**SOP# 02.354.01**

**16S Illumina library preparation**

**Author: Allison Perrotta**

**Date of Rev.: October 5, 2016**

**Page 1 of 6**

**Purpose**

This SOP describes the procedure used to create 16S libraries for Illumina sequencing from purified DNA samples.

**Scope**

For exploratory and purposes

**Regulatory References**

NA

**Responsibility**

* Responsibility of experimentalist – understanding and performing this procedure as described; reporting any deviations or problems to area supervisor; adequately documenting the procedures and results
* Area manager or supervisor – ensuring that the analyst performing this procedure is qualified; ensuring that the procedure is followed and update the procedure as necessary

**Definitions/Abbreviations**

PCR – polymerase chain reaction

M – molar, moles/liter

L – liter

mL – milliliter

uL – microliter

EB – elution buffer

Ct – cross threshold

**Related Documents**

SOP# 02.303.01 – DNA extraction clinical feces

SOP# 03.004.01 - Laminar flow hood operation and maintenance

**Required Equipment and Materials / Reagents**

* Sterile, filtered pipettor tips, any brand
* Pipetteman, for example VWR model# 89079
* Eppendorf PCR thermal block, any brand PCR thermal block can be used
* In house vacuum line, if facility does not have an in house vacuum line a stand alone vacuum pump can be used
* Ultra-clean PCR grade water, for example VWR catalog# 10126-568
* SYBR dye (Invitrogen, S7563)
* Agencourt Ampure XP, A63881 (60mL, $300)
* Roche LightCycler480 384-well plate, white
* PCR primers (IDT, all listed as 5’-3’)
  + PE16S\_V4\_U515\_F

ACACGACGCTCTTCCGATCTYRYRGTGCCAGCMGCCGCGGTAA

* + PE16S\_V4\_E786\_R CGGCATTCCTGCTGAACCGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT
  + PE\_PCR\_III-F AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
  + PE-PCR-IV-XXX (X represents a 9 base barcode sequence)

CAAGCAGAAGACGGCATACGAGATXXXXXXXXXCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

* + BMCFinal-F AATGATACGGCGACCACCGA
  + BMCFinal-R CAAGCAGAAGACGGCATACGA
* HF Phusion (NEB, M0530L)
* KAPA SYBR 2xMM , Kapa catalog# 07959362001
* Invitrogen Super magnet (16 or 8 sample capacity)
* 96 well PCR plate (VWR, catalog # 82006-704), any PCR clean 96 well plates may be used
* 8 tube PCR strip tubes (VWR, catalog # 93001-120), any PCR clean strip tubes may be used
* PCR sealing film (VWR, catalog # 82018-846), any sterile PCR sealing film may be used
* Clear QPCR plate covers (VWR catalog# 47743-942)
* Bench top vortexer (VWR, Cat# 14005-824), any benchtop vortexer that can fit the vortex adapter above can be used
* Bench top microcentrifuge, for example Eppendorf 5427R
* 190 proof Ethanol (CAS# 64-17-5)
* Elution buffer (Qiagen, cat # 19086)
* Laminar flow hood or equivalent PCR clean work area such as a biosafety cabinet
* QPCR machine, for example Roche Lightcycler480
* 10mM dNTPs (NEB, cat# N0447L)

**Precautions**

* Personal protection equipment including gloves, lab glasses, and lab coat must be worn when executing this procedure
* Ethanol is flammable and must be handled in a chemical hood

**Procedure**

1. Remove desired samples of previously extracted DNA (SOP# 02.303.01 – DNA extraction clinical feces ) from the -20°C freezer and thaw on ice.
2. Determine cycle parameters and sample normalization for Step 1
   1. Each sample will be run in triplicate
   2. Prepare Master mix as follows then place on ice:

|  |  |
| --- | --- |
| Reagent | X1 RXN (uL) |
| Ultra-clean water | 12.1 |
| HF Buffer | 5 |
| dNTPs | 0.5 |
| PE16s\_V4\_U515\_F (3uM) | 2.5 |
| PE16S\_V4\_E786\_R (3uM) | 2.5 |
| SYBR green (1/100 dilu) | 0.125 |
| Phusion | 0.25 |

* 1. Dilute all samples 1:10 in EB
  2. In a QPCR plate, aliquot 23uLs of master mix per well
  3. Aliquot 2uLs of each DNA sample into three wells (three wells/tubes per sample)
  4. Include a negative well containing master mix and 2uLs EB
  5. Mix by pipetting
  6. Seal plate with clear QPCR sealing film
  7. Spin plate down using bench top centrifuge
  8. Wrap plate in aluminum foil to shield from light
  9. Place plate on ice
  10. Place plate/tubes onto Roche LightCycler QPCR machine and run the following program:
      1. **Activation:** 95°C - 5 minutes
      2. **Amplification:**

95°C – 30 seconds

60°C – 45 seconds (1-step annealing/extension)

Measure (SYBR green setting)

