16S Amplicon Sequencing MiSeq Setup

Standard Protocol

Version 1.06

Introduction

This protocol is used for sequencing custom bacterial 16S V1-3, V4 and V3-4 amplicon libraries and Illumina Truseq/Nextera libraries.

Materials

Instruments

- MiSeq (Illumina)
- Pipettes (Range 1 uL to 1000 uL)
- MiSeq System User Guide (15027617, Illumina)

Reagents/Consumables

- Ice
- MiSeq Reagent kit v3, 600 cycles (Illumina)
- 2 N NaOH, molecular grade
- Custom primers (Read1, Read2, and Index)
- DNAse free tips (10uL, 300uL and 1000 uL)
- DNAse free tubes (1.5 mL)
- Nuclease Free H_2O (Qiagen)
- Phix control library v3, 10 nM (Illumina)
- EtOH, 70 % (molecular grade)
- Laboratory wipes
- Microscope lense cleaning wipes

Method

Input Sample Libraries

1. Library concentration must be 4 nM or above.
2. Thaw libraries and store on ice. Before use, adjust to ambient temperature.

Preparation of MiSeq Instrument and Reagents

1. Instrument Washing
    1. Press ‘Sequencing’ on the welcome screen. If washing is required the machine will let you know. [1]

2. Reboot the MiSeq to reset the memory
    1. Under "Manage Instrument" press Reboot and wait (might take 10 minutes)

[1] If the instrument has been put in standby mode a maintenance wash is required before sequencing can commence. Washing takes around 60 min.

3. Thaw reagents
    1. Place reagent cartridge and HT1 buffer in water bath at room temperature for 1 hour. After thawing store at 4°C until use. Can be stored for at least a week at 4 °C
    2. Reagent cartridge inspection (invert cartridge ten times, inspect for precipitates, tap cartridge on table to remove bubbles).
    3. Thaw sequencing primers (read 1, read 2 and index), 2N NaOH and PhiX control library at room temperature and then place on ice.
4. Prepare Sample Sheet
    1. Open a MiSeq sample sheet template in Notepad++ **(NOT in excel!!!)**
    2. Change project and sample specific info: [Header] Investigator, Project name, Experiment Name, Date; [Data] Sample_ID, Sample_Name, index and index2.
    3. Check Sample Sheet integrity by loading it into the Illumina 'Experimental Manager' If the sheet can be loaded it is compatible with the MiSeq.

Prepare Sequencing Library

3. Dilute sequencing libraries and PhiX library to 4 nM using nuclease free water (see volumes in table in appendix).
4. Prepare 0.1 N NaOH solution.
    1. 475 ul DNA H_2O and 25 ul 2.0 N NaOH
5. Denature sequencing libraries
    1. Mix 5 µL library + 5 uL 0.2 N NaOH. Final concentration is 2 nM
    2. Pipette up and down 10 times
    3. Incubate for 5 min at room temperature.
6. Dilute denatured libraries (2 nM) to 20 pM
    1. Mix 10 uL denatured library with 990 Pre-chilled HT1. Concentration is 20 pM.
7. Mix PhiX library (20 pM) with sequencing libraries (20 pM) in a 20 % to 80 % ratio
    1. Mix 120 ul PhiX library with 480 ul of different sequencing libraries (see mixing table in appendix)
    2. Place the mixed library on ice until use.

Load sample and primers on reagent cartridge

1. Adding custom sequencing primers to reagent cartridge
    1. Primer destinations:
       Read1 = well 12
       Index = well 13
       Read2 = well 14

    2. For each primer: Pierce tinfoil covering the target well with pipette 1000 µL tip, remove 100 uL of the primer solution from the well to a spintube. Add 3.4 uL of the needed primer and mix well. Transfer Primer solution back to the well it originated from and mix. Repeat for all primers.
2. Adding sample to reagent cartridge
    a. Pierce sample well with a tip and add 600 ul mixed PhiX/sequencing library.

Start sequencing run

5. Follow instructions in MiSeq System User Guide for preparing/loading the flow cell, loading the reagent cartridge, referring to the sample sheet and starting the sequencing run.

Appendix

Dilution to 4 nM

Target concentration is 4 nM and final volume must be >5 uL.

Sample	Input (nM)	V_{sample}	V_{H2O}
Library Pool			
Truseq/Nextera Library			
Phix library*	10		

***** PhiX library is 10 nM when delivered from Illumina.

Denaturation Table

Sample	Input (nM)	Denaturation	
		V_{sample}	V_{NaOH}
Library Pool	4	5	5
Truseq/Nextera Library	4	5	5
Phix library	4	5	5

Dilution to 20 pM

Sample	Input (nM)	HT1 Dilution		Final (pM)
		V_{sample}	V_{HT1}	
Library Pool	2	10	990	20
Truseq/Nextera Library	2	10	990	20
Phix library*	2	10	990	20

Sequencing Library Mixing Table

Library	%	uL
Library Pool	20	240
Truseq/Nextera Library	40	240
Phix library	20	120

Literature

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108 Suppl* , 4516–22. doi:10.1073/pnas.1000080107

Caporaso, J. G., Lauber, C. L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., … Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, *6*(8), 1621–4. doi:10.1038/ismej.2012.8**evision History**

V1.01 2013-3-19

- Protocol created based on numerous journals, the 'MiSeq System User Guide - Part # 15027617 Rev. D July 2012', Caporaso, J.G. et al., 2010. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. and Caporaso, J.G. et al., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal, 6(8), pp.1621–1624.

V1.02 2013-4-15

- Minor additions and elaborations.

V1.03 2013-8-21

- Changed input concentration from 10 nM to 4 nM and revamped the dilution scheme to be in line with the Illumina guidelines in Part # 15039740 Rev. B
- Included reference to example sample sheet
- Added hack to increase number of possible cycles from 500 to 610
- Added example of converting ng/uL to nM

V1.04 2013-8-29

- Minor changes to dilution scheme to match new recommendations for MiSeq reagent kit v3 (see Part # 15039740 Rev. C)
- Removed the hack steps where extra reagent was added. Extra reagent is not needed anymore since the new kits are designed for 2x300 bp.

V1.05 2013-9-22

- Minor corrections
- Changed NaOH working solution concentration from 0.2 to 0.1 NaOH. Provides more room for "up concentrating" low concentration samples.
- Add tables for dilution and mixing overview.
- Updated the V13 amplicon input concentration to 6 nM from 4 nM

V1.06 2015-02-24

- V13 amplicon input concentration reduced to 4 nM again.
- Streamlining and simplification of explanations.