Microbiome Translational Center

16S rRNA with MiSeq

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1 Abstract

The Purpose of this protocol is to define the steps for the preparation and sequencing of 16S rRNA gene sequence libraries using the Illumina MiSeq sequencing platform. Method adapted from Kozich, J. J. et al Appl. Environ. Microbiol. 79, 5112–20 (2013) [https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP_v4.md]

2 Materials and equipment

2.1 Reagents

- Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/clear [Bio-Rad, HSP9601]
- PCR Plate Heat Seal, foil, peelable [Bio-Rad, 1814045]
- UltraPure™ DNase/RNase-Free Distilled Water [Life Technologies, 10977015]
- Phusion High-Fidelity PCR Master Mix [NEB, M0531L]
- Quant-iT™ dsDNA Assay Kit, broad range [Life Technologies, Q33130]
- Corning™ 96-Well Solid Black Plates [Fisher Scientific, 07-200-590]
- Beckman Coulter AMPURE XP [Fisher Scientific, NC9959336]
- Misc (tips, gel)
- Biomek tips [Beckman Coulter, Biomek AP96 P250 & Span P250]
- Primers [IDT, NA]
- ZymoBIOMICS Microbial Community Standard [Zymo Research, D6300]
- TE, pH 8.0 [Life Technologies, AM9849]
- Buffer EB [Qiagen, 19086]
- Quarter Reservoir Sterile Divided by Length [Beckman Coulter, 372788]
- GEL LOAD DYE PURP (6X) 4 ML [Fisher Scientific, 50591186]
- QUICK-L 2-LOG DNA LADDER [Fisher Scientific, 50994896]
- SYBR SAFE DNA GEL STAIN [Fisher Scientific, S33102]
- AGAROSE LOW EEO 100G [Fisher Scientific, BP160100]
- NaCl (5 M) [Life Technologies, AM9760G]
- PhiX Control v3 [Illumina, C-110-3001]
- MiSeq Reagent Kit v2 (300-cycles) [Illumina, MS-102-2002]

2.2 Equipment

- Plate shaker [Benchmark, Orbishaker]

- Plate sealer [Bio-Rad, PCR Plate Sealer]
- PCR machine [Bio-Rad, C1000 Touch Thermal Cycler]
- Gel Doc [Bio-Rad, Gel Doc EZ Imager]
- Microplate reader, filter 450 nm [BioTek, Synergy HTX]
- Liquid Handler [Beckman Coulter, Biomek FXp]
- Microcentrifuge (>3000g) [Eppendorf, 5424]
- Gel cast [Thermo Scientific, Easycast B1A, B2]
- Fluorometer [ThermoFisher, Qubit® 2.0 Fluorometer]

3 Safety precautions

3.1 Human specimen handling

Universal precautions should be followed when handling human samples considered potentially infectious. Minimal protective clothing requirements are lab coat, gloves and safety glasses.

3.2 Waste

Dispose of all waste as biohazardous material.

3.3 Specific precautions

4 Method

4.1 Considerations

4.1.1 Low biomass samples

To increase the amount of input DNA for low microbial biomass samples (run a test PCR to optimize against a no-template control). Consider using a using DNA isolation kits designed to minimize kit contamination (e.g., the QIAamp UCP (UltraClean production) Pathogen Mini Kit (QIAGEN)). Consider pooling samples and run of the same extraction column to increase microbial biomass. Work in a down-flow cabinet to prevent environmental contamination.

4.1.2 Controls

DNA extraction controls: on each plate include ZymoBIOMICS Microbial Community Standard, 3 longitudinal fecal reference samples obtained from healthy volunteers and a no sample control on random positions. PCR controls: no template control and Clemente lab gut reference sample.

