Massive Analyses of cDNA Ends (MACE)

Library Preparation Kit (V2.0) – User Guide

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Material and Equipment

Equipment
Vortex mixer
Benchtop microcentrifuge for 0.2-1.5 ml tubes
Magnetic stand (suitable for 1.5 ml tubes)
Thermocycler (suitable for 0.2 ml tubes)
Sonication Device (Diagenode Bioruptor) with 0.5 ml tube holder
Calibrated single-channel pipettes
Automated microfluidic electrophoresis station (Agilent 2100 Bioanalyzer or 2200 TapeStation)
Fluorometer (Qubit)

Customer supplied consumables and labware
SPRI Beads (Agencourt AMPure XP or Agencourt SPRIselect)
PCR clean-up kit (NucleoSpin or Qiagen)
DNA High Sensitivity Assay (Qubit)
85 % Ethanol (freshly prepared)
Pipette filter tips (with aerosol barriers, nuclease-free)
1.5 ml tubes (low binding, nuclease-free)
0.2 ml tubes (nuclease-free)
0.5 ml tubes (nuclease-free) for DNA shearing (Diagenode Ref. No. WA-004-0500)

MACE Kit supplied consumables*	
For all Reactions	Nuclease-free water
	TE buffer
First Strand Reaction	Oligo ID 1, 2, 3, 4, 5, 6
	Solution 1
	Enzyme Mix 1
	Enzyme Mix 2
Second Strand Reaction	Solution 2
	Solution 3
	Solution 4
	Enzyme Mix 3
	Enzyme Mix 4
	Enzyme Mix 5
End Repair	Solution 5
	Enzyme Mix 6
Ligation	MACE Adapter
	Enzyme Mix 7
PCR Amplification	Solution 7
	Enzyme Mix 8

*Store all reagents at -20 °C.

1. cDNA Synthesis

Assess the RNA quality of each purified and DNase I treated sample with Agilent Bioanalyzer or TapeStation (RNA Kit) and quantify the concentration with the Qubit (RNA Assay).

Distribute the RNA samples (at least 2 µg in nuclease-free water) and the Oligo ID 1-6 to 0.2 ml tubes.

Sample	Sample name	ID
1		
2		
3		
4		
5		
6		

	Volume per reaction
RNA sample	10 µl
Oligo ID	2 µl

- prepare the mix of sample RNA and oligo at room temperature
- mix carefully by pipetting up and down
- incubate in a thermal cycler as follows:

  10 min at 70 °C
  cool down to 45 °C with a ramp rate of 0.1 °C per second
  hold at 45 °C until adding the First Strand Master Mix (see below)

First Strand Master Mix

	Volume per reaction
Solution 1	6 µl
Enzyme Mix 1	2 µl

- prepare the First Strand Master Mix at room temperature
- mix carefully by vortexing and spin down
- add 8 µl of the First Strand Master Mix to each sample at 45 °C
- mix carefully by pipetting up and down
- incubate in a thermal cycler as follows:

  30 min at 50 °C
  30 min at 55 °C
  15 min at 60 °C
  hold at 4 °C until adding the Enzyme Mix 2 (see below)

	Volume per reaction
Enzyme Mix 2	1 µl

- add 1 µl of Enzyme Mix 2 to each sample
- mix carefully by vortexing and spin down
- incubate in a thermal cycler as follows:

  1 hour at 37 °C
  15 minutes at 80 °C
  hold at 4 °C until adding the Second Strand Master Mix (prepared as below)

Second Strand Master Mix

	Volume per reaction
Nuclease-free water	91.5 µl
Solution 2	33 µl
Enzyme Mix 3	4 µl
Enzyme Mix 4	0.5 µl

- prepare the Second Strand Master Mix on ice
- mix carefully by vortexing and spin down
- add 129 µl of the Second Strand Master Mix to each sample of the First Stand Reaction (21 µl) on ice
- mix carefully and slowly by pipetting up and down
- incubate in a thermal cycler as follows:

  2h at 16 °C
  hold at 4 °C until adding the Solution 3 (see below)

	Volume per reaction
Solution 3	2.5 µl

- add 2.5 µl of Solution 3 to each sample
- mix carefully by vortexing and spin down
- incubate in a thermal cycler as follows:

  20 min at 75 °C
  hold at 4 °C until adding Enzyme Mix 5 (see below)

	Volume per reaction
Enzyme Mix 5	1 µl

- add 1 µl of Enzyme Mix 5 to each sample
- mix carefully by vortexing and spin down
- incubate in a thermal cycler as follows:

  30 min at 37 °C
  hold at 4 °C until adding Solution 4 (see below)

	Volume per reaction
Solution 4	2.5 µl

- add 2.5 µl of Solution 4 to each sample
- mix carefully by vortexing and spin down
- incubate in a thermal cycler as follows:

  20 minutes at 70 °C
  hold at 4 °C
- store at -20 °C or continue with cDNA purification.

