Protocol for Single Tetrad-stage Microspore Sequencing in Maize

From the article “Dissecting Meiotic Recombination based on Tetrad Analysis by Single Microspore Sequencing in Maize”

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Isolate single microspores from a single tetrad

1) In the field, select plants presenting tetrad-stage pollen, and harvest the top ~30 cm part containing tassels into a flask filled with water.
   Note 1: Under these conditions, the immature pollens would keep developing, so that fresh tetrad-stage cells can be provides from upper to lower spikelets continuously at intervals of ~30min.
2) In a spikelet there are three older and three younger anthers. Release cells from the three same-stage anthers into Isolation Buffer on a slide. Identify samples containing the last-stage tetrads, from which are easy to separate into microspores and do not adhere to the glass tools.
   Note 2: F1 tetrads have a high internal osmotic pressure. If micro-manipulating F1 tetrads, Isolation Buffer should be 27% D-sorbitol solution to balance the high pressure; if micro-manipulating tetrads from Inbred lines, much lower concentration (i.e. physiological concentration), of osmoticum is also sufficient. Do not use normal saline (NS) as Isolation Buffer, because metal ions may interfere with the amplification reaction later. From step 2) to step 5), cells should be kept in the Isolation Buffer.
3) Isolate a single tetrad into a new drop of Isolation Buffer using a thin glass pipette.
   Note 3: Use a thin glass pipette system and Programmable Microinjector PM 2000 (MDI, South Plainfield, USA) to isolate single tetrads (step 3)) and single microspores (step 5)), and to destroy the cell wall (step 4)), under 4× microscopy.
4) Repeated aspiration in and out was used to destroy the cell walls and disrupt the tetrads. Then, four microspores can be separated without cell wall.
5) One by one, place cells in less than 0.5μl Isolation Buffer into PCR wells filled with 4μl PBS (QIAGEN, Hilden, Germany). The PCR wells should be kept on ice.
   Note 4: When aspirating a microspore in the glass pipette, make sure it is still visible under the microscope. After blowing it to a PCR well, the cell should not be seen in the pipette any more, to ensure that the cell is now in the PCR well for the next reaction.

Lysis of cells

6) Add 3μl lysis Buffer (DLB : DTT = 11 : 1) into the PCR well containing a cell sample. Centrifuge briefly, then, incubate it at 65℃ for 10 min. Finally, Add 3μl Stop Buffer, centrifuge briefly, and keep the PCR well on ice.
   Note 5: Tips should not touch the surface of the sample inside PCR well when adding reagents. Do not shake the PCR well for mixing, to avoid losing the single cell sample and reducing the integrity of the DNA.
   Note 6: Step 6) to step 7) are based on standard protocol of QIAGEN REPLI-g Single Cell Kit.

MDA Amplification of single cell DNA

7) Prepare the following mixture: 29μl Reaction Buffer, 9μl sc d_2H_2O and 2μl reaction enzyme per one reaction. Add the mixture to PCR well containing 10μl sample. Centrifuge briefly and incubate at 30℃ for 8 h. Finally, incubate at 65℃ for 3min. Store it at -20℃ if not proceeding to the next step immediately.

Note 7: Similar to note 5, tips can't touch the surface of the sample inside PCR well when adding reagents. Do not shake the PCR well for mixing. These precautions avoid reducing the Integrity of DNA.
Note 8: MDA products are suitable for Single Nucleotide Variants (SNV) analysis, but not appropriate for quantification analysis (Copy Number Variations, CNV).

Quality control of whole-genome amplification products:

8) Dilute the amplification product 1:50. Use the following 10 markers to amplify the diluted DNA by PCR:

