# 16S Library Multiplex Preparation (Automated) - in progress

16S Library Multiplex Preparation (Automated)

Note:

* This protocol is for preparing 16S libraries on Rosie (the Alm lab's epMotion 5075) and should only be used to prepare 96 well sample plates for multiplexing.
* At the beginning of EACH DAY using Rosie spray and wipe down the deck with RNAse AWAY and 70% EtOH then UV the deck for 10-15minutes before starting protocols for the day.
* If you are preparing only one plate for sequencing please use the PE\_16s\_V4\_U515\_F primer in the first step PCR. If you are planning on combining multiple 96 well plates for sequencing (**4 or more recommended**) please use the PE\_16s\_V4\_F\_Bar## primer for your first step PCR.
* When needed all plates (except those being stored directly after a PCR step) should be stored with aluminum foil plate covers

Materials:

* Agencourt Ampure XP, A63881 (60mL, $300)
* 2 Roche LichtCycler480 384-well plate (Axygen, PCR-384-LC480-W)
* 1:100 dilution of SYBR stock (Invitrogen S7563, 10,000x)
* Step 1/ Initial QPCR Primers ( PE\_16s\_v4U515-F)
* Step 2 primers ( PE-III-PCR-F, PE-IV-PCR-XXX; NOTE - PE-IV-PCR-XXX primers should be used as a pre-pipetted 96well plate)
* Final QPCR primers (BMC Final F, BMC Final R)
* HF Phusion (NEB, M0530L)
* KAPA SYBR 2xMM for final QPCR (Kapa biosystems, KK4611)
* Invitrogen Super magnet (16 sample capacity: DynaMag-5, 12303, $750; -OR- 8 sample capacity: DynaMag-Spin, 12320, $358.79)
* 96 well magnet plate (Agencourt, SPRIPlate 96R Super Magnet Plate, A32782, ~$734.00 ($660 with academic discount))
* up to 10 boxes of 300uL filtered epTIPS (Eppendorf, Cat# 0030014456)
* up to 13 boxes of 300uL unfiltered epTIPS (Eppendorf, bulk Cat# 960050620)
* 8 boxes of 50uL filtered epTIPS (Eppendorf, Cat# 0030014413)
* up to 10 30mL reagent reservoirs (Eppendorf, 250 reservoirs Cat# 960051500)
* up to 2 100mL reagent reservoirs (Eppendorf, 250 reservoirs Cat# 960051511)
* Aluminum foil freezer plate covers (VWR, 89049-034)
* PCR plate covers (VWR, 951022115)

**Determination of  Step 1 Cycle Time and Sample Check:**

Rosie Protocol Used: '16s Stp1 384 QPCR.dws', please note that this protocol can be run for 1 or 2 sample plates at once (there is a prompt half way through the protocol that will ask if you are running two plates)

Materials used:

* Contents of MM
* 384 well Lichtcycler QPCR plate
* Clear PCR plate covers
* 50uL filtered epTIPS x 2
* 300uL non-filtered epTIPS x 1
* 30mL reagent reservoir x 1

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **X1 RXN (uL)** | **X220 RXN (uL)** | **X431 RXN (uL)** |
| **H2O** | **12.1** | **2,662** | **5,215.1** |
| **HF Buffer** | **5** | **1,100** | **2,155** |
| **dNTPs** | **0.5** | **110** | **215.5** |
| **PE16s\_V4\_U515\_F (3uM)** | **2.5** | **550** | **1,077.5** |
| **PE16S\_V4\_E786\_R (3uM)** | **2.5** | **550** | **1,077.5** |
| **Template** | **2** | **-** | **-** |
| **SYBR green (1/100 dilu)** | **0.125** | **27.5** | **53.9** |
| **Phusion** | **0.25** | **55** | **107.8** |

Run this step in duplicate or triplicate to best estimate proper cycling time

Initial QPCR Program (Roche Lighcycler 480):  
Heat:  
98°C – 30 seconds  
Amplify:  
98°C – 30 seconds  
52°C – 30 seconds  
72°C – 30 seconds  
Cool:  
4°C - continuous  
Use Ct (bottom of curve, not mid-log) of curves to determine dilutions for step 1 amplification (Illumina Library Construction/Tools/Attachments/16s primer\_amp\_QPCR sheets.xls)  
Breakdown of QPCR amplification math (done to normalize each sample):  
○   delta Ct = Sample Ct - lowest Ct in sample set  
○   fold = 1.75^(delta Ct)  
○   dilution needed = fold  
○  note - that input is 2uL per RXN so sample with lowest Ct gets 2uL undiluted  
**Please note** – samples may fail due to too little, too much material, or a poor reaction. It is recommended that failed samples be re-run before moving forward

Sample Normalization (one plate at a time):

Rosie Protocol Used: 'Initial Dilu-Sample2.dws' and 'Initial Dilution-H2O.dws'

- The above protocols import volume information using the Initial Dilution Sample and H2O pages from the Illumina Library Construction/Tools/Attachments/16s primer\_amp\_QPCR sheets.xls **saved as two individual CSV files** and imported to Rosie.