4.2 Setup

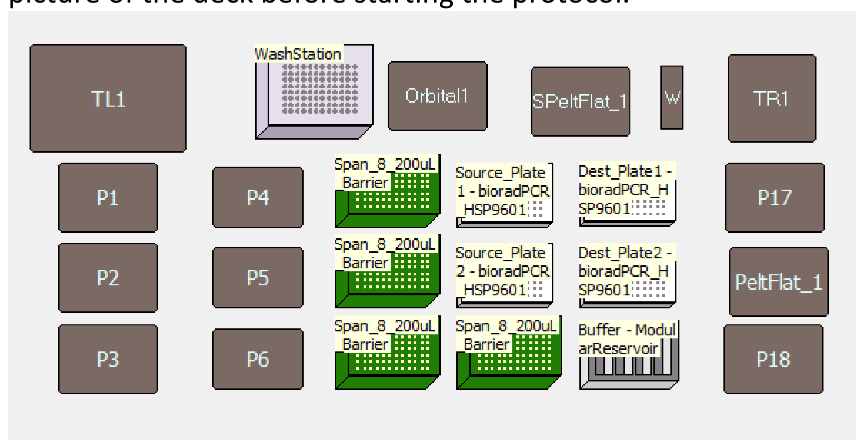
4.2.1 Primer dilution

1. Reconstitute indexed primer stock to 100 uM in TE (see Appendix for primer design).
2. Reconstitute sequence primers to 100 uM in EB (see Appendix for primer design).
3. Dilute Sx5xx primers to 10 uM (140 ul stock + 1360 ul EB), mix by vortexing.
4. Dilute Sx7xx primers to 10 uM (100 ul stock + 900 ul EB), mix by vortexing.
5. Add 2x 720 ul late for Sx5xx and 1x1000 ul for Sx7xx to 96 deep well plate
6. With a multichannel transfer 50 ul of Sx5xx and 50 ul of Sx7xx to 4 PCR 96 well plates (See Appendix for plate layout) according to the scheme: Plate 1: A701 – A712 with A501 – A508; Plate 2: A701 – A712 with B501 – B508; Plate 3: B701 – B712 with B501 – B508; Plate 4: B701 – B712 with A501 – A508.

7. Seal plates for 3 sec @ 180C.
8. Vortex plates for 3 min at 300 rpm and spin at 1000g for 1 min.
9. Barcode plates and store at -80C

4.2.2 Dilute DNA

10. Quantify the DNA in each sample using the Quant-IT dsDNA Assay Kit, Broad Range (MTCP007-Quant-iT_dsDNA_Assay).
    Note: If the concentration is less than 50ng/µL by the Qubit® dsDNA BR Assay Kit, the samples should be reanalyzed using the Qubit® dsDNA HS Assay Kit.
11. Standardize all samples to 2 ng/ul using BioMek [protocol: MTC/2_DiluteDNA]. Specify dilutions in template DNA_Dil.csv. Make sure to load the correct DNA_Dil.csv file for both steps and setup deck as per figure below. Fill the first well of the Modular Reservoir with EB. Home the axis before use under the instrument menu. Make sure all PCR plates are labeled/barcoded and take a picture of the deck before starting the protocol.



12. After the protocol completes, vortex plates for 3 min at 300 rpm and spin at 1000g for 1 min.
13. Seal plates for 3 sec @ 180C.
14. Store plates at -80C.

4.3 PCR

4.3.1 Master mix (96 samples)

15. Combined the following:

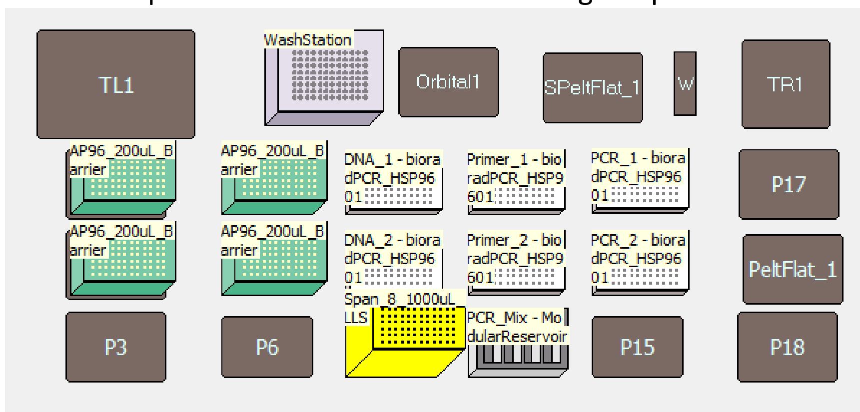
Master Mix:	**96**	**samples**
Reagent	ul	Total
H2O	8.0	845
2xPhusion	12.5	1320

4.3.2 Reaction

16. Barcode PCR plates.
17. Transfer Master Mix with repeat pipette to PCR plate.
18. Transfer DNA with multichannel to PCR plate.
19. Transfer Paired Primers with multichannel to PCR plate.

