation: g-Tube for ULI PacBio. This protocol was adapted from Sanger Tree of Life HMW DNA Extraction: Automated Plant MagAttract v.3 to further improve sample lysis. These improvements have been made through a combination of preheating the lysis buffer, delaying the addition of RNase A to later on in sample lysis and increasing centrifugation of the lysate, which have led to a reduction in tissue clumping, minimised oxidative damage of the DNA, reduced Proteinase K inhibition of RNase A and increased purity of the HMW DNA due to the exclusion of aggregated, insoluble or sedimented contaminants.

Acronyms

HMW: high molecular weight
SPRI: solid-phase reversible immobilisation
LI: low input
ULI: ultra-low input

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Keywords: HMW DNA extraction, magnetic bead extraction, MagAttract, automated DNA extraction, KingFisher, plant DNA extraction, solid phase reversible immobilisation, reference genome, long read sequencing

GUIDELINES

- For the lysis buffer master mix, prepare enough for n+1 samples to allow for pipetting errors**.**
- Keep samples on dry ice to maintain temperature and prevent nucleic acid degradation until the lysis buffer is ready to be added to them.
- For the 0.45X SPRI, the DNA and AMPure beads should not sit together in the sample plate for more than 5 minutes before starting the KingFisher™ Apex.
- An experienced operator can expect to comfortably process up to 32 samples, with approximately 2–3 hours handling time over a start to finish period of 4-5 hours. This estimation includes the utilisation of the KingFisher™ Apex for both the extraction and SPRI protocols, and excludes subsequent QC checks.

Additional Notes:

- FluidX tubes are used throughout the Tree of Life programme in order to track samples, therefore rather than the microcentrifuge tubes which have been mentioned in this protocol for DNA storage, all routine DNA extracts are stored in FluidX tubes.
- Both the KingFisher™ Apex protocol scripts and the KFX.files have been made available for this protocol – the KFX.files require 'BindIx software for KingFisher Apex' to allow the KingFisher™ Apex protocols to be viewed on a PC or laptop. Alternatively, the files can be transferred directly onto a KingFisher™ Apex instrument using a USB flash drive.

MATERIALS

- 1.5 mL DNA Lo-Bind microcentrifuge tubes (Eppendorf Cat. no. 0030108051)
- 2 mL DNA Lo-Bind microcentrifuge tubes (Eppendorf Cat. no. 0030108078)
- Thermo Fisher KingFisher™ 96-well Deep-well plates (Thermo Fisher Cat. no. 95040450)
- Thermo Fisher KingFisher™ 200 µL standard 96-well Plate (Thermo Fisher Cat. no. 97002084)
- Thermo Fisher KingFisher™ 96 Tip Comb (Thermo Fisher Cat. no. 97002570)
- Qiagen MagAttract HMW DNA extraction kit (Qiagen Cat. no. 67563)
- Dry ice
- 1x phosphate-buffered saline (PBS)
- 100% absolute ethanol
- 15 mL or 50 mL centrifuge tubes
- AMPure PB beads (Pacific Biosciences Cat. no. 100-265-900)
- Buffer EB (Qiagen Cat. no. 19086)

Equipment:

- Pipettes for 0.5 to 1000 µL and filtered tips
- Wide-bore tips (200 µL and 1000 µL, filtered if available)
- Thermo Fisher KingFisher™ Apex instrument (Cat. no. 5400930)

- Eppendorf ThermoMixer C (Cat. no. 5382000031)
- Eppendorf SmartBlock 2.0 mL (Cat no. 5362000035)
- Eppendorf SmartBlock 50 mL (Cat no. 5365000028)
- Vortexer (Vortex Genie™ 2 SI-0266)
- Mini centrifuge (Cat. no. SS-6050)
- Eppendorf Centrifuge 5425/5425 R (Cat. no. 5405000263)
- DynaMag™-2 magnetic rack (Cat. no. 12321D)
- Timer

