MiSeq Dual Index Amplicon Sequencing Sample Prep

1) Perform the first PCR using the primer constructs
   llumina adapter-N_4-*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	***	/
Elongation	72°C	2 min	

Hybridization conditions are primer-dependent, but should be similar to those used for the primers without adapters. For elongation time, 15 s/kb should suffice.

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 cleaning” protocol.

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

Multiplex_fwd

AATGATACGGCGACCACCGAGA{TCTACAC}-[i5 index]-ACACTCTTTCCCTACACGACG

Multiplex_rev

CAAGCAGAAGACGGCATACGAGAT-[i7 index]-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

The curly brackets denote bases that will not be read in the sequencer. See notes at the bottom for multiplexing indexes combination guidelines.

Each well should contain 23 µL of the clean PCR product above, 25 µL os Phusion Mastermix and 1.5 µL of each primer (10 µM).

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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 necessary. 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 transferring 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 at least 15 µL and a concentration of 2-10 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 (5% is enough on an up-to-date machine; 30% may be required in older settings).

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INDEXES

i7		**i5**	
N701	TAAGGCGA	N501	TAGATCGC
N702	CGTACTAG	N502	CTCTCTAT
N703	AGGCAGAA	N503	TATCCTCT
N704	TCCTGAGC	N504	AGAGTAGA
N705	GGACTCCT	N505	GTAAGGAG
N706	TAGGCATG	N506	ACTGCATA
N707	CTCTCTAC	N507	AAGGAGTA
N708	CAGAGAGG	N508	CTAAGCCT
N709	GCTACGCT	**NB!** the i7 barcodes should be reverse complemented in the primers you order, to be read correctly upon sequencing!	
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	**i7**	**i5**
1	Any	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	[option1] N501 + N502 [option2] N503 + N504 [option3] N505+ N506
> 12	N701 + N702 + N704 + N703 + N705 + N706 + any other	[option1] N501 + N502 + any other [option2] N503 + N504 + any other [option3] N505+ N506 + any other

If doing less than twelve samples, consider also using the single index protocol.

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