Reagent	Unit	
DNA (2ng/ul)	2.0	ul
Paired primers	2.5	ul
Master Mix	20.5	ul
Total	25	ul

20. Repeat for up to four 96 well plates. Seal plates, vortex briefly and spin down
    Note: Alternatively use Biomek Protocol MTC/3_SetupPCR. Setup the deck as indicated below and take a picture of the deck before starting the protocol.



4.3.3 PCR program

21. Place in thermocycler and run the program below

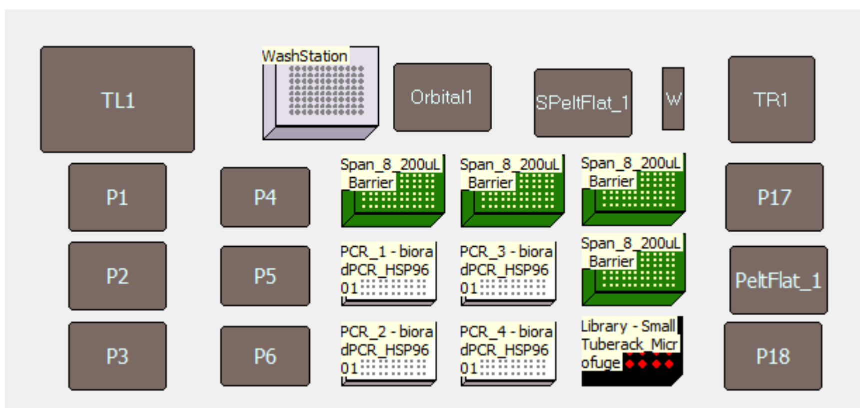
Step	Temp	Time
Denaturation	98	0:30
25 cycles	98	0:10
	50	0:30
	72	0:30
Extension	72	2:00
Hold	4	Inf

4.4 Gel Electrophoresis

22. 1 or 2 random rows of 12 should be selected from each PCR plate and run on a gel to confirm success of the PCR.
23. Use 5 ul of sample, 1 ul of 6X loading dye in a 1% agarose gel (bring to boil 0.5g agarose in 50 ml 1xTAE, cool down and add 5 ul of 10.000x SYBR safe).
24. Run at 100v for 30 minutes alongside a 2-log ladder.
25. Photograph gel using the EZ Imager. Check to be sure there is a band for every well

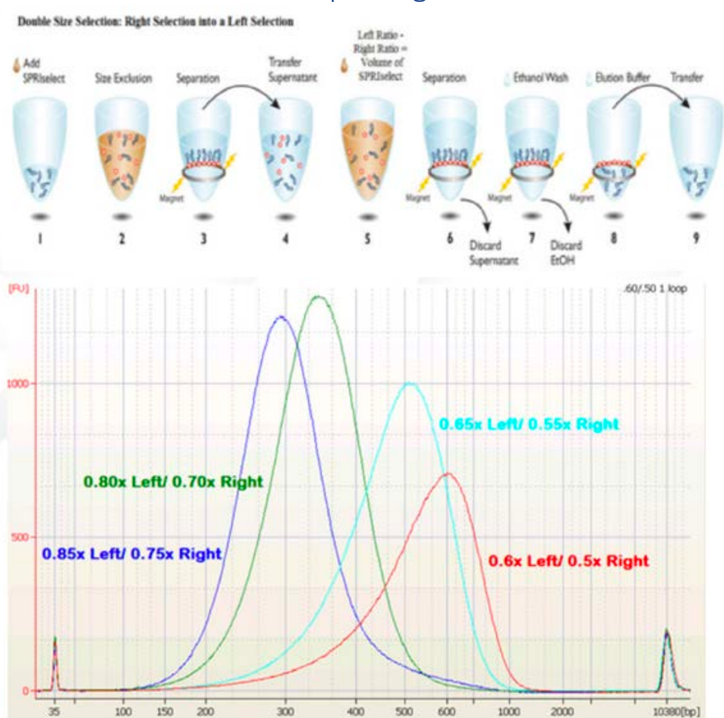
4.5 Normalization and Pooling

26. Quantify the DNA in each sample using the Quant-IT dsDNA Assay Kit, Broad Range (MTCP007-Quant-iT_dsDNA_Assay).
27. Combine in ~equal proportions with Biomek (Protocol MTC/4_CombineLibrary) following the Library.csv template. Setup deck as below and take a picture before starting. Place a barcoded library Eppendorf tube in position A1 of the Small TubeRack. Transfer a maximum of 10ul for any sample including NTC controls.



