Preparing *Arabidopsis* Genomic DNA for Size-Selected ~20 kb SMRTbell™ Libraries

This protocol can be used to prepare purified *Arabidopsis* genomic DNA for size-selected SMRTbell templates with average insert sizes of 10 to 20 kb. **We recommend starting with 20-40 grams of three-week-old *Arabidopsis* whole plants, which can generate >100 µg of purified genomic DNA.**

Before You Begin

To perform this procedure you **must** have the following supplies, materials, and instrumentation:

Item	Vendor
NanoDrop® Spectrophotometer (Series 2000, 2000c, 3300, or 8000)	Thermo Scientific
Qubit® Fluorometer	Invitrogen (P/N Q32857)
g-TUBE® microcentrifuge tubes	Covaris
AMPure® PB Beads	Pacific Biosciences
Water bath set at 74°C on day 1, 50°C on day 2, and 37°C on day 3	Any Major Laboratory Supplier (MLS)
Liquid nitrogen	
4Coors™ Clean porcelain mortar and pestle (1 per sample) 00 mL	Sigma-Aldrich
Falcon™ tubes	
Centrifuge chilled to 4°C	
Kimwipes® Lint-Free Tissues	MLS
Nylon mesh filters: 200 µm, 100 µm and 40 µm	Aquasample
Plastic funnel	
Genomic-tip 500/G kit	QIAGEN
DNA Buffer Set	QIAGEN
Nucleus Isolation Buffer (Must be cooled to 0°C for > 1 hour before use): 10 mM Tris pH 9.5 10 mM EDTA 100 mM KCL 500 mM Sucrose 4 mM Spermidine 1 mM Spermine 0.1% BME	
NIB + 10% Triton X-100 (Must be cooled to 0°C for > 1 hour before use):	
Carlson Lysis Buffer (Pre-warm in 74°C bath ~ 1 hour before use): 100 mM Tris pH 9.5 2% CTAB 1.4 M NaCl 1% PEG 6000 20 mM EDTA	
2-Mercaptoethanol (BME)	Sigma-Aldrich
Chlorform:Isoamyl alcohol 24:1, > 175 mL	Sigma-Aldrich
3 M NaOAc	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
70% Ethanol, ice cold	Sigma-Aldrich

Proteinase K, > 600 mAU/mL solution	QIAGEN
RNase A, 100 mg/mL	
TE + 100 mM NaCl	
Pulsed-Field Gel Electrophoresis (PFGE) system (BluePippin™/Pippin Pulse System)	Sage Science

Procedure

STEP	Day 1	Notes
1: Grind the Tissue	1. Cut 10 grams of leaf tissue from plants and transfer to the mortar. 2. Add liquid nitrogen (enough to cover the tissue) and grind tissue. 3. When the liquid nitrogen has evaporated, continue grinding for approximately 30 seconds, to flour-like fineness. 4. Add more liquid nitrogen and grind again until the nitrogen is gone, then continue grinding for an additional 30 seconds. 5. Add more liquid nitrogen and grind a third time as above. 6. Transfer the powder to a chilled flask or beaker on ice. 7. Add 100 mL of ice cold NIB and mix by swirling. Mixture should be smooth, not clumpy. Repeat the above steps until all tissue is ground.	
2: Filter to remove particulate material	Note: Make sure there is no ice in the plant extract before filtering. 1. Place a clean Falcon tube on ice to receive the filtered material. 2. Place the funnel on top of Falcon tube. 3. Place a nylon 200 µm mesh filter into the funnel. 4. Filter the liquid through the nylon mesh into a clean tube. Note: Do NOT force through the filter. 5. Wash the filter with ~ 10 mL of cold clean NIB (for the first filtration only). 6. Repeat the filtering steps above for 100 µm filter and then the 40 µm filter. 7. Add 1/20 volume of cold NIB + 10% Triton to the filtrate 8. Divide into cold centrifuge tubes, ~5 grams of leaf material per tube , 4 to 8 tubes. 9. Centrifuge at 4°C at 2000 g for 10 minutes to pellet the nuclei. 10. Remove and discard the supernatant from all tubes. Note: it is possible to stop here and freeze the pellets at 80°C, but preferable to continue through the CTAB treatment and stop in alcohol precipitation.	

3: CTAB Treatment	Note: Make sure the Carlson Lysis Buffer is pre-warmed to 74°C. 1. Add 10 mL of Carlson Lysis Buffer + 25 µl of BME to each pellet. 2. Combine 2 pellet tubes (2 x 10 mL) into one tube, so there are half the number of tubes. 3. Incubate the closed tubes at 74°C for 2 hours, swirl every 30 minutes until finished. 4. Cool the tubes to room temperature.	
4: Double Extraction with Chloroform/Isoamyl 24:1	1. Add 20 mL (equal volume) of 24:1 chloroform/isoamyl alcohol to each tube. 2. Invert gently several times to mix. 3. Centrifuge 5000 g for 10 min at 4°C. 4. Transfer aqueous upper phase (non-green) to a new tube. 5. Repeat extraction by adding 20 mL of 24:1 chloroform/isoamyl to the aqueous phase from above. 6. Invert lightly to mix several times. 7. Centrifuge 5000 g for 10 min at 4°C. 8. Transfer aqueous upper phase to new tube.	
5: DNA Precipitation	1. To each 20 mL double extracted aqueous phase from above: a. Add 2 mL of 3 M NaOAc b. Mix gently c. Add 22 mL of isopropanol d. Mix gently 2. Precipitate at 4°C overnight.	