2. Purification and Pooling

Purify each cDNA sample with silica columns for DNA purification (NucleoSpin PCR Clean-up or QIAquick PCR Purification Kit). Elute with 30 µl TE Buffer in low binding tubes.

Quantify the concentration of each cDNA sample with the Qubit DNA High Sensitivity Assay. Use 2 µl of cDNA for quantification.

For pooling we recommend to use equal amounts of cDNA for each sample (at least 10 ng of cDNA per sample, but not more than 150 ng in total). Store the pooled sample at -20 °C or continue with fragmentation.

3. Fragmentation

- Adjust the sample volume with TE Buffer to 100 µl total volume
- Transfer to the 0.5 ml tubes for DNA Shearing

Sonication should be performed as described by the manufacture protocol. The aim is to get an average DNA fragment size of 200 bp.

We developed a standard protocol using the Diagenode Bioruptor (UCD 300) sonication device with the tube holder for 0.5 ml tubes (Ref. No. UCD-pack 0.5) and the corresponding Bioruptor 0.5 ml Microtubes for DNA Shearing (Ref. No. WA-004-0500).

For the majority of the samples this protocol allows you to shear your DNA to the preferable size of 200 bp, however actual results may vary depending on the amount and the quality of your starting material.

Getting started:

1. Switch on the Bioruptor Water Cooler and equilibrate to 4 °C.
2. If necessary add TE Buffer to fill up the sample to 100 µl, transfer to the 0.5 ml Bioruptor Microtubes and store the DNA samples on ice for 5 minutes before sonication.
3. Spin down shortly before starting the sonication.

4. The tube holders should always be completely filled with tubes. Never leave empty spaces in the tube holder. Fill the empty spaces with tubes containing the same volume of water (100 µl).

Sonication program:

- Power setting: L position (Low)
- 30 s ON, 30 s OFF
- 4 °C
- 15-30 cycles
- Briefly centrifuge samples after every 5 cycles of sonication

Keep aliquots (1-2 µl) for Bioanalyzer or TapeStation (DNA High Sensititvity Kit), check fragmentation before purification, re-fill the volume to 100 µl with nuclease-free water.

Assess the fragment size on the Agilent Bioanalyzer or TapeStation (DNA High Sensitivity Kit).

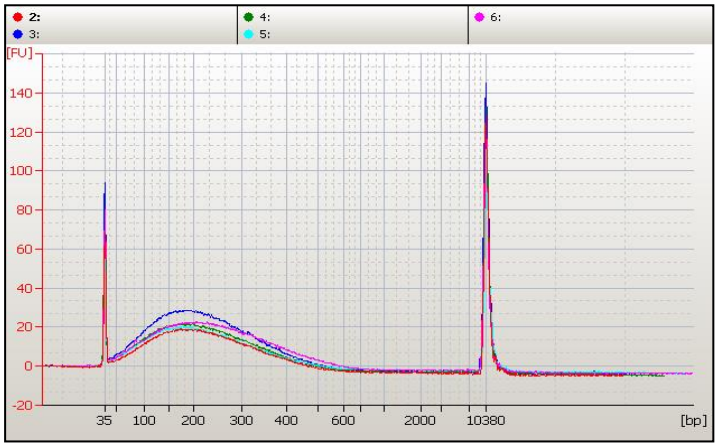

Figure 1: Example of DNA size distribution of different samples after 20 cycles of sonication.

4. Bead Clean up

You need:

- SPRI Beads
- 85 % Ethanol
- Elution Buffer
- 2x 1.5 ml low binding tube
- Magnetic stand

SPRI Beads: use a ratio of 1.0 volumes of your sample to 1.2 volumes of the beads

1. Remove the SPRI beads from storage (4 °C) and equilibrate to room temperature for at least 30 min.
2. Mix the SPRI beads thoroughly by vortexing for 30 s before pipetting.
3. Add 120 µl of SPRI beads to 100 µl of the sample (use 1.5 ml low binding tubes).
4. Gently pipette the entire volume up and down 10 times to mix thoroughly.
5. Incubate for 5 minutes at room temperature.
6. Place the tube on the magnetic stand for 5 minutes at room temperature until the liquid appears completely clear.
7. Carefully remove the supernatant (optional: keep the supernatant). Take care not to disturb the beads.
8. Add carefully 180 µl of freshly prepared 85 % Ethanol without disturbing the beads. Do not remove the tube from the magnetic stand.
9. Incubate 1 min at room temperature, then remove and discard the supernatant. Take care not to disturb the separated magnetic beads.
10. Briefly centrifuge the tube to collect the remaining Ethanol at the bottom of the tube and place it back on the magnetic stand.
11. Remove the remaining Ethanol and air-dry the beads for 1 min on the magnetic stand with the cap open.
12. Take the tube from the magnetic stand and add 16 µl nuclease-free water.
13. Gently pipette the entire volume up and down 10 times to mix thoroughly. Ensure that the beads are completely rehydrated and resuspended.
14. Incubate for 2 min at room temperature.
15. Place the tube on the magnetic stand for 5 min and save the supernatant to a fresh 1.5 ml low binding tube.