28.

4.6 Double size selection and cleanup using AMPure XP Beads



4.6.1 Right side selection

This step is used to bind the large, unwanted fragments to the beads. The supernatant will contain the desired fragments.

29. Add 77 µl (0.77X) resuspended AMPure XP beads to 100 µl DNA solution. Mix well on a vortex mixer or by pipetting up and down at least 10 times.
30. Incubate for 5 minutes at room temperature.
31. Place the tube on a magnetic rack to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully <u>transfer 100 ul of the supernatant</u> to a new. Discard beads that contain the large fragments.

4.6.2 Left side selection

This step will bind the desired fragment sizes to the beads. Unwanted smaller fragment sizes will not bind to the beads.

32. Add 33 µl (0.33X) resuspended AMPure XP beads to 100 ul of the supernatant, mix well and incubate for 5 minutes at room temperature.
33. Put the tube on a magnetic rack to separate beads from supernatant. After the solution is clear (approximately 3 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads).
34. Add 200 µl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
35. Repeat Step above once.
36. Keeping the tube on the magnetic rack, with the cap open, air dry the beads for 5 minutes. **Caution:** Do not overdry the beads. This may result in lower recovery of DNA target.
37. Remove the tube from the magnet. Elute DNA target from beads into 42 µl sterile 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature.
38. Put the tube in a magnetic rack until the solution is clear, approximately 3 minutes.
39. Transfer approximately 40 µl of the supernatant to a barcoded clean tube.

4.7 Gel Electrophoresis

40. Use 5 ul of sample (before and after cleanup), 1 ul of 6X loading dye in a 1% agarose gel (bring to boil 0.5g agarose in 50 ml 1xTAE, cool down and add 5 ul of 10.000x SYBR safe).
41. Run at 100v for 30 minutes alongside a 2-log ladder.
42. Photograph gel using the EZ Imager. Compare before and after AMPure purification ensure that there is a single band after cleanup. Alternatively, use a BioAnalyzer or DNA ScreenTape to evaluate Library.

4.8 Dilution

43. Quantify Library sample (Qubit HS, follow manufactures protocol) and normalize to 15 nM using the following formula (library size V4 386 bp).

$$\frac{(\text{concentration in ng}/\mu\text{l})}{(660\ \text{g/mol} \times \text{average library size in bp})} \times 10^6 = \text{concentration in nM}$$

44. Barcode tube and store library at -80C or send for MiSeq 2x250bp sequencing.

5 Appendix

5.1 Primers

5.1.1 Overall design considerations:

- The sequencing primers must have a melting temperature near 65°C. This can be achieved by altering the pad sequence.
- The index sequences must balance the number of bases at each position. The index sequences listed here have a 25% ATGC composition at each site. If you are going to cherry pick indices from the list, make sure that you have even representation.

5.1.2 Generic PCR primer design:

AATGATACGGCGACCACCGAGATCTACAC <i5><pad><link><16Sf> VX.N5??
CAAGCAGAAGACGGCATACGAGAT <i7><pad><link><16Sr> VX.N7??
Generic read 1 primer design: <pad><link><16Sf> VX.read1

Generic read 2 primer design: <pad><link><16Sr> VX.read2
Generic index read primer design: Reverse complement of (<pad><link><16Sr>) VX.p7_index

The listed sequences in the generic design, above, are the adapter sequences to allow annealing of the amplicons to the flow cell. The i5 and i7 sequences are the 8-nt index sequences. The pad is a 10-nt sequence to boost the sequencing primer melting temperatures. The link is a 2-nt sequence that is anti-complementary to the known sequences. The 16Sf and 16Sr are the gene specific primer sequences. Primers are purchased from IDT with no special purification. This system should work for any other region of the 16S rRNA gene or any other gene. The only thing to change would be the 16Sf/16Sr sequences and confirm that when combined with the pad sequence that the melting temperature is near 65°C.

