egSEQ® RNA Library Preparation

RNA Library Preparation
Designed for Illumina® Next Generation Sequencing

User Guide

Version 1.01
31st July 2023

For Research Use Only.
Not for in-vitro diagnostic use unless otherwise specified.

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Overview of Workflow

Materials Provided

The ingredients required for performing egSEQ universal library preparation include:

- egSEQ RNA Library Prep Kit
- egSEQ Adapter & UDI Primer

Library Preparation Module

Item	Screw Cap	Components	Storage	Volume	
				16 rxn	96 rxn
egSEQ RNA Library Prep Kit	Red	Fast Frag Buffer	-20°C ± 5°C	72 µL	432 µL
	Brown	Fast First Strand Buffer		108 µL	640 µL
	Green	Fast First Strand Enzyme		36 µL	216 µL
	Orange	Fast Second Strand Buffer with dUTP		540 µL	2*1584 µL
	Orange	Fast Second Strand Enzyme		88 µL	540 µL
	Blue	Fast Ligation Buffer		540 µL	2*1584 µL
	Blue	Fast Ligase Mix		88 µL	540 µL
	White	PCR Master Mix with UDG		450 µL	2*1350 µL

Adapter & UDI Primer Module

Item	Screw Cap	Components	Storage	Volume	
				96*1 rxn	96*10 rxn
egSEQ Adapter & UDI Primer 1-96 (up to 384 indexes)	Blue	Adapter (15 µM)	-20°C ± 5°C	540 µL	4*1350 µL
	/	UDI Primer N (10 µM each)*		8 µL each	96*75 µL

UMI Adapter & UDI Primer Module

Item	Screw Cap	Components	Storage	Volume	
				96*1 rxn	96*10 rxn
egSEQ UMI Adapter & UDI Primer 1-96 (up to 384 indexes)	Blue	Insert UMI Adapter (15 µM)	-20°C ± 5°C	540 µL	4*1350 µL
	/	UDI Primer N (10 µM each)*		8 µL each	96*75 µL

Other Required Materials

The materials below are recommended by Edinburgh Genetics. Please select appropriate materials based on experience and availability.

Reagents Required

Item	Recommendation	Supplier Catalogue #
Ethanol Absolute	-	-
Nuclease-Free Water	Nuclease-Free Water	Ambion (AM9930)
Magnetic Beads (for Purification) *	egSEQ Pure Beads	Edinburgh Genetics (EG1200)
	Agencourt AMPure XP Kit	Beckman Coulter (A63880)
Fragment Analyser	Agilent DNA 1000 Kit	Agilent (5067-1504)
Nucleic Acid Quantification Assay	Qubit dsDNA HS Assay Kit	Thermo Fisher (C47257)

* Not to be confused with egSEQ Cap Beads. Choose only one of the recommended reagents.

Equipment Required

Item	Recommendation	Supplier Catalogue #
96-well Magnetic Stand, 0.2 mL block	DynaMag-96 Side	Thermo Fisher (12331D)
Fragment Analyser	Agilent 2100 Bioanalyzer	Agilent (G2939AA)
Nucleic Acid Quantifier	Qubit® 4.0 Fluorometer	Thermo Fisher (Q33238)
Vortex Mixer	-	-
Mini Centrifuge	-	-
Ice Block	-	-
Thermal Cycler	-	-

*Consumables Required**

Item	Recommendation	Supplier Catalogue #
Qubit tubes, 0.5 mL	Qubit® assay tubes	Thermo Fisher (Q32856)
PCR tubes, 0.2 mL	-	-
8-tubes strip, 0.2 mL	-	-
Pipette tips, 10 µL	-	-
Pipette tips, 200 µL	-	-

* References to PCR tube(s) in this guide also apply to plate(s). You may choose to use suitable PCR plate(s).

Before You Begin

Before beginning the experiment, please read the following notes carefully and where necessary confirm requirements are met:

- Please use RNase-Free equipment during the library construction process.

- Before the experiment, please confirm whether the self-prepared reagents (such as absolute ethanol) meet the experimental conditions. If necessary order additional quantities.

