Amplicon Indexing for Illumina with Clean Up

Material

Instrument / equipment/ hardware/ software	Application
Eppendorf Thermal Cycler	DNA amplification
Agilent Bioanalyzer / Caliper GX / Fragment Analyzer	Fragment size determination of finished libraries
twin.tec PCR plate 96, skirted (Eppendorf)	Sample containment (setup)
NUNC deep well plates	Reagent container
ABgene 2.2 mL storage plate Mk.II	Liquid waste container
BioRad Microseal B plate seal	Plate seal for PCR reactions
AlumaSeal II plate seal	Plate seal for storage

* This is what we use in our lab, it can be exchanged by compatible materials or adjusted to your lab settings. Some of these materials are used because of automation of the protocols.

Reagents

Kit/ Reagent	Vendor/ Catalog #	Storage conditions	Shelf-life	CAS number/ hazard label abbreviation
KAPA HiFi HotStart ReadyMix	Roche KK2602	-20°C	12 months	Tetramethylammonium chloride 75-57-0 / **Xi, Xn, T**
MagSi-NGS prep plus	Nordic Biosite/Tataa MDKT00010075	4°C	stated on bottle	not hazardous
Elution buffer (EB)	Qiagen 19086	RT	N/A	not hazardous

10 mM Tris-HCl, pH 8.5				
BSA	Thermo Scientific B14	-20°C	stated on tube	not hazardous
Ethanol	VWR 20823.290	RT	stated on bottle	64-17-5 / **F, Xn**
custom primers	IDT N/A	-20°C / -80°C*	N/A	not hazardous

* Working solutions are stored at -20°C, stock solutions at -80°C

DNA input requirements

Samples must be normalized to one **unique DNA yield and volume (50 µl)** across all samples. **Be aware, yield ≠ concentration!** *Since you are in control of how much beads to add and the elution volumes you should allow this concentration/volume to be within limits*

Example concentrations and volumes:

- 20 µl with a concentration of 1 ng/µl -> yield of 20 ng
- 20 µl with a concentration of 4.5 ng/µl -> yield 90 ng
- 30 µl with a concentration of 0.5 ng/µl -> yield of 15 ng
- 40 µl with a concentration of 2 ng/µl -> yield of 80 ng

Bead Cleanup 1

Determination of bead volume

The volume of MagSI beads to be added to the samples depends on the sample volume and amplicon length. Please use the following table to determine the multiplication factor for the bead volume:

Amplicon length	**Multiplication factor**
100-150 bp	x 2
150-250 bp	x 1.5
250-400 bp	x 1.2

> 400 bp	x 1

- Calculate the bead volume to add as follows:
  [Bead Volume to add to sample] = [Volume in µl] * [Multiplication Factor]
- Example calculations:
  - With 40 µl of an amplicon with the length 123 bp
    - 40 µl * multiplication factor 2 = **80 µl beads need to be pipetted to the samples**
  - With 50 µl of an amplicon with the length of 450 bp
    - 40 µl * multiplication factor 1 = **40 µl beads need to be pipetted to the samples**

Determination of elution volume

The elution volume depends on the total yield calculated previously. Generally, the eluted concentration should be ~0.8 ng/µl assuming a loss of 20% in the bead purification and with a **minimal elution volume of 20 µl independent of yield.**
The final volume can therefore be calculated as follows:

[Elution Volume] = [User Yield] * [Purification Loss] / [Aimed Concentration]

- Example calculation:
  - user send in a total yield of 40 ng
    - elution volume = 40 * 0.8 (purification loss) / 0.8 (aimed final concentration) = **40 µl will be added to a fixed volume for the cleanup step (see below).**

Use the determined bead and elution volumes and follow manufacturer's instructions

PCR 2 (outer PCR)

Primer sequences

i5 indexing primer: Illumina handle1 – [index] – Illumina adapter1:
AATGATACGGCGACCACCGCGCTC{TACAC}-[index]-ACACTCTTTCCCTACACGACG
*The brackets denote bases that will not be read in the sequencer
i7 indexing primer: Illumina handle2 – [index] – Illumina adapter2:
CAAGCAGAAGACGGCATACGAGAT-[index]-GTGACTGGAGTTCAG

Reagent Preparation for PCR 2

Single reaction:
Sample volume: 6 ul
Master mix 1: 10 ul HotStart 2x Master Mix
Indexing primer Mix: 4 ul

Add the master mix and corresponding index per well and amplify with the following conditions.

Initial denaturation	98 °C	2 min
Denaturation	98 °C	20 s
Annealing	55 °C	30 s
Elongation	72 °C	30 s
GO TO step 2 for 8 cycles (max 10 cycles)		
Final elongation	72 °C	2 min

Bead Cleanup 2

Follow the manufacturer's instructions. Standard conditions/sample for this clean up is: sample volume = 18 ul; bead volume = 21.6 ul; elution volume = 20 ul.