MiSeq Amplicon Sequencing Sample Prep

1) Perform the first PCR using the primer constructs
   llumina adapter-4N-*forward primer*
   ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N_4-*fwd_primer*
   and Illumina adapter-*reverse primer*
   AGACGTGTGCTCTTCCGATCT-*rev_primer*

Optimization of the protocol may be required, but a starting point is 2.5 µL of each primer (10 µM), 1-10 ng of template DNA, 25 µL of Phusion Mastermix (ThermoScientific) and dH_2O to a total volume of 50 µL.

Cycling conditions are:

Denaturation	98°C	30”	
Denaturation	98°C	10”	\
Hybridization	***	30”	|- 20x
Elongation	72°C	4”	/
Elongation	72°C	2 min	

Hybridization conditions are primer-dependent, but should be similar to those used for the primers without adapters. Kapa HiFi polymerase (Kapa Biosystems) can be considered with the template is particularly refractory to amplification, following the instructions from the manufacturer.

2) A cleaning step must be performed to eliminate loose primers and eventual primer dimers or low molecular weight unspecific products. This can be achieved with magnetic beads, a spin column or a gel. If carrying out this procedure in SciLifeLab Stockholm, a CA-cleaning in the MBS is recommended. See “CA purification” protocol”.

3) A second PCR is conducted for attaching standard Illumina handles and index primers.

Multiplex_1.01
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

Multiplex_2.01 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Index_primer CAAGCAGAAGACGGCATACGAGAT-X_6-GTGACTGGAGTTC

In SciLifeLab Stockholm, these can be found in the PhD-student lab in floor 3, in the common reagent freezer. There are 12 standard Illumina barcodes. Different combinations can be achieved, but will have to be ordered separately.

Each well should contain 20 µL of the clean PCR product above, 25 µL os Phusion Mastermix, 1 µL of each primer (10 µM) and 2 µL dH_2O.

Cycling conditions are as follows:

Denaturation	98°C	30”	
Denaturation	98°C	10”	\
Hybridization	62°C	30”	|- 10x
Elongation	72°C	5”	/
Elongation	72°C	2 min	

4) Repeat step (2) twice. If using magnetic beads, adapt the bead amount for the larger amount of DNA now present. Beads will interfere with the downstream steps, so remove them first by placing the plate in a magnetic stand, leaving it undisturbed for at least 3 minutes and then

transfering the reaction product to a fresh plate.

5) Quantify sample concentration and length. For concentration, a fluorometric measurement such as Qubit (Invitrogen) or PicoGreen (Invitrogen) should be preferred over an auto-fluorescence method such as NanoDrop (Thermo Scientific).

6) Measure the average fragment length. BioAnalyzer (Agilent) HS is recommended, but a simple agarose gel may suffice.

7) Pool all samples together for a total volume of c. 15 µL and a concentration of 2 nM / barcoded template. The molarity of a sample can be calculated using the formula:

$$Concentration*10^6/656.6*Length$$

8) Samples are now ready to be handled by facility personnel. When using Illumina for amplicon sequencing it is always required to spike the sample mix with random DNA, such as PhiX (15-40%, for a final read output of 50%).

INDEXES

N701	TAAGGCGA
N702	CGTACTAG
N703	AGGCAGAA
N704	TCCTGAGC
N705	GGACTCCT
N706	TAGGCATG
N707	CTCTCTAC
N708	CAGAGAGG
N709	GCTACGCT
N710	CGAGGCTG
N711	AAGAGGCA
N712	GTAGAGGA

These are the standard Nextera indexes. To make sure that the sequencer detects signal in multiple channels, combine them as follows:

Plex	**Barcode**
1	Any
2	[option1] N701 + N702 [option2] N702 + N704
3	[option1] N701 + N702 + N704 [option2] N703 + N705 + N706
4 to 5	[option1] N701 + N702 + N704 + any other [option2] N703 + N705 + N706 + any other
6	N701 + N702 + N704 + N705 + N706
7-12	[option1] N701 + N702 + N704 + any other [option2] N703 + N705 + N706 + any other