Maker	Primer Sequence	Tm
maker_chr1	5'-AAGTGGTGAGGTAAGCCTGC-3'/5'-ATAGGAGACACCCTGGGCAT-3'	60℃
maker_chr2	5'-CTCTTCCAATCGGGTTTGC-3'/5'-AATTGCACATAACAGAGGCG-3'	56℃
maker_chr3	5'-CTCAGGAGGAGGAAATGTGG-3'/5'-CTTCTGTCCGTGAAGGATGG-3'	60℃
maker_chr4	5'-AAGAACAGCATTGTCGTCACC-3'/5'-GTCCAGCGTCAGAGCTTACC-3'	58℃
maker_chr5	5'-AAAGCACTTACATCATGGGAAAC-3'/5'-TTGGTGTAGCTCCGATTTG-3'	58℃
maker_chr6	5'-CATAGTCCGATCTTGGTGACG-3'/5'-CATACAGGGAGTCACGGTCC-3'	60℃
maker_chr7	5'-AGCACCAGGAAGTTGTGAGG-3'/5'-CCAACTCGATACGAAGAGCC-3'	60℃
maker_chr8	5'-TCCAAGTCCCATGGCAGAAC-3'/5'-CCATGCTAGCTGATCGTCGT-3'	58℃
maker_chr9	5'-CCCGAGTTGCATGGAAGACA-3'/5'-CGTGCGTTTGAATTGGCTGA-3'	60℃
maker_chr10	5'-CCTTCTAATTAAAGTCAAAGCCA-3'/5'-CAACACCCAACATCCGTGCT-3'	58℃

The PCR mixture contains 1ul diluted DNA, 1ul forward primer (5mM), 1μl reverse primer (5mM), 1.5μl 10×PCR Buffer (Takara), 0.3μl dNTP (10mM), 0.15μl rTaq (Katara), 10.05μl d_2H_2O. Perform PCR with the following cycling parameters:

Process	Temperature	Duration	Cycles
Initial denaturation	94℃	3 min	1
Denaturation	94℃	30s	35
Annealing	Tm*	30s	
Extension	72℃	1 min	
Stop reaction	72℃	7 min	1
Store	4℃	Forever	1

*Tm is based on the GC content of primers. See the last table.

Detect the PCR product by 2% Agarose gel electrophoresis. Sample with no less than 8 of the 10 markers successfully amplified, is considered to be of a quality sufficient to make a

library.

Measuring DNA concentration of amplification product

9) Add 4μl amplification product into 100μl d_2H_2O. Measure the DNA concentration by Nanodrop 2000. The concentration should be approximately 40~50 ng/μl.

DNA Library construction

10) Dilute DNA to 1.2μg in 60μl. Then fragment it by sonication (Bioruptor UCD-200, Seraing, Belgium) at ~5℃for a few cycles (30s sonication and 30s rest per one cycle), so that most of the DNA fragments range from 300bp to 500bp.
    Note 9: Detection of fragments' size is by 2% Agarose gel electrophoresis; if most of the fragment are still larger than 500bp, continue sonicating a few more cycles until the suitable size of fragments are generated. The number of cycles depends on when the fragments reach 300bp to 500bp; the mean number of cycles would be ten.
    Note 10: Step 11) to Step 18) are based on the standard protocol of TruSeq DNA Sample Prep v2 Kit (Illumina, San Diego, US).
11) End repair: Add 40μl End Repair Mix and 10μl Resuspension Buffer to 50μl of DNA fragmented. After mix thoroughly, incubate it at 30℃ for 30min.
12) Purification #1st by Agencourt Ampure XP beads (Beckman Coulter, cat. no. A 63881): Keep the Beads at room temperature (RT) and vortex briefly. Add 160μl Beads to 100μl of the end repaired sample. Mix thoroughly, then incubate at RT for 15min. Place this sample on a magnetic stand at RT for 15min. Aspirate and discard the supernatant using a 200μl pipette. Add 200μl fresh 80% EtOH, incubate at RT for 30s, remove and discard the supernatant. Add 200μl fresh 80% EtOH again to complete the washing, incubate at RT for 30s, remove and discard the supernatant. Let the sample dry at RT for ~5-10min. Remove the sample containing Beads from the magnetic stand, then add 17.5μl Resuspension Buffer to the Beads. Mix thoroughly and incubate at RT for 2min. Place the sample again on the magnetic stand at RT for 5min. Finally, transfer 15μl of the supernatant into a new PCR well for the next step.
13) Adenylate 3' end: Add 2.5μl Resuspension Buffer and 12.5μl A Tailing Mix to the PCR well containing 15μl of sample purified as described above. Mix thoroughly, and incubate at 37℃ for 30min.
14) Ligate Adapters: Add 2.5μl Resuspension Buffer, 2.5μl Index and 2.5μl Ligation Mix to the PCR well containing 30μl of A Tailing sample. Mix thoroughly, and incubate at 30℃ for 10min. Then add 5μl Stop Buffer immediately, and mix thoroughly.
15) Purification #2nd using Agencourt Ampure XP beads (Beckman Coulter, cat. no. A 63881): Place the Beads at room temperature (RT) and vortex. Add 42.5μl Beads to 42.5μl of the Ligation sample. Mix thoroughly, then incubate at RT for 15min. Place the sample on a magnetic stand at RT for 5min. Remove and discard the supernatant using a 200μl pipette. Add 200μl fresh 80% EtOH, incubate at RT for 30s, remove and discard the supernatant. Add 200μl fresh 80% EtOH again to complete the washing, incubate at RT for 30s, remove and