- Some experimental steps during the experiment cannot be suspended. Please follow the specific operational instructions and do not pause the experiment at stages which are not marked on this protocol.

- Buffers, primers and other reagents preserved at -20 ° C must be thawed on ice and fully mixed before use. These cannot be dissolved by high-temperature heating and other methods.

- If the reagent amount used in each experiment is small, aliquoting is recommended to avoid quality changes caused by repeated freeze-thaw.

- Minimise the number of freeze-thaw cycles of samples to avoid RNA degradation as this can lead to small RNA fragments and poor sequencing quality.

- The sample quality has a great influence on the experimental results.

- Please use the enzyme/enzyme mix immediately after pulse-spin down (≤ 600 g).

- Please take out the magnetic beads from 4° C freezer and incubate at room temperature for 30 minutes before use.

- A260/A280 = 1.8 to 2.1 is generally accepted as "pure" for RNA samples. If the ratio is lower/higher, it may indicate the presence of protein or genomic DNA.

- Wear gloves and a mask when handling reagents and preparing RNA libraries. Keep RNA samples on ice and the lab environment clean to avoid RNA degradation.

Step 1: RNA Fragmentation

Reagents Required	Equipment Required
• Fast Frag Buffer	• Thermal Cycler

1.1. Thaw RNA samples and Fast Frag Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

1.2. Prepare reaction mixture on ice as indicated below.

Component	Volume per Reaction
RNA	13 µL (10 ng~1 µg in total)
Fast Frag Buffer	4 µL
Total Volume	**17 µL**

1.3. Mix well by gently pipetting or vortexing and centrifuge briefly.

1.4. Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 105°C	
94°C	**7 min**
4°C	**Hold**

1.5. When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 2 immediately.

Step 2: Reverse Transcription

Reagents Required:
- Fast First Strand Buffer
- Fast First Strand Enzyme

Equipment Required:
- Thermal cycler

2.1. Thaw Fast First Strand Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

2.2. Place First Strand Enzyme on ice. Mix well by rotating and then centrifuge briefly. Place back on ice.

2.3. Prepare reaction mixture on ice as indicated below.

Component	Volume per Reaction
Sample from STEP 1.5	17 µL
Fast First Strand Buffer	6 µL
Fast First Strand Enzyme	2 µL
Total Volume	**25 µL**

2.4. Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

2.5. Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 105°C	
25°C	10 min
42°C	15 min
70°C	15 min
4°C	**Hold**

2.6. When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 3 immediately.

Step 3: 2nd Strand Synthesis & A-Tailing

Reagents Required:
- Fast Second Strand Buffer with dUTP
- Fast Second Strand Enzyme

Equipment Required:
- Thermal Cycler

3.1. Thaw Fast Second Strand Buffer with dUTP on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

3.2. Place Fast Second Strand Enzyme on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

3.3. Prepare reaction mixture on ice as indicated below.

Component	Volume per Reaction
Sample from STEP 2.6	25 µL
Fast Second Strand Buffer with dUTP	30 µL
Fast Second Strand Enzyme	5 µL
Total Volume	**60 µL**

3.4. Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

3.5. Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 105°C	
16°C	30 min
72°C	15 min
4°C	**Hold**

3.6. When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 4 immediately.

Step 4: Adapter Ligation

Reagents Required:
- Adapter
- Fast Ligation Buffer
- Fast Ligase Mix

Equipment Required:
- Thermal cycler

4.1. Thaw Adapter on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

4.2. Dilute the Adapter (15 µM) according to the table below.

RNA Input	Adapter Concentration	Dilution Time
100 ng ~ 500 ng	7.5 µM	2
50 ng	3.75 µM	4
25 ng	1.5 µM	10
10 ng	0.75 µM	20

4.3. Thaw Fast Ligation Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

4.4. Place Fast Ligase Mix on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

4.5. Prepare reaction mixture on ice as indicated below.

Component	Volume per Reaction
Sample from STEP 3.6	60 µL
Fast Ligation Buffer	30 µL
Fast Ligase Mix	5 µL
Adapter (Diluted)	5 µL
Total Volume	**100 µL**

If handling many samples at the same time, prepare the reaction mixture without Adapter. It is suggested to mix the Adapter and the products from STEP 3.6 first, and then add the reaction mixture to reduce adapter self-ligation.

