egSEQ® Enzymatic Library Preparation

Enzymatic Library Preparation

Designed for Illumina® Next Generation Sequencing

User Guide

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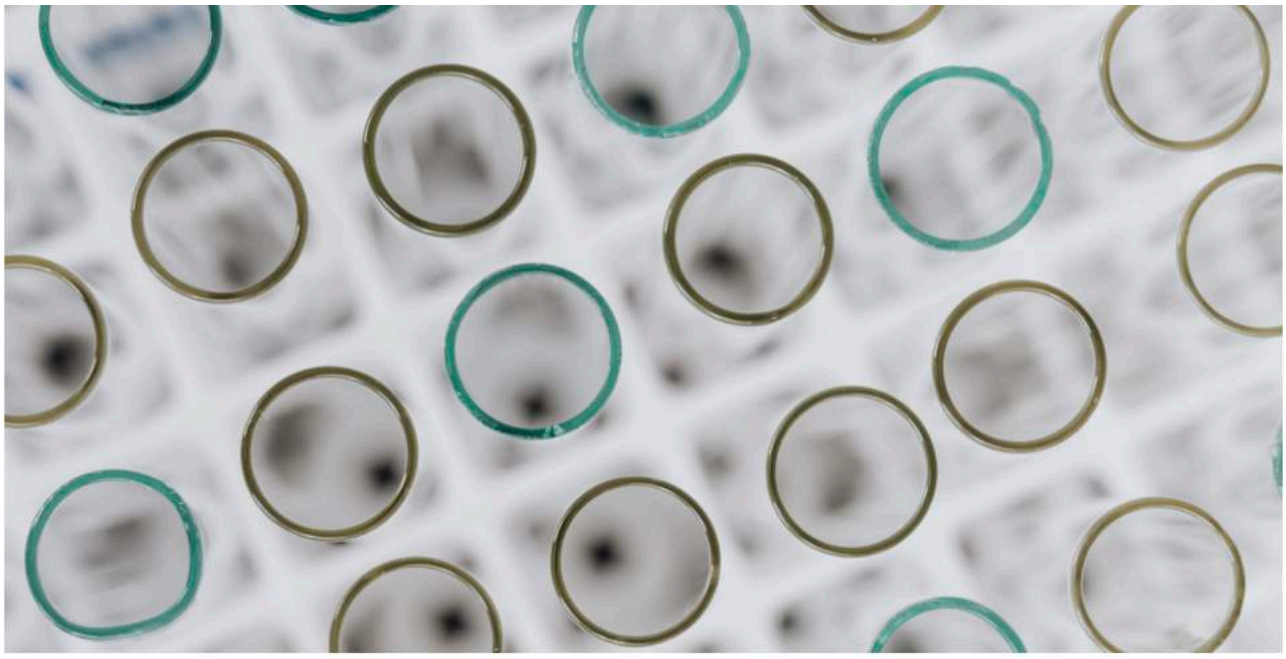

