1.1 Preparation 1st strand reaction buffer and random primer mix

1.1.1 5’

- 1st strand reaction buffer ● 8 uL
- Random primer ● 2 uL
- NF water 10 uL

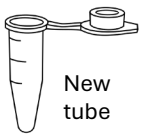
Mix well
Keep on ice

1.2 mRNA isolation, fragmentation, and priming

1.2.1 Dilute total RNA with NF water to 50 uL 5’

1.2.2 3’

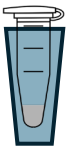
- Oligo dT beads 20 uL
- RNA binding buffer 2X 100 uL



New tube

1.2.3 Pipetting 6x
Spin down

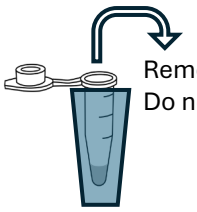
1.2.4 5’



Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear

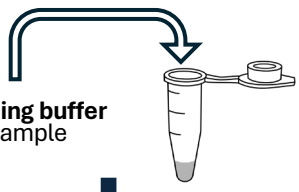
1.2.5 3’



Remove supernatant
Do not disturb the beads

1.2.6 Remove from rack

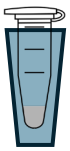
1.2.7 3’



RNA binding buffer
100 uL / sample

Pipetting 6x

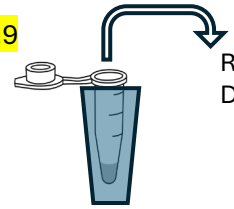
1.2.8 5’



Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear

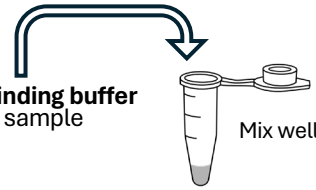
1.2.9 3’



Remove supernatant
Do not disturb the beads

Remove from rack

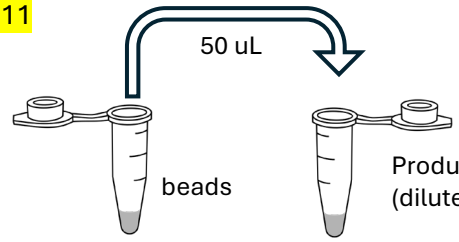
1.2.10 3’



RNA binding buffer
50 uL / sample

Mix well by pipetting 6x

1.2.11 5’



50 uL

beads

Product of step 1.2.1 (diluted RNA)

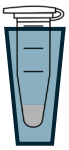
1.2.12 Thermocycler (heated lid >75 °C)
65°C 5’
4°C ∞ 7’

1.2.13 Remove from thermocycler

1.2.14 5’

Mix well by pipetting
Incubate RT 5’

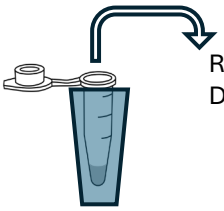
1.2.15



Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear 5’

1.2.16 5’

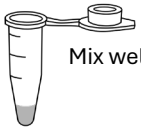


Remove supernatant
Do not disturb the beads

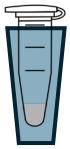
1.2.17 Remove from rack

1.2.18 5’

Wash buffer 200 uL

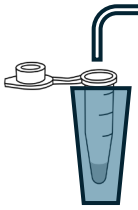


Mix well by pipetting

1.2.19  5’

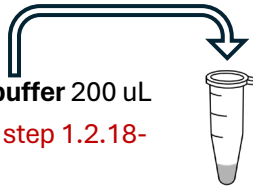
Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear

1.2.20  5’

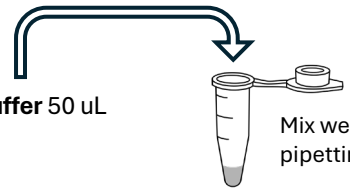
Remove supernatant
Do not disturb the beads

1.2.21 Remove from rack

1.2.22  5’

Wash buffer 200 uL
(repeat step 1.2.18-1.2.21)

Mix well by pipetting

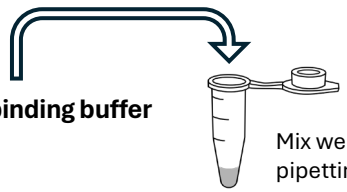
1.2.23  5’