STEP	Day 2	Notes
1	1. Centrifuge at 4500 g for 90 minutes. 2. Discard supernatant. 3. Wash pellets with 70% ice cold ethanol, 40 mL/tube. 4. Spin at 4500 g for 90 min. 5. Discard supernatant. 6. Invert tubes on Kimwipes tissues. 7. Air dry 10 minutes, or until there is no residual ethanol in the tube.	
2: Prepare for QIAGEN® Genomic-Tip	1. GENTLY resuspend DNA pellets in a *total* of 10 mL of QIAGEN buffer G2 / sample (volume/tube = 10 mL/# of tubes). 2. Add RNase A (100 mg/mL) to the resuspended DNA pellets, 2 µl/mL of G2, or 20 µl total RNase for 10 mL of G2. 3. Swirl the tubes to well mix. 4. Incubate ~ 5 minutes at room temperature. 5. Add proteinase K to each tube, 10 µl proteinase K/mL of G2, or 100 µl total for a 10 mL sample. 6. Again swirl to mix well. 7. Incubate in a water bath at 50°C for 1 hour. 8. Cool to room temperature. Note: Consider setting up QIAGEN Genomic Tips during incubation and cooling. 9. Spin briefly to bring all liquid to the bottom of the tubes. 10. Pool all the tubes for a sample into one tube, or 10 mL/sample. 11. Pre-heat ~ 20 mL/sample QIAGEN buffer QF in a 50°C water bath.	

3: **QIAGEN Genomic-Tip DNA Purification**	SET-UP 1. Label Genomic Tip 500/G column(s) with the sample name(s). 2. Label 6 x 50 mL Falcon tubes /sample as follows: Column equilibration, Sample flow through, Wash 1, Wash 2, Wash 3, and Eluted Sample, with the sample name. Note: Save all tubes until DNA has been recovered from the column. 3. Place the column mounted in the column holder on top of the "Column equilibration" tube. 4. Add 10 mL of QIAGEN buffer QBT to the column. 5. Drain by gravity flow. 6. Move the column to the "Sample flow through" tube. 7. Load the room temp RNase and proteinase treated 10 mL sample on the column. 8. Allow the sample to load and the column to empty by gravity flow. COLUMN WASH 1. Move the column to the "Wash 1" tube. 2. Wash the column with 15 mL of QIAGEN buffer QC. 3. Load and empty the column by gravity flow. 4. Repeat the washing until the column has been washed 3 times, using "Wash 2" and "Wash 3" tubes. ELUTION 1. Move the column to the "Eluted Sample" tube. 2. Add 15 mL of 50°C QIAGEN buffer QF (from water bath). 3. Allow the column to drain by gravity to elute the sample.	
4: **DNA Precipitation**	1. Add 10.5 mL (0.7 volumes) of room-temperature isopropanol to the eluted DNA. 2. Swirl the tube to mix. Note: DNA precipitate should be visible in the form of white cotton-like threads. 3. Precipitate at 4°C overnight.	

STEP	Day 3	Notes
1	1. Centrifuge at >4500 g for 90 minutes at 4°C. 2. Discard supernatant. 3. Add 40 mL of ice cold 70% Ethanol. 4. Centrifuge at > 4500 for > 30 minutes at 4°C. 5. Discard supernatant. 6. Invert tubes on Kimwipes tissues. 7. After 5 minutes turn tubes on side and let dry until there is no ethanol remaining, ~10 minutes. 8. Add 500 µl to 1 mL of TE + 100 mM NaCl. 9. Swirl tubes to mix. 10. Incubate at 37°C overnight, swirling occasionally if possible.	

STEP	Day 4	Notes
1: **DNA Quantitation and QC**	1. Swirl the tube and keep overnight at 37°C. 2. Swirl tube once. 3. Spin tube to collect all of the liquid at the bottom of the tube. 4. Quantitate DNA with Nanodrop and Qubit systems. Note: The numbers should match. 5. QC size by running on a pulsed field gel electrophoresis system. 6. Aliquot the DNA in ~ 50 µg per tube. 7. Store DNA at -20°C.	

SMRTbell™ Library Preparation

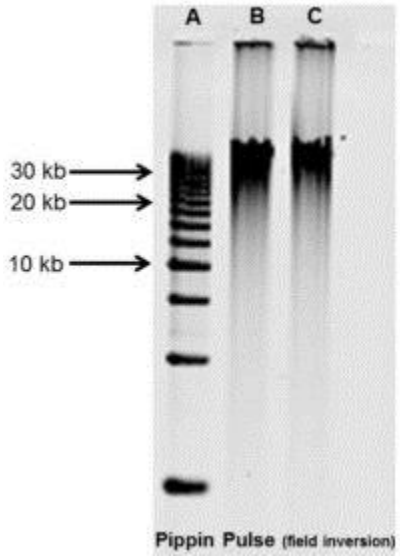
1. Prior to shearing, purify with 0.5X AMPure PB beads to remove the salt.
2. Shear to 20 kb with Covaris® g-TUBE device, following the **Guidelines for Preparing Size-Selected ~20kb SMRTbell™ Templates** protocol.
3. Size select using the Blue Pippin system, using a lower size limit of 7 to 8 kb and an upper size limit of 50 kb.

Example Results

DNA Yield

Strain	Input	Conc (ng/µl)	Volume (µl)	Total DNA (µg)
B	30 grams leaf tissue	312	600	187
C	30 grams leaf tissue	273	600	164

PFGE Gel QC



Pippin Pulse gel of DNA purified from 2 strains of *Arabidopsis*. Lane A, 2.5 kb ladder; lanes B and C, *Arabidopsis* DNA.

Conditions: Pippin Pulse (SW version 1.2), (Pulsed-Field Gel Electrophoresis) 9 hour run waveform type 10-48 kb