5. End Repair

	Volume
Solution 5	6 µl

- add 6 µl of Solution 5 to the purified sample (16 µl) at room temperature
- mix carefully by vortexing and spin down

	Volume
Enzyme Mix 6	3 µl

- add 3 µl of Enzyme Mix 6 to the sample at room temperature
- mix carefully by vortexing and spin down
- incubate in a thermal cycler as follows:

  30 min at 20 °C
  20 min at 75 °C
  hold at 4 °C

6. Adapter Ligation

	Volume
MACE Adapter	1 µl

- add 1 µl of MACE Adapter to the sample at room temperature
- mix carefully by vortexing and spin down

	Volume
Enzyme Mix 7	1 µl

- add 1 µl of Enzyme Mix 7 to the sample at room temperature
- mix carefully by vortexing and spin down
- incubate in a thermal cycler as follows:

  15 min at 20 °C
  hold at 4 °C

7. Bead Clean up

You need:

- SPRI Beads
- 85 % Ethanol
- Elution Buffer
- 2x 1.5 ml low binding tube
- Magnetic stand

SPRI Beads: use a ratio of 1.0 volumes of your sample to 1.2 volumes of the beads

16. Remove the SPRI beads from storage (4 °C) and equilibrate to room temperature for at least 30 min.
17. Mix the SPRI beads thoroughly by vortexing for 30 s before pipetting.
18. Add 120 µl of SPRI beads to 100 µl of the sample (use 1.5 ml low binding tubes).
19. Gently pipette the entire volume up and down 10 times to mix thoroughly.
20. Incubate for 5 minutes at room temperature.
21. Place the tube on the magnetic stand for 5 minutes at room temperature until the liquid appears completely clear.
22. Carefully remove the supernatant (optional: keep the supernatant). Take care not to disturb the beads.
23. Add carefully 180 µl of freshly prepared 85 % Ethanol without disturbing the beads. Do not remove the tube from the magnetic stand.
24. Incubate 1 min at room temperature, then remove and discard the supernatant. Take care not to disturb the separated magnetic beads.
25. Briefly centrifuge the tube to collect the remaining Ethanol at the bottom of the tube and place it back on the magnetic stand.
26. Remove the remaining Ethanol and air-dry the beads for 1 min on the magnetic stand with the cap open.
27. Take the tube from the magnetic stand and add 25 µl nuclease-free water.
28. Gently pipette the entire volume up and down 10 times to mix thoroughly. Ensure that the beads are completely rehydrated and resuspended.
29. Incubate for 2 min at room temperature.
30. Place the tube on the magnetic stand for 5 min and save the supernatant to a fresh 1.5 ml low binding tube.

8. PCR Amplification

	Volume
Solution 7	25 µl
Enzyme Mix 8	1 µl

- Prepare the PCR Mix and mix well.
- Add 26 µl of the Master Mix to 24 µl template.
- mix carefully by vortexing and spin down
- place the tubes in the thermocycler.

Thermocycler program:

Cycle Steps	Temperature	Time	Cycles
Initial Denaturation	95 °C	5 min	1
Denaturation	98 °C	20 s	maximal 14
Annealing	65 °C	20 s	
Extension	72 °C	60 s	
Final Extension	72 °C	5 min	1
Hold	4 °C	∞	

The number of cycles needed for optimal yield may vary depending on the amount of cDNA.

We recommend choosing the optimal cycle number based on a Test PCR.

Optional: Check different number of cycles (e.g. 8, 10, 12, 14) with a test PCR.

To set up a test PCR use 2.5 µl (1/10 Volume) of template, 12.5 µl Solution 7, 0.5 µl Enzyme Mix 8 and fill up with nuclease-free water to 25 µl. Run the PCR with a pausing step after the extension at 72 °C at 8, 10, 12 and 14 cycles. Remove 5 µl of the reaction volume during the pausing step (30 seconds) and place it in a tube with 1 µl loading dye (ThermoFisher R0631) on ice. Load all points and a DNA ladder (ThermoFisher SM0331) on a 2 % Agarose gel (thickness of 0.5 cm), run it for 40 min at 5 V/cm and stain it with Ethidium bromide. Choose the number of cycles with the weakest but clearly visible band.