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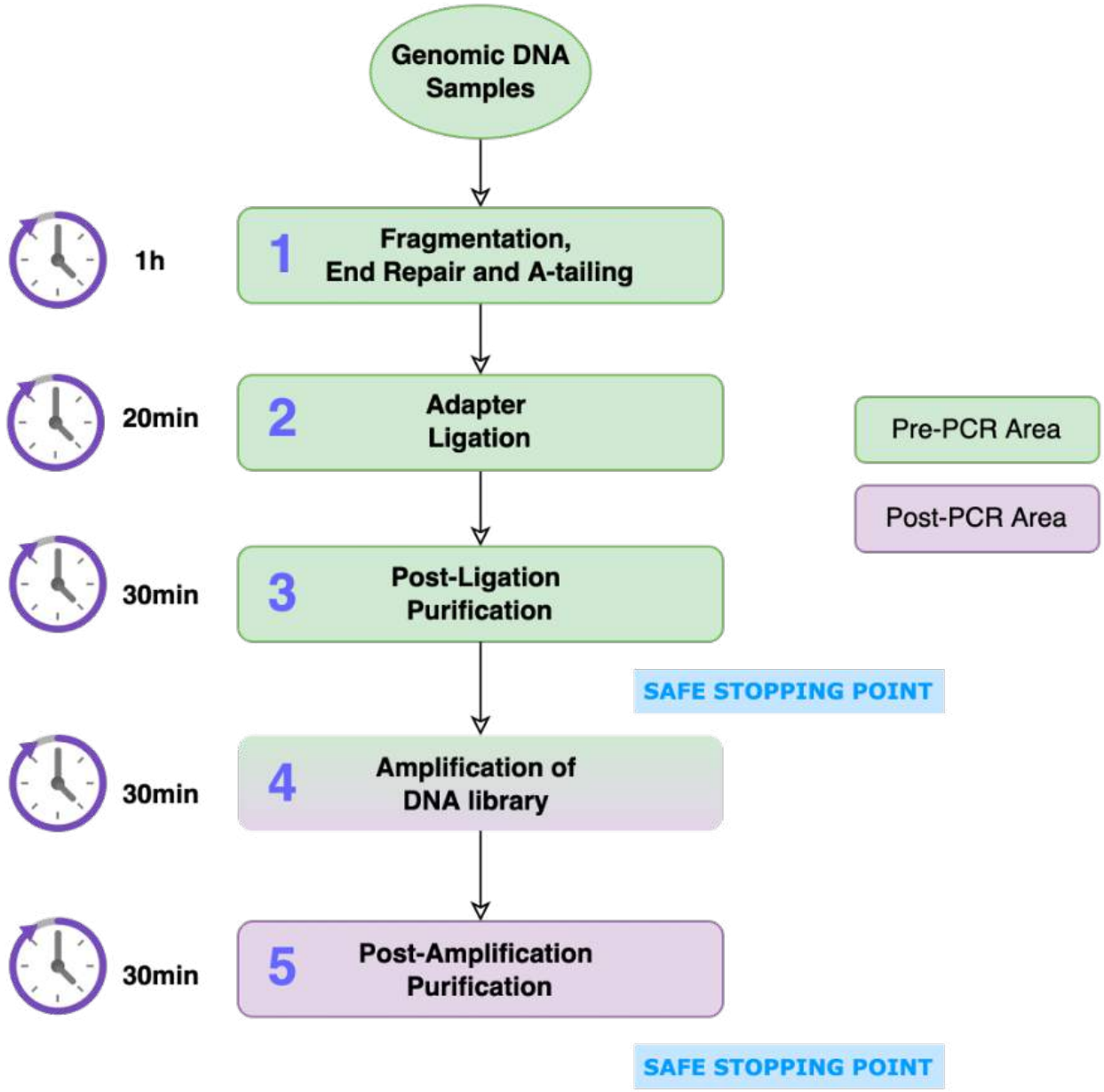
Overview of Workflow

There are 5 steps in the workflow, outlined below with estimated time to completion for each step.



Materials Provided

The ingredients required for performing egSEQ enzymatic library preparation include:

- egSEQ Enzyme DNA Library Prep Kit
- egSEQ Adapter & UDI Primer

egSEQ Enzyme DNA Library Prep Kit

Item	Screw Cap	Components	Storage	Volume	
				16 rxn	96 rxn
egSEQ Enzyme DNA Library Prep Kit	Yellow	Fragment & ERA Buffer	-20°C ± 5°C	180 µL	1080 µL
	Yellow	Fragment & ERA Enzyme Mix		180 µL	1080 µL
	Blue	Ada Ligation Buffer		540 µL	2*1584 µL
	Blue	Ada Ligase		180 µL	1080 µL
	White	PCR Master Mix		450 µL	2*1350 µL

egSEQ Adapter & UDI Primer

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*10 µL

* N is the index number.

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:

- The kit is suitable for various sample types: gDNA (extracted from blood, Saliva, Oropharyngeal swabs, fresh tissue and frozen tissue and cell) and FFPE DNA. The recommended DNA input is 1 ng to 1 µg.

- DNA dissolved in Nuclease-Free Water or Tris-HCl is recommended and the elution buffer should be free of EDTA (refer to *Appendix 2* for EDTA removing method).

- A260/A280 = 1.8 to 2.0 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of protein contamination. If the A260/A230 ratio is lower than 2.0, it may indicate the presence of guanidine. If the purity of DNA is not as expected, purification using magnetic beads before library preparation is recommended.

- Qubit dsDNA High Sensitivity Assay Kit (or equivalent) should be used to measure dsDNA concentration. Agilent 2100 Bioanalyzer (or equivalent) is used to assess the fragment size of each library.

- Prepare the reaction mixture on ice or an ice block.

- A 0.2 mL magnetic stand will be used.

- egSEQ Pure Beads or Agencourt AMPure XP are the recommended magnetic beads for purification in this protocol. If an alternative is used, please assess the volume of magnetic beads used for purification via experimentation beforehand.

- Before the experiment, confirm whether the self-prepared reagents (such as absolute ethanol) meet the experimental conditions and are within shelf life. These are listed in the *Other Required Materials* section below. Prepare additional batches as required.

- Suitable stopping points are highlighted clearly in the instructions. Refrain from stopping at other stages of the experiment.