Tris buffer 50 uL

Mix well by pipetting

1.2.24 Thermocycler
(heated lid >90 °C)
80°C 2’
25°C ∞  5’

1.2.25 Remove from thermocycler when reach 25°C

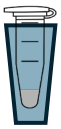
1.2.26  5’

RNA binding buffer
50 uL

Mix well by pipetting

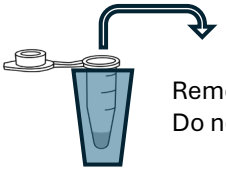
1.2.27  5’

Incubate RT 5’

1.2.28  5’

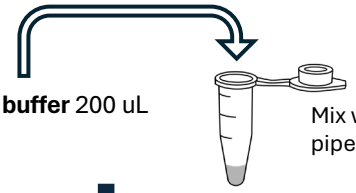
Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear

1.2.29  5’

Remove supernatant
Do not disturb the beads

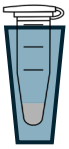
1.2.30 Remove from rack

1.2.31  5’

Wash buffer 200 uL

Mix well by pipetting

1.2.32 Spin down briefly

1.2.33  5’

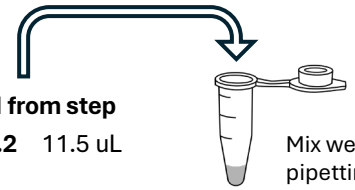
Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear

1.2.34  5’

Completely remove supernatant.
Do not disturb the beads.
Spin down the tube, place on the magnetic rack and with 10uL tip remove all of the wash buffer

1.2.35 Remove from rack

1.2.36  5’

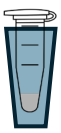
MM from step 1.1.2 11.5 uL

Mix well by pipetting

1.2.37 Thermocycler
(heated lid 105°C)
94°C 15’
4°C ∞,  17’

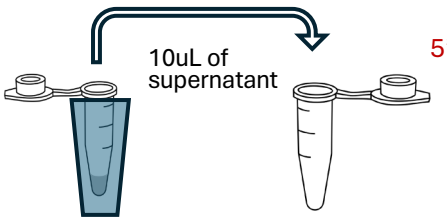
immediately transfer the tube to ice for 1 minute as soon as it cool enough to handle

Quickly Spin down

1.2.38  5’

Magnetic rack

Incubate RT 2- 5’
Or until sol. is clear

1.2.39  5’

10uL of supernatant

1.2.40 

Place on ice, immediately proceed to 1st strand cDNA synthesis

1. 3 First strand cDNA synthesis

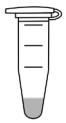
1.3.1 — 5’

- Product of 1.2.40 10 uL
- NF water 8 uL
- **NEBNext 1st strand cDNA Synthesis enzyme mix** 🟣 2 uL

1.3.2 — Mix well by pipetting

1.3.3 — 50’

Thermocycler (heated lid >80 °C)
25°C 10’
42°C 15’
70°C 15’
4°C ∞



1. 4 Second strand cDNA synthesis

1.4.1 — 5’

- Product of 1.3.4 20 uL
- **NEBNext 2nd strand synthesis reaction buffer** 🟡 8 uL
- **NEBNext 2nd strand synthesis enzyme mix** 🟡 4 uL
- NF water 48 uL

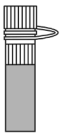
1.4.2 — Mix well by pipetting (on ice!!)

1.4.3 — 60’

Thermocycler (heated lid : off)
16°C 1 hour

1. 5 Purification of dsDNA

1.5.1



Vortex Ampure beads to resuspend well

1.5.2 — 10’

144 uL

Mix well by pipetting or vortexing

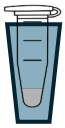
1.4.3 product

1.5.3

Incubate in RT for 5’

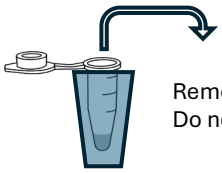
Spin down

1.5.4 — 5’



Wait until sol.clear

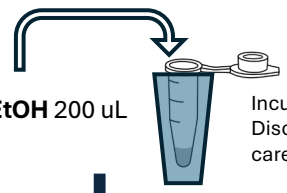
5’



Remove supernatant
Do not disturb the beads

1.5.5 — 3’