- please run the H2O protocol first

Materials used:

* 50uL filtered epTIPS x 2
* 30mL reagent reservoir x 1
* EB
* 1 Axygen 96 well skirted PCR plate

Library Preparation:

**Step 1 (one plate at a time)**

**Rosie Protocol Used:** '16s Step 1 PCR.dws'

Materials used:

* 4 Axygen 96 well skirted PCR plates
* 1 row of a 300uL non-filtered epTIPS box
* 1 box of 50uL filtered epTIPS
* 1 30mL reagent reservoir
* Clear PCR plate covers

Please Note: Samples are run as four 25uL reactions that are pooled at end of cycling

1st step Master Mix 25uL RXN (MM1)

|  |  |  |
| --- | --- | --- |
| **Reagent** | **X1 RXN (uL)** | **X431 RXNs (uL)** |
| **H2O** | **12.25** | **5,279.8** |
| **HF Buffer** | **5** | **2,155** |
| **dNTP** | **0.5** | **215.5** |
| **PE16S\_V4\_U515\_F -OR- PE16S\_v4\_F\_Bar##  (3uM)** | **2.5** | **1077.5** |
| **PE16S\_V4\_E786\_R (3uM)** | **2.5** | **1077.5** |
| **Template** | **2** | **-** |
| **Phusion** | **0.25** | **107.8** |

16SStep 1 Cycling Program:  
Heat:  
98°C – 30 seconds  
Amplify:  
98°C – 30 seconds  
52°C – 30 seconds  
72°C – 30 seconds  
Cool:  
4°C - continuous  
Run amplification cycle number determined via QPCR (no more than 20 cycles allowed)

After cycling pool duplicates, now have 1x 100uL reaction per sample:

**Rosie Protocol Used:** '4x to 96pool.dws'

Materials used:

* 1 Axygen 96 well skirted PCR plate
* 1 box of 300uL filtered epTIPS

96 well SPRI Clean Up

**Rosie Protocol Used:** 'SingleSPRI.dws'

Materials used:

* SPRI beads (8.5mL)
* 70% EtOH (30.2ml)
* EB (4.1mL)
* 96 well magnet plate
* 3 30mL reagent reservoirs
* 1 100mL reagent reservoir (for waste)
* 1 Axygen 96 well skirted PCR plate
* 3 boxes of 300uL filtered epTIPS
* 5 boxes of 300uL un-filtered epTIPS

Step 2 (one plate at a time):

**Rosie Protocol Used:** '16s Step2 PCR.dws'

Materials used:

* 4 Axygen 96 well skirted PCR plates
* 1 pre-pipetted PE-PCR-IV-XXX barcode plate
* 3 boxes 50uL filtered epTIPS
* 1 30mL reagent reservoir
* Clear PCR plate covers

Please Note: Samples are run as four 25uL reactions that are pooled at end of cycling

|  |  |  |
| --- | --- | --- |
| **Reagents** | **X1 RXN (uL)** | **X431 RXNs (uL)** |
| **H2O** | **8.65** | **3,728.15** |
| **HF Buffer** | **5** | **2,155** |
| **dNTPs** | **0.5** | **215.5** |
| **PE-PCR-III-F (3uM)** | **3.3** | **1,422.3** |
| **PE-PCR-IV-XXX (3uM)** | **3.3** | **-** |
| **Template** | **4** | **-** |
| **Phusion** | **0.25** | **107.75** |

16s Step 2 Cycling Program:

Heat:  
98°C – 30 seconds  
Amplify:  
98°C – 30 seconds  
83°C – 30 seconds  
72°C – 30 seconds  
Cool:  
4°C - continuous  
Run 7 cycles of amplification

After cycling pool duplicates, now have 1x 100uL reaction per sample:

**Rosie Protocol Used:** '4x to 96pool.dws'

Materials used:

* 1 Axygen 96 well skirted PCR plate
* 1 box of 300uL filtered epTIPS

**\***\* Now you have a choice!**\***\*\*

**#1 - If your sample plate is a mixture of sample types (stool and saliva for example) or you have reason to suspect something went wrong**

**#2 - If your sample plate is all the same sample type all samples can be pooled 1:1 after**

**Choice #1:**

96 well SPRI Clean Up

**Rosie Protocol Used:**'SingleSPRI.dws'

Materials used:

* SPRI beads (8.5mL)
* 70% EtOH (30.2ml)
* EB (4.1mL)
* 96 well magnet plate
* 3 30mL reagent reservoirs
* 1 100mL reagent reservoir (for waste)
* 1 Axygen 96 well skirted PCR plate
* 3 boxes of 300uL filtered epTIPS
* 5 boxes of 300uL un-filtered epTIPS

Final QPCR (this is a copy of the BMC QPCR for quality control, it can be modified to use normal SYBR green as well):  
Once you have a substantial or all of your samples prepared you can run a final QPCR to determine dilutions and volumes for multiplexing. This step also confirms that the library preparation was successful!