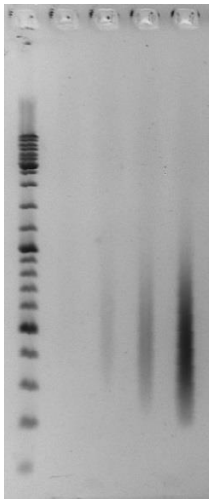

Figure 2: Ethidium bromide stained Agarose gel of a Test PCR with 8, 10, 12 and 14 cycles. Lane 1: GeneRuler DNA Ladder Mix (500 ng). Lane 2-5: Test PCR with 8, 10, 12 and 14 cycles. In this case the massive PCR was performed with 10 cycles.

9. Bead Clean-up

You need:

- SPRI Beads
- 85 % Ethanol
- Elution Buffer
- 4 x 1.5 ml low binding tube
- Magnetic stand

Pool the 5 PCR reactions into one 1.5 ml low binding tube (sample volume is 250 µl), mix and centrifuge down. Pipette 250 µl to another 1.5 ml tube.

SPRI Beads: use a ratio of 1.0 volumes of your sample to 0.85 volumes of the beads

1. Remove the SPRI beads from storage (4 °C) and equilibrate to room temperature for at least 30 min
2. Mix the SPRI beads thoroughly by vortexing for 30 s before pipetting.
3. Add 85 µl of SPRI beads to 100 µl of the sample.
4. Gently pipette the entire volume up and down 10 times to mix thoroughly.
5. Incubate for 5 minutes at room temperature.
6. Place the tube on the magnetic stand for 5 minutes at room temperature until the liquid appears completely clear.
7. Carefully remove the supernatant (optional: keep the supernatant). Take care not to disturb the beads.
8. Add carefully 500 µl of freshly prepared 85 % Ethanol without disturbing the beads. Do not remove the tube from the magnetic stand.
9. Incubate 1 min at room temperature, then remove and discard the supernatant. Take care not to disturb the separated magnetic beads.
10. Briefly centrifuge the tube to collect the remaining Ethanol at the bottom of the tube and place it back on the magnetic stand.
11. Remove the remaining Ethanol and air-dry the beads for 1 min on the magnetic stand with the cap open.
12. Take the tube from the magnetic stand and add 100 µl nuclease-free water.
13. Gently pipette the entire volume up and down 10 times to mix thoroughly. Ensure that the beads are completely rehydrated and resuspended.
14. Incubate for 2 min at room temperature.
15. Place the tube on the magnetic stand for 5 min and save the supernatant to a fresh 1.5 ml low binding tube.
16. Add 85 µl fresh SPRI beads to 100 µl of the sample.

17. Gently pipette the entire volume up and down 10 times to mix thoroughly.
18. Incubate for 5 minutes at room temperature.
19. Place the tube on the magnetic stand for 5 minutes at room temperature until the liquid appears completely clear.
20. Carefully remove the supernatant (optional: keep the supernatant). Take care not to disturb the beads.
21. Add carefully 200 µl of freshly prepared 85 ml Ethanol without disturbing the beads. Do not remove the tube from the magnetic stand.
22. Incubate 1 min at room temperature, then remove and discard the supernatant. Take care not to disturb the separated magnetic beads.
23. Briefly centrifuge the tube to collect the remaining Ethanol at the bottom of the tube and place it back on the magnetic stand.
24. Remove the remaining Ethanol and air-dry the beads for 1 min on the magnetic stand with the cap open.
25. Take the tube from the magnetic stand and add 20 µl nuclease-free water.
26. Gently pipette the entire volume up and down 10 times to mix thoroughly. Ensure that the beads are completely rehydrated and resuspended.
27. Incubate for 2 min at room temperature.
28. Place the tube on the magnetic stand for 5 min and save the supernatant to a fresh 1.5 ml low binding tube
29. Store the sample at -20 °C or continue.

Assess the library quality on the Agilent Bioanalyzer or TapeStation (DNA High Sensitivity Kit). If a peak at around 80 bp (primers) or around 150 bp (adaptor-dimer) is shown in the Agilent traces, repeat the SPRI clean-up (step 10).

Assess the library concentration with the Qubit (High Sensitivity Kit). Dilute the library to the required concentration for Illumina sequencing (2 or 4 nM) according to the Qubit and Bioanalyzer measurement.

To process the raw sequencing data upload the bcl or fastq files with the Data Uploader after login at http://tools.genxpro.net.

10. Appendix

Oligo ID	Index Sequence
1	ATCACGAT
2	CGATGTAT
3	TTAGGCAT
4	TGACCAAT
5	ACAGTGAT
6	GCCAATAT

Table 1: Oligo ID Sequence (Illumina TruSeq Index)

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MACE Library Prep Kits are covered by issued and/or pending patents.

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