5.1.3 Sequence primers

Name		**pad**	**link**	**16Sf**
Read1		TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
		pad	**link**	**16Sr**
Read2		AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
		pad	**link**	**16Sr**
ReadI		AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT

5.1.4 Forward index primers

Name		adapter	i5	pad	link	16Sf
SA501		AATGATACGGCGACCACCGAGATCTACAC	ATCGTACG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA502		AATGATACGGCGACCACCGAGATCTACAC	ACTATCTG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA503		AATGATACGGCGACCACCGAGATCTACAC	TAGCGAGT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA504		AATGATACGGCGACCACCGAGATCTACAC	CTGCGTGT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA505		AATGATACGGCGACCACCGAGATCTACAC	TCATCGAG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA506		AATGATACGGCGACCACCGAGATCTACAC	CGTGAGTG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA507		AATGATACGGCGACCACCGAGATCTACAC	GGATATCT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SA508		AATGATACGGCGACCACCGAGATCTACAC	GACACCGT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB501		AATGATACGGCGACCACCGAGATCTACAC	CTACTATA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB502		AATGATACGGCGACCACCGAGATCTACAC	CGTTACTA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB503		AATGATACGGCGACCACCGAGATCTACAC	AGAGTCAC	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB504		AATGATACGGCGACCACCGAGATCTACAC	TACGAGAC	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB505		AATGATACGGCGACCACCGAGATCTACAC	ACGTCTCG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB506		AATGATACGGCGACCACCGAGATCTACAC	TCGACGAG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB507		AATGATACGGCGACCACCGAGATCTACAC	GATCGTGT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SB508		AATGATACGGCGACCACCGAGATCTACAC	GTCAGATA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC501		AATGATACGGCGACCACCGAGATCTACAC	ACGACGTG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC502		AATGATACGGCGACCACCGAGATCTACAC	ATATACAC	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC503		AATGATACGGCGACCACCGAGATCTACAC	CGTCGCTA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC504		AATGATACGGCGACCACCGAGATCTACAC	CTAGAGCT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC505		AATGATACGGCGACCACCGAGATCTACAC	GCTCTAGT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC506		AATGATACGGCGACCACCGAGATCTACAC	GACACTGA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC507		AATGATACGGCGACCACCGAGATCTACAC	TGCGTACG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SC508		AATGATACGGCGACCACCGAGATCTACAC	TAGTGTAG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD501		AATGATACGGCGACCACCGAGATCTACAC	AAGCAGCA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD502		AATGATACGGCGACCACCGAGATCTACAC	ACGCGTGA	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD503		AATGATACGGCGACCACCGAGATCTACAC	CGATCTAC	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD504		AATGATACGGCGACCACCGAGATCTACAC	TGCGTCAC	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD505		AATGATACGGCGACCACCGAGATCTACAC	GTCTAGTG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD506		AATGATACGGCGACCACCGAGATCTACAC	CTAGTATG	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD507		AATGATACGGCGACCACCGAGATCTACAC	GATAGCGT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
SD508		AATGATACGGCGACCACCGAGATCTACAC	TCTACACT	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA

5.1.5 Reverse index primers

Name		adapter	i7	pad	link	16Sr
SA701		CAAGCAGAAGACGGCATACGAGAT	AACTCTCG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA702		CAAGCAGAAGACGGCATACGAGAT	ACTATGTC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA703		CAAGCAGAAGACGGCATACGAGAT	AGTAGCGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA704		CAAGCAGAAGACGGCATACGAGAT	CAGTGAGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA705		CAAGCAGAAGACGGCATACGAGAT	CGTACTCA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA706		CAAGCAGAAGACGGCATACGAGAT	CTACGCAG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA707		CAAGCAGAAGACGGCATACGAGAT	GGAGACTA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA708		CAAGCAGAAGACGGCATACGAGAT	GTCGCTCG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA709		CAAGCAGAAGACGGCATACGAGAT	GTCGTAGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA710		CAAGCAGAAGACGGCATACGAGAT	TAGCAGAC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA711		CAAGCAGAAGACGGCATACGAGAT	TCATAGAC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SA712		CAAGCAGAAGACGGCATACGAGAT	TCGCTATA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB701		CAAGCAGAAGACGGCATACGAGAT	AAGTCGAG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB702		CAAGCAGAAGACGGCATACGAGAT	ATACTTCG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB703		CAAGCAGAAGACGGCATACGAGAT	AGCTGCTA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB704		CAAGCAGAAGACGGCATACGAGAT	CATAGAGA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB705		CAAGCAGAAGACGGCATACGAGAT	CGTAGATC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB706		CAAGCAGAAGACGGCATACGAGAT	CTCGTTAC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB707		CAAGCAGAAGACGGCATACGAGAT	GCGCACGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB708		CAAGCAGAAGACGGCATACGAGAT	GGTACTAT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB709		CAAGCAGAAGACGGCATACGAGAT	GTATACGC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB710		CAAGCAGAAGACGGCATACGAGAT	TACGAGCA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB711		CAAGCAGAAGACGGCATACGAGAT	TCAGCGTT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SB712		CAAGCAGAAGACGGCATACGAGAT	TCGCTACG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC701		CAAGCAGAAGACGGCATACGAGAT	ACCTACTG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC702		CAAGCAGAAGACGGCATACGAGAT	AGCGCTAT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC703		CAAGCAGAAGACGGCATACGAGAT	AGTCTAGA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC704		CAAGCAGAAGACGGCATACGAGAT	CATGAGGA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC705		CAAGCAGAAGACGGCATACGAGAT	CTAGCTCG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC706		CAAGCAGAAGACGGCATACGAGAT	CTCTAGAG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC707		CAAGCAGAAGACGGCATACGAGAT	GAGCTCAT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC708		CAAGCAGAAGACGGCATACGAGAT	GGTATGCT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC709		CAAGCAGAAGACGGCATACGAGAT	GTATGACG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC710		CAAGCAGAAGACGGCATACGAGAT	TAGACTGA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC711		CAAGCAGAAGACGGCATACGAGAT	TCACGATG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SC712		CAAGCAGAAGACGGCATACGAGAT	TCGAGCTC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD701		CAAGCAGAAGACGGCATACGAGAT	ACCTAGTA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD702		CAAGCAGAAGACGGCATACGAGAT	ACGTACGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD703		CAAGCAGAAGACGGCATACGAGAT	ATATCGCG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD704		CAAGCAGAAGACGGCATACGAGAT	CACGATAG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD705		CAAGCAGAAGACGGCATACGAGAT	CGTATCGC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD706		CAAGCAGAAGACGGCATACGAGAT	CTGCGACT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD707		CAAGCAGAAGACGGCATACGAGAT	GCTGTAAC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD708		CAAGCAGAAGACGGCATACGAGAT	GGACGTTA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD709		CAAGCAGAAGACGGCATACGAGAT	GGTCGTAG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD710		CAAGCAGAAGACGGCATACGAGAT	TAAGTCTC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD711		CAAGCAGAAGACGGCATACGAGAT	TACACAGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT
SD712		CAAGCAGAAGACGGCATACGAGAT	TTGACGCA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT

5.1.6 Primer plate layout

		SA701	SA702	SA703	SA704	SA705	SA706	SA707	SA708	SA709	SA710	SA711	SA712
	Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
SA501	A												
SA502	B												
SA503	C												
SA504	D												
SA505	E												
SA506	F												
SA507	G												
SA508	H												

		SA701	SA702	SA703	SA704	SA705	SA706	SA707	SA708	SA709	SA710	SA711	SA712
	Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
SB501	A												
SB502	B												
SB503	C												
SB504	D												
SB505	E												
SB506	F												
SB507	G												
SB508	H												

		SB701	SB702	SB703	SB704	SB705	SB706	SB707	SB708	SB709	SB710	SB711	SB712
	Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
SA501	A												
SA502	B												
SA503	C												
SA504	D												
SA505	E												
SA506	F												
SA507	G												
SA508	H												

		SB701	SB702	SB703	SB704	SB705	SB706	SB707	SB708	SB709	SB710	SB711	SB712
	Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
SB501	A												
SB502	B												
SB503	C												
SB504	D												
SB505	E												
SB506	F												
SB507	G												
SB508	H												