KingFisher™ Apex DNA Extraction Protocol Script:

KFX file: Qiagen MagAttract Standard.kfx

1. Pick Up Tip - Tip Plate
2. DNA Binding - Sample Plate
   Pre-collect beads: Off
   Release beads: Off
   Heating & Cooling: Off
   Mixing 1# 00:05:00 Fast
   Postmix: Off
   Collect beads: On 5 Count 2 Seconds
3. Collect Beads 1 - Sample Plate
   Collect beads: Count 5 Collect time: 1 Second
4. Wash 1 - MW1 Wash 1 Plate
   Pre-collect beads: Off
   Release beads: On 00:00:10 Bottom mix
   Heating & Cooling: Off
   Mixing 1# 00:01:00 Fast
   Postmix: Off
   Collect beads: On 5 Count 1 Second
5. Collect Beads 2 - MW1 Wash 1 Plate
   Collect beads: Count 5 Collect time: 1 Second
6. Wash 2 - MW1 Wash 2 Plate
   Pre-collect beads: Off
   Release beads: On 00:00:10 Bottom mix
   Heating & Cooling: Off
   Mixing 1# 00:01:00 Fast
   Postmix: Off
   Collect beads: On 5 Count 1 Second
7. Collect Beads 3 - MW1 Wash 2 Plate
   Collect beads: Count 5 Collect time: 1 Second
8. Wash 3 - PE Wash 1 Plate

Pre-collect beads: Off
Release beads: On 00:00:10 Bottom mix
Heating & Cooling: Off
Mixing 1# 00:01:00 Fast
Postmix: Off
Collect beads: On 5 Count 1 Second

9. Collect Bead 4 - PE Wash 1 Plate
   Collect beads: Count 5 Collect time: 1 Second
10. Wash 4 - PE Wash 2 Plate
    Pre-collect beads: Off
    Release beads: On 00:00:10 Bottom mix
    Heating & Cooling: Off
    Mixing 1# 00:01:00 Fast
    Postmix: Off
    Collect beads: On 5 Count 1 Second
11. Collect Bead 5 - PE Wash 2 Plate
    Collect beads: Count 5 Collect time: 1 Second
12. Water Rinse - NFW Plate
    Pre-collect beads: Off
    Release beads: Off
    Heating & Cooling: Off
    Mixing 1# 00:00:00
    Postmix: Off
    Collect beads: On 5 Count 1 Second
13. Dry - NFW Plate
    Duration: 00:01:00 Dry Type: Above Well
14. Elute 1 - Elution Plate 1 Plate
    Pre-collect beads: Off
    Release beads: On 00:00:00
    Heating & Cooling: On 25°C Pre-heat: Off
    Mixing 1# 00:01:00 Paused Looping: 1
    2# 00:05:00 Slow Tip Position: Above Well
    Postmix: Off
    Collect beads: On 3 Count 1 Seconds
15. Elute 2 - Elution Plate 2 Plate
    Pre-collect beads: Off
    Release beads: On 00:00:00
    Heating & Cooling: On 25°C Pre-heat: Off
    Mixing 1# 00:01:00 Paused Looping: 1
    2# 00:05:00 Slow Tip Position: Above Well
    Postmix: Off
    Collect beads: On 3 Count 1 Seconds
16. Leave Tip - NFW Plate

KingFisher™ Apex 0.45X SPRI Protocol Script:

KFX file: Pre-shear 0.45X SPRI.kfx

1. Pick Up Tip - Tip Plate
2. Mix - Sample Plate
   Pre-collect beads: Off
   Release beads: On 00:00:00
   Heating & Cooling: Off
   Mixing:
   1# 00:01:00 Slow
   2# 00:01:00 Medium
   3# 00:08:00 Paused
   Looping: 1 Tip position: Tip edge in liquid
   Postmix: Off
   Collect beads: On 10 Count 30 Seconds
3. Wash 1 - Ethanol Wash Plate
   Pre-collect beads: On
   Release beads: Off
   Heating & Cooling: Off
   Mixing 1# 00:00:30 Slow
   Postmix: Off
   Collect beads: Off
4. Wash 2 - Ethanol Wash Plate
   Pre-collect beads: Off
   Release beads: Off
   Heating & Cooling: Off
   Mixing 1# 00:00:30 Slow
   Postmix: Off
   Collect beads: Off
5. Dry - Ethanol Wash Plate
   Duration: 00:01:00 Above well
6. Elute - Elution Plate
   Pre-collect beads: Off
   Release beads: On 00:01:00 Slow
   Heating & Cooling: On 37°C Preheat: On
   Mixing: 1# 00:07:00 Slow
   2# 00:08:00 Paused
   Looping: 1 Tip position: Tip edge in liquid
   Postmix: Off
   Collect beads: On 4 Count 30 Seconds
7. Leave Tip - Ethanol Wash Plate

Protocol PDF:

Sanger Tree of Life HMW DNA Extraction_ Automated Plant MagAttract v.4.pdf

SAFETY WARNINGS

- The operator must wear a lab coat, powder-free nitrile gloves and safety specs to perform the laboratory procedures in this protocol. Cotton glove liners are strongly recommended when handling the samples on dry ice.
- Waste needs to be collected in a suitable container (e.g. plastic screw-top jar or Biobin) and disposed of in accordance with local regulations.
- Liquid waste needs to be collected in a suitable container (e.g. glass screw-top jar) and disposed of in accordance with local regulations.
- Do not open the door of the KingFisher™ Apex instrument whilst it is in operation.

BEFORE START INSTRUCTIONS

- Add 100% ethanol to the MW1 and PE wash buffers as per manufacturer's instructions.
- AMPure PB beads are stored in the fridge at 4 °C – take them out 30 minutes before starting the 0.45X SPRI KingFisher™ Apex protocol to bring them to room temperature.

Sample lysis

1 Set one heat block with a 50 mL SmartBlock to 65 °C and another heat block with a 2 mL SmartBlock to 55 °C.

	Reagent	Volume per sample
	Phosphate-buffered saline (PBS)	200 µL
	Buffer AL	150 µL

2 Prepare a lysis buffer master mix in a 50 mL centrifuge tube:

3 Place the lysis buffer on the 65 °C heat block and incubate at 400 rpm for at least 20 minutes. Keep at temperature until added to the sample.

4 Transfer 50 mg of cryogenically disrupted tissue from each sample to 2 mL microcentrifuge tubes.

- Ensure the disrupted tissue is completely disrupted into a fine powder; avoid matted/clumped powder. This is crucial for optimal DNA yield and integrity; poorly disrupted tissue drastically decreases lysis and extraction efficiency.
- Any samples containing poorly disrupted tissue 'chunks' should be flagged as requiring reprocessing and further cryogenically disrupted.

5 Transfer the samples to a pre-chilled cold block on wet ice and incubate for 10 minutes to equilibrate temperature.

6 Add 20 µL Proteinase K (for n+1 samples) to the preheated lysis buffer immediately prior to initiating lysis, swirling the centrifuge tube to mix.

7 Add 370 µL of the preheated lysis buffer plus Proteinase K to each sample, immediately homogenising the lysate by mixing with 5 rapid pulse vortexes, and place on the 55 °C heat block at 600 rpm for 15 minutes.

8 After 5 minutes incubation, resuspend any severely aggregated samples by pipette mixing with a wide-bore pipette tip.

9 After the initial 15 minute incubation, add 4 µL RNase A to each sample and mix thoroughly by inversion until any aggregated, insoluble or sedimented tissue particles are resuspended.

10 Incubate samples for a further 45 minutes on the heat block at 55 °C at 600 rpm.

11 During this incubation, samples should be occasionally mixed (every 5–15 minutes) by inversion to resuspend sedimented particles.Do not mix the samples by inversion for the final 15 minutes of lysis, allowing aggregated, insoluble or sedimented tissue particles to settle at the bottom of the tube.