80% EtOH 200 uL

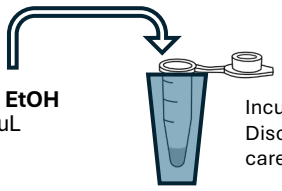


Incubate for 30’’
Discard supernatant carefully

1.5.6

3’

80% EtOH 200 uL



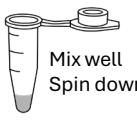
Incubate for 30’’
Discard supernatant carefully

1.5.7 — 10’

Dried-up beads 5-10’
Remove from rack

1.5.8 — 7’

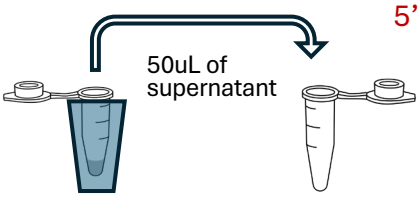
0.1x TE Buffer 53 uL



Mix well
Spin down, incubate for 2’

Place the tube on magnetic rack
Wait until sol.clear

1.5.9 — 5’



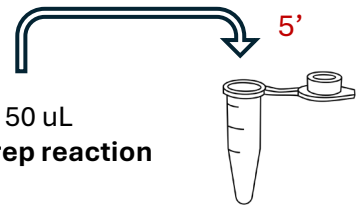
50uL of supernatant

Safe stop point

1.6 End Preparation of cDNA library

1.6.1 — 5’

- Product of 1.5.9 50 uL
- **NEBNext End Prep reaction buffer** 7 uL
- **NEBNext End Prep enzyme mix** 3 uL



1.6.2 — Mix well by pipetting (set the 100ul pipette to 50 uL

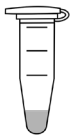
1.6.3 — 60’

Thermocycler (heated lid >75 °C)
20°C 30’
65°C 30’
4°C ∞

Proceed immediately to adaptor ligation!!

1.7 Adaptor Ligation

1.7.1 — Dilute **NEBNext adaptor** in ice-cold **Adaptor Dilution Buffer,** and keep on ice — 1’



1.7.2 — 5’

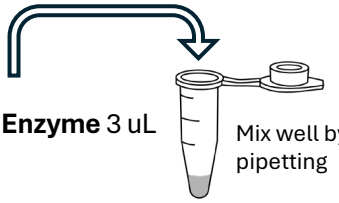
- Product of 1.6.4 60 uL
- Diluted adaptor (step 1.7.1) 2.5 uL
- **NEBNext Ligation Enhancer** 1 uL
- **NEBNext Ligation Master Mix** 30 uL

1.7.3 — Mix well by pipetting (set the 100ul pipette to 80 uL)
Spin down

1.7.4 — Thermocycler 20°C 15’ — 15’

1.7.5 — 5’

USER Enzyme 3 uL — Mix well by pipetting



Product of 1.7.4

1.7.6 — Thermocycler (heated lid >45°C)
37°C 15’ — 15’

Purification process!!

1. 8 Purification of Ligation Reaction

1.8.1 — 87 uL — 5’

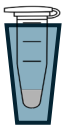
Mix well by pipetting or vortexing

1.7.6 product

1.8.2 — Incubate in RT for 10’ — 10’

Spin down

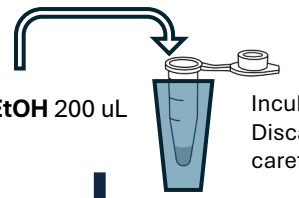
1.8.3 — 5’



Wait until sol.clear
Remove the supernatant

1.8.4 — 1’

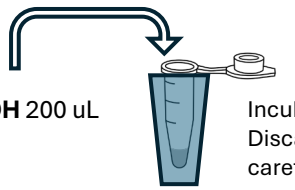
80% EtOH 200 uL



Incubate for 30’’
Discard supernatant carefully

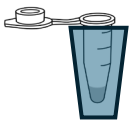
1.8.5 — 1’

80% EtOH 200 uL



Incubate for 30’’
Discard supernatant carefully

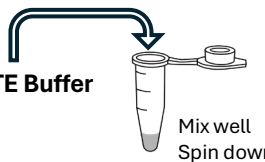
1.8.6-1.8.7 — 5’



Spin the tube
Put back in the rack
Dried-up completely

1.8.8 — 5’