Step 1: Fragmentation, End-Repair and A-Tailing

Reagents Required	Equipment Required
• Fragment & ERA Buffer • Fragment & ERA Enzyme Mix • Nuclease-Free Water	• Thermal cycler • Mini Centrifuge

1.1. Thaw *Fragment & ERA Buffer* on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

1.2. Place *Fragment & ERA Enzyme Mix* on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

Please set the thermal cycler program before the next step. When the thermal cycler block reaches 4°C, pause the program. The purpose of this step is to quickly start the program after step 1.4 to reduce the reaction time outside the program.

Temperature	Time
Heat lid temperature 85°C	
4°C	1 minutes
37°C	**X* minutes**
65°C	30 minutes
4°C	**Hold**

* Please choose the suitable fragmentation time according to the final library peak size, sample type and sample quality, as set out below:

Final Library Peak size	Fragment Peak Size	gDNA	FFPE DNA
500bp	200~1500 bp	10 minutes	5 minutes
300bp	200~1200 bp	12 minutes	8 minutes
280bp	200~800 bp	15 minutes	5-12 minutes
270bp	200~600 bp	20 minutes	10-15 minutes

The same fragmentation time may not produce a consistent fragment size. To produce a consistent-size fragment, size selection step (Step 3.3.2) is recommended after **Adapter Ligation**.

1.3. Add DNA samples to the PCR tube.

The kit allows library preparation from DNA input amounts ranging from 1 ng to 1 µg. The recommended input DNA amount is more than 50 ng.

1.4. Prepare reaction mixture on ice as indicated below. Mix well by gently pipetting (do not vortex violently) and centrifuge briefly.

Component	Volume per Reaction
DNA	X µL
Nuclease-Free Water	(40 - X) µL
Fragment & ERA Buffer	10 µL
Fragment & ERA Enzyme Mix	10 µL
Total Volume	**60 µL**

Please proceed with this step on ice to avoid the effects of temperature change.

1.5. Place the PCR tube on the thermal cycler and start the program.

1.6. Proceed to STEP 2 immediately when the program finishes.

Step 2: Adapter Ligation

Reagents Required:
- Ada Ligation Buffer
- Ada Ligase
- Nuclease-Free Water
- Adapter (15 µM)

Equipment Required:
- Thermal cycler
- Mini Centrifuge

2.1. Thaw the *Adapter (15 µM)* on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

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

DNA Input	Adapter Concentration	Dilute Times
50 ng to 1 µg	15 µM	/
25 ng	7.5 µM	2
10 ng	3 µM	5
5 ng	1.5 µM	10
2.5 ng	750 nM	20
1 ng	300 nM	50

2.3. Thaw *Ada Ligation Buffer* on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

2.4. Place *Ada Ligase* on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

2.5. Prepare the reaction mixture on ice as indicated below.

Component	Volume
Sample from STEP 1	60 µL
Adapter (diluted)	5 µL
Ada Ligation Buffer	30 µL
Nuclease-Free Water	5 µL
Ada Ligase	10 µL
Total Volume	**110 µL**

If there are many samples, prepare the master mix without the adapter. Mix the adapter and the sample from STEP 1 first, then add the master mix to reduce adapter self-ligation.

2.6. Mix well by gently pipetting (do not vortex violently) and centrifuge briefly.

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

Temperature	Time
Shut down heat lid	
22°C	15 minutes
4°C	**Hold**

2.8. Proceed to STEP 3 immediately when the program finishes.

Step 3: Post Ligation Purification

Reagents Required:	Equipment Required:
• Magnetic Beads	• Magnetic Stand
• 80% Ethanol (freshly prepared)	• Vortex Mixer
• Nuclease-Free Water	• Mini Centrifuge

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

3.2. Vortex the magnetic beads for 30 seconds to mix well, and then equilibrate the magnetic beads to room temperature for 30 minutes.

3.3. Choose one method from below (3.3.1 **or** 3.3.2).

3.3.1. If there is no special requirement for the DNA fragment size, please follow the steps below (recommended):

a. Add 88 µL (0.8×) magnetic beads to each ligated sample from STEP 2. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

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

3.3.2. If there is any special requirement for the DNA fragment size, please follow the steps below (Caution: DNA fragment size selection will generate more consistent DNA fragment sizes but potentially more than 50% library loss):

a. Add A µL of magnetic beads to each ligated sample from STEP 2. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

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

c. Keep the PCR tube containing the magnetic beads on a magnetic stand. Transfer (A-3) µL of clear supernatant containing each library to a clean PCR tube.

d. Add B µL of magnetic beads to clear supernatant. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

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

Size Selection					
A (µL)	38 µL	32 µL	28 µL	23 µL	16 µL
B (µL)	16 µL	16 µL	12 µL	12 µL	12 µL
DNA fragment size	200 bp	250 bp	320 bp	380 bp	450 bp

egSEQ Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please choose the suitable volume according to the library type.

If using a different brand, assess the volume of magnetic beads used for purification via pre-experiments.