|  |  |  |
| --- | --- | --- |
| **Reagents** | **X1 RXN (uL)** | **X220 RXNs (uL)** |
| **H2O** | **7.2** | **1,584** |
| **PE Seq Primer-F (10uM)** | **0.4** | **88** |
| **PE Seq Primer-R (10uM)** | **0.4** | **88** |
| **KAPA SYBRgreen MM** | **10** | **2,200** |
| **Template 2** | **2** | **-** |

Final QPCR Program (Opticon or Lichtcycler)

Activation:  
95°C - 5 minutes  
Amplification:  
95°C – 30 seconds  
60°C – 45 seconds

Run 35 cycles of amplification

Use mid-log phase of curves to determine volumes for multiplexing (use Illumina Library Construction/Tools/Attachments/16s primer\_amp\_QPCR sheets.xls)  
**Please note** – samples may fail due to too little, too much material, or a poor reaction. It is recommended that failed samples be re-run before moving forward  
Breakdown of QPCR multiplexing math (done to normalize each sample):  
○  delta Ct = Sample Ct - lowest Ct in sample set  
○  fold = 1.75^(delta Ct)  
○  ratio = 1/fold  
○  volume to mix b/c of ratio = X\*ratio (X = minimum desired volume per sample)  
○  how to dilute = fold  
○  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

Multiplexing:

Rosie protocol used: '96 to 1 pool\_postSPRI.dws'

Materials used:

* 2 1.5mL eppendorf tubes (one for samples and one for negatives)
* 1 box of 300uL filtered epTIPs

- The above protocol imports volume information using the Final Multiplex page from the Illumina Library Construction/Tools/Attachments/16s primer\_amp\_QPCR sheets.xls **saved as a CSV files** and imported to Rosie

- Please note that the tube rack takes up two deck positions, this is not shown on Rosie's deck display

**Choice 2:**

Multiplexing:

Rosie protocol used: '96 to 1 pool\_preSPRI.dws'

Materials used:

* 2 1.5mL eppendorf tubes (one for samples and one for negatives)
* 1 box of 300uL filtered epTIPs

- The above protocol imports volume information using the Final Multiplex page from the Illumina Library Construction/Tools/Attachments/16s primer\_amp\_QPCR sheets.xls **saved as a CSV files** and imported to Rosie

- Please note that the tube rack takes up two deck positions, this is not shown on Rosie's deck display

SPRI (for individual tubes of pooled samples):

Materials used:

* SPRI beads (85% of total sample volume)
* 70% EtOH (2mLs per sample tube)
* EB (200uL per sample tube)
* Invitrogen Super magnet (16 or 8 sample capacity)

- allow SPRI beads to warm to RT

- Aliquot SPRI beads into tubes containing sample mixture (SPRI volume should be 85% of sample volume)

- Vortex well and incubate for 13 minutes at RT

- Separate on magnet for 2 minutes

- While on magnet, remove/discard SN

- Wash beads 2x with 1mL of 70% EtOH

- Air dry beads for 15-20 minutes on magnet

- remove tubes from magnet, add 200uL of EB to tubes

- vortex well, incubate for 7 minutes at RT (still off the magnet)

- Separate on magnet for 2 minutes

- transfer SN to new tube

Final QPCR  
Once you have a substantial or all of your samples prepared you can run a final QPCR to determine dilutions and volumes for multiplexing. This step also confirms that the library preparation was successful

|  |  |
| --- | --- |
| Reagents | X1 RXN (uL) |
| H2O | 7.2 |
| PE Seq Primer – F (10uM) | 0.4 |
| PE Seq Primer – R (10uM) | 0.4 |
| KAPA SYBRgreen MM | 10 |
| Template | 2 |

Final QPCR Program (Opticon or Lichtcycler)

Activation:  
95°C - 5 minutes  
Amplification:  
95°C – 30 seconds  
60°C – 45 seconds

Run 35 cycles of amplification

Use mid-log phase of curves to determine volumes for multiplexing (use Illumina Library Construction/Tools/Attachments/16s primer\_amp\_QPCR sheets.xls)  
**Please note** – samples may fail due to too little, too much material, or a poor reaction. It is recommended that failed samples be re-run before moving forward  
Breakdown of QPCR multiplexing math (done to normalize each sample):  
○  delta Ct = Sample Ct - lowest Ct in sample set  
○  fold = 1.75^(delta Ct)  
○  ratio = 1/fold  
○  volume to mix b/c of ratio = X\*ratio (X = minimum desired volume per sample)  
○  how to dilute = fold  
○  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

Sample Multiplexing and Submission for Sequencing:  
- Run a Bioanalyzer DNA HighSensitivity on all sample poolings as well as final multiplexed lane to confirm library size (~450bp) and concentration

Please note - a peak at ~120bp is usually primer dimers, if this peak is large you will need to repeat the SPRI clean up to remove it or will need to gel purify your sample.

- Aliquot ~20uL of the final mix and submit for sequencing