discard the supernatant. Let the sample dry at RT for ~5-10min. Keep this sample containing Beads away from the magnetic stand, then add 22.5µl Resuspension Buffer. Mix thoroughly and incubate at RT for 2min. Place the sample on the magnetic stand at RT for 5min. Finally, transfer 20µl of the supernatant into a new PCR well for the next step.

16) Size selection by MinElute Gel Extraction Kit (QIAGEN, Hilden, Germany, cat. no. 28604): After 2% Agarose gel electrophoresis, excise gel segment containing the main target band, which should be limited in size range of 100bp. Add 900µl QG Buffer to 2ml tube containing 300mg gel. Incubate it at RT until gel melts completely. Add 300µl isopropanol, and mix thoroughly. Place a MinElute column in a new 2ml collection tube. Transfer 750µl of the melted sample in the MinElute column, centrifuge for 1min at 13000 rpm and discard the flow-through. Repeatedly transfer another 750µl and centrifuge. Add 500µl QG Buffer to the column, centrifuge for 1min and discard flow-through. Add 750µl PE Buffer to the column, stand for 5min, then centrifuge for 1min and discard flow-through. Centrifuge for 1min one more time to remove residual ethanol completely. Place the column into a clean 1.5ml tube. Let it dry at RT for 5min. Add 23µl EB Buffer onto the center of column membrane, incubate it at RT for 3min, then centrifuge it for 1 min. The flow-through is collected and added onto the center of the same column membrane, incubate it at RT for 3min, then centrifuge for 1 min. Now the flow-thorough is the sample purified for PCR reaction.

17) PCR amplification of library: Add 5µl Primer and 25µl PCR Mix to 20µl of the Purified sample above. Mix thoroughly, and perform PCR with the following cycling parameters:

Process	Temperature	Duration	Cycles
Initial denaturation	98℃	30s	1
Denaturation	98℃	10s	
Annealing	60℃	30s	10
Extension	72℃	30s	
Stop reaction	72℃	300s	1
Store	4℃	Forever	1

18) Purification #3rd by Agencourt Ampure XP beads (Beckman Coulter, cat. no. A 63881): Keep the Beads at room temperature (RT) and vortex. Add 50µl Beads to 50µl of the PCR sample. Mix thoroughly, then incubate it at RT for 15min. Place this PCR sample on a magnetic stand at RT for 5min. Remove and discard the supernatant using a 200µl pipette. Add 200µl fresh 80% EtOH, incubate at RT for 30s, remove and discard the supernatant. Add 200µl fresh 80% EtOH again to complete the washing, incubate at RT for 30s, remove and discard the supernatant. Let the sample dry at RT for ~5-10min. Place the sample with the Beads away from the magnetic stand, then add 22.5µl Resuspension Buffer to the Beads. Mix thoroughly and incubate at RT for 2min. Place the sample on the magnetic stand at RT for 5min again. Finally, transfer 20µl of the supernatant into a new PCR well; it is the library. Store it at -20℃, up to two months. Go on to qualify and quantify it for pair-end sequencing on Illumina Hiseq 2000 platform.