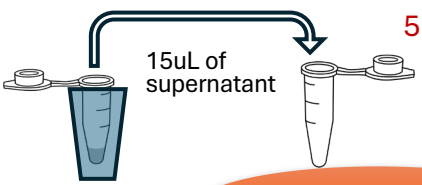
0.1x TE Buffer 53 uL



Mix well
Spin down, incubate for 2’

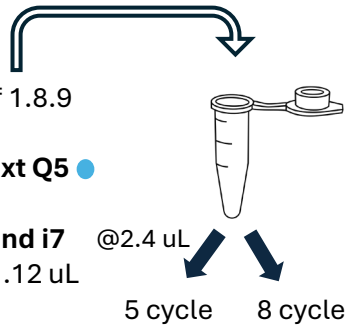
Put back in the rack

1.8.9 — 5’



Safe stop point

Small Scale PCR reaction



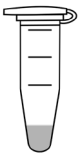
- Product of 1.8.9 1.68 uL
- **2x NEBNext Q5** 2.8 uL
- **10 uM i5 and i7 primers** 1.12 uL

@2.4 uL

5 cycle 8 cycle

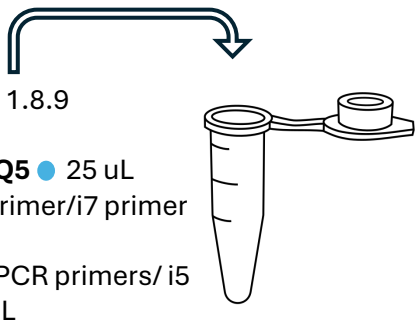
Thermocycler
98°C 30''
98°C 10''
65°C 1'15''
65°C 5'
4°C ∞

} 5 and 8 cycle



1.9 PCR Enrichment of Adaptor-ligated DNA

1.9.1



- Product of 1.8.9 15 uL
- **NEBNext Q5** 25 uL
- Index (X) primer/i7 primer 5 uL
- Universal PCR primers/ i5 primer 5 uL

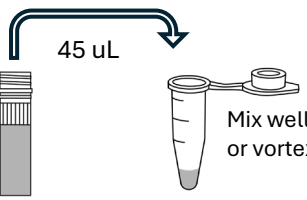
1.9.3

Thermocycler lid heated 105°C
98°C 30''
98°C 10''
65°C 1'15''
65°C 5'
4°C ∞

} 7-15 cycle

1. 10 Purification of Ligation Reaction

1.10.2

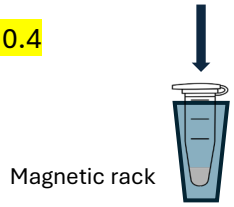


1.9.3 product

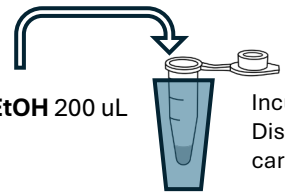
1.10.3

Incubate in RT for 5'

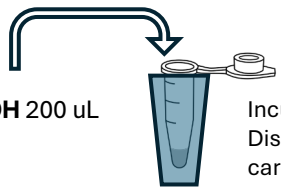
1.10.4



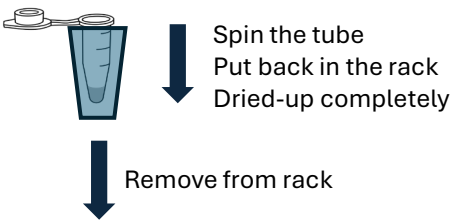
1.10.5



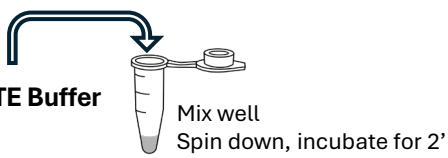
1.10.6



1.10.7

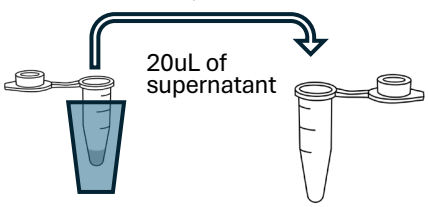


1.10.8



Put back in the rack

1.10.9



Safe stop point

Bioanalyzer check

qPCR (quantification)