Protocol: XENO-LIBERA

❖ General Guidelines Before Starting

- Ensure reaction components are added in the order listed.
- Thoroughly mix the reactions by pipetting up and down 15-20 times.

A. 3' Adapter Ligation

1. On ice, prepare the 3' Adapter Ligation reaction and mix by pipetting.

Component	Volume
DNase/RNase-free water	0.3 µl
Adapter 1	1 µl
RNase Inhibitor	1 µl
Ligase A	1 µl
Ligase A Buffer	2.7 µl
PEG	11 µl
Template RNA (added in step 2)	10 µl
Total Volume	27 µl

2. Add template RNA to each tube containing the 3' ligation Master Mix and mix by pipetting.
3. Incubate for 1 hour at 28°C, and then for 20 min at 65°C, and then hold at 4°C for at least 5 min.
4. Proceed immediately to 5' ligation.

B. 5' Adapter Ligation

1. On ice, prepare the 5' Adapter Ligation reaction and mix by pipetting.

Component	Volume
Product from "A. 3' Adapter Ligation'	27 µl
DNase/RNase-free water	2 µl
Ligase B Buffer	8 µl
RNase Inhibitor	1 µl
Ligase B	1 µl
Adapter 2	1 µl
Total Volume	40 µl

2. Incubate for 30 min at 28°C, and then for 20 min at 65°C, and then hold at 4°C.
3. Proceed immediately to reverse transcription.

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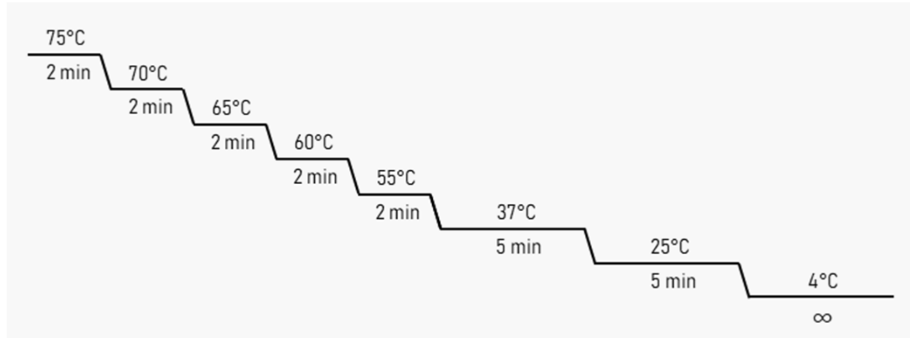
C. Reverse Transcription

1. Add 2 µl AD cleanup to each tube and mix by pipetting.
2. Incubate the tubes with the following program.



3. On ice, prepare the reverse transcription reaction and mix by pipetting.

Component	Volume
Product from "B. 5' Adapter Ligation"	42 µl
RT Primer (SRI 1~12)	2 µl
DNase/RNase-free water	2 µl
RT Buffer	12 µl
RNase Inhibitor	1 µl
RTase	1 µl
Total Volume	60 µl

4. Incubate for 1 hour at 50°C, and then for 15 min at 70°C, and then hold at 4°C for at least 5 min.

D. Clean-up

1. Mix 1 volumes (100 µl) of product from "C. Reverse Transcription" with 5 volumes (500 µl) of DNA Binding Buffer (DB).

   *Note : add 40 µl DNase/RNase-free distilled water to the reverse transcription product
2. Transfer the mixture to the Library spin column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
3. Add 700 µl of Washing Buffer P1 to the column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
4. Add 700 µl of Washing Buffer P2 to the column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
5. Add 700 µl of Washing Buffer P3 to the column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
6. Centrifuge the column at 12,300 x *g* for 2 min to dry the membrane.
7. Transfer the column to a new 1.5 ml microcentrifuge tube and add at least 40 µl of Elution Buffer(EB) or DNase/RNase-free distilled water to the center of the membrane.
8. Centrifuge the column at 12,300 x *g* for 1 min to elute the DNA.

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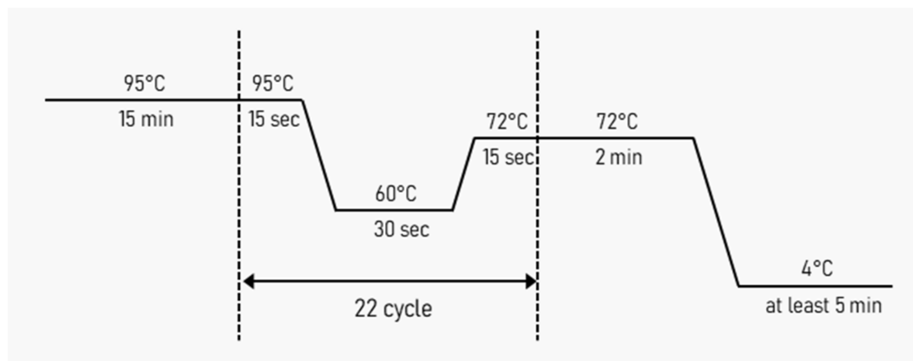
E. Amplification

1. On ice, prepare the amplification reaction and mix by pipetting.

Component	Volume
Product from "D. Clean-up"	20 µl
AMP Buffer	16 µl
HS Taq	1 µl
Forward Primer	2 µl
Reverse Primer	2 µl
DNase/RNase-free Water	39 µl
Total Volume	80 µl

2. Incubate the tubes with the following program.



F. Clean-up

1. Mix 1 volumes (100 µl) of product from "E. Amplification" with 3 volumes (300 µl) of DNA Binding Buffer (DB).
   *Note : add 20 µl DNase/RNase-free distilled water to the amplification product
2. Transfer the mixture to the Library spin column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
3. Add 700 µl of Washing Buffer P1 to the column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
4. Add 700 µl of Washing Buffer P2 to the column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
5. Add 700 µl of Washing Buffer P3 to the column and centrifuge at 2,000 x *g* for 1 min. Discard the flow-through.
6. Centrifuge the column at 12,300 x *g* for 2 min to dry the membrane.
7. Transfer the column to a new 1.5 ml microcentrifuge tube and add at least 40 µl of Elution Buffer(EB) or DNase/RNase-free distilled water to the center of the membrane.
8. Centrifuge the column at 12,300 x *g* for 1 min to elute the DNA.

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