* + 1. Run 35 cycles of amplification
    2. Cool to 4°C
  1. Export and save Ct data to an excel spread sheet
  2. Normalize each sample by dilution using the Ct values
     1. Note: This is a relative normalization, do not take 1:10 dilution of original material into account in this calculation or samples will be too dilute
     2. delta Ct = Sample Ct - lowest Ct of non-negative sample in sample set (maximum of 20 cycles)
     3. fold = 1.75^(delta Ct)
     4. ratio = 1/fold
     5. volume to mix b/c of ratio = 2uL\*ratio
     6. how to dilute = fold
     7. note - sample with lowest Ct will get an undiluted 2uls added to step 1 PCR

1. Step 1 – 16S amplification
   1. Each sample will be run as four 25uL reactions that are pooled back into one 100uL volume per sample after cycling
   2. Prepare master mix as follows and place on ice:

|  |  |
| --- | --- |
| Reagent | X1 RXN (uL) |
| Ultra-clean water | 12.25 |
| HF Buffer | 5 |
| dNTP | 0.5 |
| PE16S\_V4\_U515\_F  (3uM) | 2.5 |
| PE16S\_V4\_E786\_R (3uM) | 2.5 |
| HF Phusion | 0.25 |

* 1. Aliquot 23uLs of master mix into PCR plate/tubes
  2. Aliquot 2uLs of normalized DNA sample (four wells/tubes for each sample)
  3. Mix by pipetting
  4. Seal plate with PCR sealing film
  5. Spin plate/tubes down using bench top centrifuge
  6. Place plate/tubes onto PCR thermal block and run the following program:
     1. Heat: 98°C – 30 seconds
     2. Amplify:

 98°C – 30 seconds

52°C – 30 seconds

72°C – 30 seconds

* + 1. Repeat Amplification steps X number of times (X determined by Ct number used in normalization above, can not be greater than 20)
    2. Cool: 4°C
  1. Vortex and spin down samples using bench top vortexor and centrifuge
  2. Pool replicates into one 100uL volume per sample
  3. Clean PCR reactions with Ampure beads (SOP XXX)

1. Step 2 – Addition of required Illumina sequences
   1. Each sample will be run as four 25uL reactions that are pooled back into one 100uL volume per sample after cycling
   2. Prepare master mix as follows and place on ice:

|  |  |
| --- | --- |
| Reagents | X1 RXN (uL) |
| Ultra-clean water | 8.65 |
| HF Buffer | 5 |
| dNTPs | 0.5 |
| PE-PCR-III-F (3uM) | 3.3 |
| PE-PCR-IV-XXX (3uM) | 3.3 |
| HF Phusion | 0.25 |

* 1. Aliquot 21uLs of master mix into PCR plate/tubes
  2. Aliquot 4uLs of DNA from Step 1 (four wells/tubes for each sample)
  3. Mix by pipetting
  4. Seal plate with PCR sealing film
  5. Spin plate/tubes down using bench top centrifuge
  6. Place plate/tubes onto PCR thermal block and run the following program:
     1. Heat: 98°C – 30 seconds
     2. Amplify:

 98°C – 30 seconds

83°C – 30 seconds

72°C – 30 seconds

* + 1. Repeat Amplification steps 7 times
    2. Cool: 4°C
  1. Vortex and spin down samples using bench top vortexor and centrifuge
  2. Pool replicates into one 100uL volume per sample
  3. Clean PCR reactions with Ampure beads (SOP XXX)

1. Final QPCR to quality check libraries and normalize
   1. Each sample will be run in triplicate
   2. Prepare Master mix as follows then place on ice:

|  |  |
| --- | --- |
| Reagents | X1 RXN (uL) |
| Ultra-clean water | 7.2 |
| BMC Final – F (10uM) | 0.4 |
| BMC Final – R (10uM) | 0.4 |
| KAPA SYBRgreen MM | 10 |

* 1. Dilute libraries 1:10 in EB
  2. Aliquot 18uLs of master mix into QPCR plate/tubes
  3. Aliquot 2uLs of diluted libraries into plate wells/tubes (three wells/tubes per sample)
  4. Place plate/tubes onto Roche LightCycler QPCR machine and run the following program:
     1. **Activation:** 95°C - 5 minutes
     2. **Amplification:**

95°C – 30 seconds

60°C – 45 seconds (1-step annealing/extension)

Measure (SYBR green setting)

* + 1. Run 35 cycles of amplification
    2. Cool to 4°C
  1. Export and save Ct data to an excel spread sheet
  2. Normalize each sample by dilution using the Ct values
     1. Note: This is a relative normalization, do not take 1:10 dilution of original material into account in this calculation or samples will be too dilute
     2. delta Ct = Sample Ct - lowest Ct of non-negative sample in sample set
     3. fold = 1.75^(delta Ct)
     4. ratio = 1/fold
     5. volume to mix b/c of ratio = X\*ratio (X = minimum desired volume per sample)
     6. how to dilute = fold
     7. note - sample with lowest Ct will get an undiluted Xuls added to final multiplex, X can be raised or lowered to accommodate the needed volume of other samples
  3. Use normalization data above to dilute and pool samples as needed into one final library mix

1. Pooled library can now be submitted for Illumina sequencing at the MIT BioMicro Center or the Broad Sequencing pipeline

**Version History**

This is the first version of this document

**Worksheets**

Worksheet 1 - 16S library preparation – initial

Worksheet 2 - 16S library preparation - final

**Appendix**

NA