Loading and Running the KingFisher™ Apex for DNA Extraction

12 Whilst samples lyse, label nine 1 mL 96-well deep-well KingFisher™ plates and fill the number of wells required for the number of samples in each plate as follows:

	Plate	Reagent(s) required
	Tip plate	96-well tip comb (no reagent)
	Elution 2	200 µL Buffer AE
	Elution 1	200 µL Buffer AE
	NFW Wash	500 µL nuclease-free water
	PE Wash 2	700 µL Buffer PE
	PE Wash 1	700 µL Buffer PE
	MW1 Wash 2	700 µL Buffer MW1
	MW1 Wash 1	700 µL Buffer MW1
	Sample plate	15 µL Suspension G magnetic beads + 280 µL Buffer MB

13 Once samples have completed lysing, remove sample tubes from the heat block and allow the lysate to settle to the bottom of the tube for 5 minutes.

14 Centrifuge the samples for 10 minutes at 8,000 rpm at room temperature.

15 Using a wide-bore pipette tip, set the volume to 380 µL, gently transfer lysate from the sample tubes to individual wells in the sample plate, taking care to avoid aspirating the pelleted tissue particles.

16 Select the required DNA extraction protocol in the protocol list on the KingFisher™ Apex (details in KingFisher™ Apex DNA Extraction Protocol Script/attached KFX file in the Materials section) and select using the play button.

17 Load the filled plates onto the instrument following the instructions provided on screen.

18 Prior to loading the “Sample Plate”, the instrument will prompt to remove the “Tip Plate”. Once the final plate is loaded, the protocol will automatically begin; this takes approximately 50 minutes.

19 Once the protocol has completed, follow the on-screen instructions to remove plates from the instrument.

20 Inspect the elution plates for any magnetic beads in the wells. In the rare instance of magnetic beads remaining in the eluate (possible in viscous samples), these samples will need to be transferred to a 1.5 mL microcentrifuge tube and placed on a magnetic rack. Allow around 5 minutes for the beads to migrate and take the clear eluate containing the DNA using a wide-bore pipette tip.

21 Using a 200 µL multi-channel pipette and wide-bore tips, pipette eluates from Elution Plate 2 into Elution Plate 1, and gently pipette mix 5–10 times with wide-bore tips to fully homogenise DNA in the eluate. Elution Plate 1 with the combined eluates is now the ‘Sample Plate’ for the 0.45X SPRI.

Loading and Running the KingFisher™ Apex for the 0.45X SPRI

22 Set-up the KingFisher plates for the 0.45X SPRI as detailed below:

	Plate	Plate type	Reagent(s) required
	Tip Plate	1 mL Deep-well	96-well tip comb (no reagent)

	Plate	Plate type	Reagent(s) required
	Sample Plate (Elution Plate 1 from DNA Extraction Protocol)	1 mL Deep-well	380 µL DNA + 171 µL AMPure PB beads
	Ethanol Wash Plate	1 mL Deep-well	1000 µL 80% EtOH (freshly made)
	Elution Plate	200 µL standard	135 µL Buffer EB

23 Select the required 0.45X SPRI protocol in the protocol list on the KingFisher™ Apex (details in KingFisher™ Apex 0.45X SPRI Protocol Script/attached KFX file in the Material section) and select using the play button.

24 Load the filled plates onto the instrument following the instructions provided on screen.

25 Once the final plate is loaded, the protocol will automatically begin; this will take approximately 40 minutes.

26 Once the protocol has completed, follow the on-screen instructions to remove plates from the instrument.

27 Using a wide-bore pipette tip, transfer the 130 µL of eluate from the elution plate into microcentrifuge tubes.

28 Incubate the DNA at room temperature overnight and perform the required QC the following morning.

29 Store the DNA at 4 °C.