3.4. Keep the PCR tube containing magnetic 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 seconds.

3.5. Keep the PCR tube containing magnetic 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 seconds. Remove and discard the clear supernatant.

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

3.7. Keep the PCR tube containing magnetic beads on the magnetic stand at room temperature for 3 to 5 min to dry the bead pellet. Do not over-dry the bead pellet.

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

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

3.10. Transfer 20 µL of clear supernatant containing each library to a clean PCR tube. Proceed to STEP 4.

This is a safe stop point and samples can be stored at -20°C for up to one month.

Step 4: Amplification of Library DNA

Reagents Required:	Equipment Required:
• PCR Master Mix • UDI Primer	• Thermal cycler • Mini Centrifuge

4.1. Thaw *PCR Master Mix* on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

4.2. Thaw *UDI Primer* on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

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

Component	Volume per reaction with Unique Dual Identifier
Sample from STEP 3	20 µL
PCR Master Mix	25 µL
UDI Primer N (10 µM each)	5 µL
Total Volume	**50 µL**

Ensure that the index number N of indexed primer are marked down.

4.4. Mix well by gently pipetting (do not vortex violently) and centrifuge briefly.

4.5. 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	2 min	1
98°C	20 sec	N Cycles
60°C	30 sec	
72°C	30 sec	
72°C	1 min	1
4°C	**Hold**	**1**

DNA Input	PCR Cycles N (with Step 3.3.1)	PCR Cycles N (with Step 3.3.2)
1 µg	3~4	3~4
500 ng	4~5	4~6
200 ng	5~6	6~7
100 ng	6~7	7~8
50 ng	7~9	8~10
20 ng	9~11	10~12
10 ng	11~13	12~15
1 ng	15~17	17~19

4.6. When the PCR amplification finished, please proceed to STEP 5.

Amplified libraries from STEP 4 can be stored at -20°C temporarily. However it is recommended to proceed to STEP 5 on the same day as STEP 4 completion.

Step 5: Post Amplification Purification

Reagents Required:
- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water
- Qubit dsDNA HS Assay (or equivalent)

Equipment Required:
- Magnetic stand
- Vortex mixer
- Mini Centrifuge
- Fragment Analyser
- Nucleic Acid Quantifier

5.1. Vortex the magnetic beads for 30 seconds to mix well. Equilibrate the magnetic beads to room temperature for 30 minutes.

5.2. Add 50 µL (1.0×) of magnetic beads to each amplified sample from STEP 4. Pipet or vortex to mix well. Incubate at room temperature for 5 minutes.

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

5.4. Keep the PCR tube containing magnetic 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 seconds.

5.5. Keep the PCR tube containing magnetic 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 seconds. Remove and discard the clear supernatant.

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

5.7. Keep the PCR tube containing magnetic beads on the magnetic stand at room temperature for 3 to 5 minutes to dry the bead pellet. Do not over-dry.

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

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

5.10. 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.

5.11. Quantify each library using Qubit dsDNA High Sensitivity Assay or equivalent. Analyse the fragment size of each library using a suitable fragment analyser such as Agilent 2100 Bioanalyzer.

The experiment ends here!

Appendix 1: EDTA Removal

Reagents Required:	Equipment Required:
• Magnetic Beads • 80% Ethanol (freshly prepared) • Nuclear-Free Water	• Magnetic Stand • Vortex Mixer • Mini Centrifuge • Fragment Analyser

If the sample has a very high concentration (e.g. 100 ng/μL) and the volume used for library construction is small (e.g. 2μL), EDTA removal can be ignored.

1. The recommended method for removing EDTA is purification using magnetic beads.

2. Vortex the magnetic beads for 30 seconds to mix well. Equilibrate the magnetic beads to room temperature for 30 minutes.

3. Add 2× volume magnetic beads to the DNA sample. For example, add 100 μL magnetic beads if the DNA sample is 50 μL.

4. Pipet or vortex to mix well. Incubate at room temperature for 5 minutes.

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

6. Keep the PCR tube containing magnetic 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 seconds.

7. Keep the PCR tube containing magnetic 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 seconds. Remove and discard the clear supernatant.

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

9. Keep the PCR tube containing magnetic beads on the magnetic stand at room temperature for 3 to 5 minutes to dry the bead pellet. Do not over-dry the bead pellet.

10. Add 42 µL of Nuclease-Free Water. Remove the PCR tube containing magnetic beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 minutes.

11. Spin briefly on a mini centrifuge. Place the PCR tube containing magnetic beads on a magnetic stand for 2 minutes and allow the magnetic beads to fully separate from the supernatant.

12. Transfer 40 µL of clear supernatant containing sample to a clean PCR tube. Libraries can be stored at -20°C for up to one month.

13. Quantify each library using Qubit dsDNA High Sensitivity Assay or equivalent.

Technical Support

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