4.6. Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

4.7. Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Shut down heat lid	
20°C	15 min
4°C	**Hold**

4.8. When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 5 immediately.

Step 5: Post-Ligation Purification

Reagents Required:
- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water

Equipment Required:
- Magnetic stand

egSEQ Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Choose the suitable volume according to the library type and assess the volume of beads used for purification via pre-experiments if not using the recommended brand.

5.1. Prepare the 80% ethanol for purification. Place the 80% ethanol at room temperature.

5.2. Vortex the magnetic beads for 30 sec to mix well, and equilibrate the magnetic beads to room temperature for 30 min.

5.3. Add 45 µL (0.45×) of magnetic beads to each ligated sample from STEP 4.8. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

5.4. Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 3 min).

5.5. Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec.

5.6. Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec. Remove and discard the clear supernatant.

5.7. Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

5.8. Keep the PCR tube containing beads on the magnetic stand at room temperature for 3 ~ 5 min to dry the bead pellet. Do not overdry the bead pellet.

5.9. Add 22 µL of Nuclease-Free Water. Remove the PCR tube containing beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 min.

5.10. Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 2 min).

5.11. Transfer 20 µL of clear supernatant containing each library to a clean PCR tube. Please proceed to STEP 6.

Step 6: Amplification of Library DNA

Reagents Required:
- PCR Master Mix with UDG
- UDI Primer

Equipment Required:
- Thermal Cycler

6.1. Thaw PCR Master Mix with UDG on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

6.2. Thaw UDI Primer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

6.3. Prepare PCR reaction mixture on ice as indicated below.

Component	Volume per Reaction
Sample from STEP 5.11	20 µL
PCR Master Mix with UDG	25 µL
UDI Primer N (10 µM each)	5 µL
Total Volume	**50 µL**

Ensure that the index number N of indexed primers are noted.

6.4. Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

6.5. Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time	PCR Cycles
Heat lid temperature 105°C		
98°C	1 min	1
98°C	10 s	N cycles
60°C	30 s	
72°C	30 s	
72°C	5 min	1
4°C	Hold	

Input	PCR Cycles N (with Step 3.3.1)
500 ng	7
250 ng	7
100 ng	9
50 ng	10
25 ng	11
10 ng	12

The PCR cycle number is adjusted according to RNA input.

6.6. When the program has completed, mix thoroughly and centrifuge briefly. Proceed to STEP 7 immediately.

Step 7: Post-Amplification Purification

Reagents Required:
- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water

Equipment Required:
- Magnetic Stand

egSEQ Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Choose the suitable volume according to the library type and assess the volume of beads used for purification via pre-experiments if not using the recommended brand.

7.1. Prepare the 80% ethanol for purification. Place the 80% ethanol at room temperature.

7.2. Vortex the magnetic beads for 30 sec to mix well. Equilibrate the beads to room temperature for 30 min.

7.3. Add 45 µL (0.9×) of magnetic beads to each amplified sample from STEP 6.6. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

7.4. Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 3 min).

7.5. Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec.

7.6. Keep the PCR tube containing beads on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec. Remove and discard the clear supernatant.

7.7. Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

7.8.Keep the PCR tube containing beads on the magnetic stand at room temperature for 3 ~ 5 min to dry the bead pellet. Do not overdry the bead pellet.

7.9. Add 30 µL of Nuclease-Free Water. Remove the PCR tube containing beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 min.

7.10. Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant.

7.11. Transfer 28 µL of clear supernatant containing each library to a clean PCR tube. Libraries can be stored at -20°C for up to one month.

7.12. Quantify each library using Qubit dsDNA High Sensitivity Assay Kit. Analyze the fragment size of each library using Agilent 2100 Bioanalyzer.

The experiment ends here.

Technical Support

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