EMP 16S Illumina Amplicon Protocol

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Abstract

The 16S protocol detailed here is designed to amplify prokaryotes (bacteria and archaea) using paired-end 16S community sequencing on the Illumina platform. Primers 515F-806R target the V4 region of the 16S SSU rRNA.

For running these libraries on the MiSeq and HiSeq, please make sure you read the supplementary methods of Caporaso et al. (2012). You will need to make your sample more complex by adding 5-10% PhiX to your run.

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Guidelines

16S amplification primers

The current primers have been modified from the original 515F-806R primer pair (Caporaso et al., 2011) in the following ways:

1. Barcodes are now on the forward primer of the new 515FB-806RB primer pair. This enables the usage of various reverse primer constructs to obtain longer amplicons (tested on 806RB and 926R).
2. Degeneracy was added to both the forward and reverse primers to remove known biases against Crenarachaeota/Thaumarchaeota (515FB, also called 515F-Y, Parada et al., 2016) and the marine and freshwater Alphaproteobacterial clade SAR11 (806RB, Apprill et al., 2015).

The primer sequences without linker, pad, barcode, or adapter are as follows:

- Current, 2015-present (fwd-barcoded: 515FB-806RB):
  `FWD:GTGYCAGCMGCCGCGGTAA; REV:GGACTACNVGGGTWTCTAAT`
- Original, pre-2015 (rev-barcoded: 515F-806R):
  `FWD:GTGCCAGCMGCCGCGGTAA; REV:GGACTACHVGGGTWTCTAAT`

Note: Studies in the Qiita database will have `library_construction_protocol`

as `515f/806rbc` if original primers or `515fbc/806r` if current primers ("bc" stands for barcode).

Ordering primers:

The primer sequences in this protocol are always listed in the 5′ -> 3′ orientation. This is the orientation that should be used for ordering. See the page Primer Ordering and Resuspension for more information. Primers and primer constructs were designed by Greg Caporaso (2011, 2012). Modifications to primer degeneracy were done by the labs of Jed Furhman (Parada et al., 2016) and Amy Apprill (Apprill et al., 2015). Forward-barcoded constructs were redesigned by Walters et al. (2016) based upon the original constructs generated by Caporaso et al. (2012).

- Illumina 16S Primer Constructs (shorter: 515FB–806RB)
- Illumina 16S Primer Constructs (longer: 515FB–926R)

515FB forward primer, barcoded:

Field descriptions (space-delimited):

1. 5′ Illumina adapter
2. Golay barcode
3. Forward primer pad
4. Forward primer linker
5. Forward primer (515FB)

```
AATGATACGGCGACCACCGAGATCTACACGCT XXXXXXXXXXXX TATGGTAATT GT
GTGYCAGCMGCCGCGGTAA
```

806RB reverse primer:

Field descriptions (space-delimited):

1. Reverse complement of 3′ Illumina adapter
2. Reverse primer pad
3. Reverse primer linker
4. Reverse primer (806RB)

```
CAAGCAGAAGACGGCATACGAGAT AGTCAGCCAG CC GGACTACNVGGGTWTCTAAT
```

PCR reaction mixture

Reagent	Volume
PCR-grade water	13.0 µL
PCR master mix (2x)	10.0 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Template DNA	1.0 µL
Total reaction volume	25.0 µL

Notes:

- PCR-grade water from Sigma (cat. no. W3500) or MoBio (cat. no. 17000-11)
- Platinum Hot Start PCR Master Mix (2x) from ThermoFisher (cat. no. 13000014)
- Final master mix concentration in 1x reaction: 0.8x
- Final primer concentration in 1x reaction: 0.2 µM

Thermocycler conditions

- Primers: 16S V4 515f-806rB
- Amplicon size: ~390 bp
- Cycle times are longer for 384-well thermocyclers.

Temperature	Time, 96-well	Time, 384-well	Repeat
94 °C	3 min	3 min	
94 °C	45 s	60 s	x35
50 °C	60 s	60 s	x35
72 °C	90 s	105 s	x35
72 °C	10 min	10 min	
4 °C	hold	hold	

16 S sequencing primers

Read 1 sequencing primer:

Field descriptions (space-delimited):

1. Forward primer pad
2. Forward primer linker
3. Forward primer

```
TATGGTAATT GT GTGYCAGCMGCCGCGGTAA
```

Read 2 sequencing primer:

Field descriptions (space-delimited):

1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer

```
AGTCAGCCAG CC GGACTACNVGGGTWTCTAAT
```

References

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Protocol

Step 1.

Amplify samples in triplicate, meaning each sample will be amplified in 3 replicate 25-µL PCR reactions.

Step 2.

Pool triplicate PCR reactions for each sample into a single volume (75 µL).

NOTES
Luke Thompson 15 Mar 2018

Do not combine amplicons from different samples at this point.

Step 3.

Run amplicons from each sample on an agarose gel.

NOTES
Luke Thompson 15 Mar 2018

Expected band size for 515f-806r is ~300-350 bp. Low-biomass samples may yield faint or no visible bands; alternative methods such as a Bioanalyzer could be used to verify presence of PCR product.

Step 4.

Quantify amplicons with Quant-iT PicoGreen dsDNA Assay Kit (follow manufacturer's instructions).

REAGENTS
Quant-iT PicoGreen dsDNA Assay Kit P11496 by Thermo Fisher Scientific

PROTOCOL

. Quanti-iT™ Pico Green dsDNA Assay (Invitrogen P7589)

CONTACT: Bonnie Poulos

Pico Green dsDNA Assay

Step 4.1.

Warm Quant-iT PicoGreen reagent to room temp in the dark.

REAGENTS

Quant-iT PicoGreen dsDNA kit P7589 by Thermo Scientific

NOTES

Bonnie Poulos 12 Oct 2015

PicoGreen reagent is diluted in dimethylsulfoxide (DMSO) which solidifies at refrigerator temperatures. It must be completely liquified before use by allowing it to come to room temperature. Vortex solution briefly to mix well and centrifuge for 5 sec to bring liquid to bottom of tube; then dispense for use in the assay. PicoGreen reagent is also light-sensitive, so reagent should be protected from light.

Pico Green dsDNA Assay

Step 4.2.

Prepare 1XTE buffer from 20X stock solution using nuclease-free water: will need 200 µl/well (for diluting standards, samples and PicoGreen).

NOTES

Bonnie Poulos 12 Oct 2015

Prepare 1X TE by pipetting 2.5 mL of 20X stock TE into a sterile 50 mL centrifuge tube and filling to 50 mL mark with molecular biology grade water. Invert tube to mix.

Pico Green dsDNA Assay

Step 4.3.

Dilute DNA standard to either “High” 2 µg/mL (1:50 of λ DNA stock) or “Low” 50 ng/mL (1:1000 of λ DNA stock).

NOTES

Bonnie Poulos 12 Oct 2015

It is best to run standards in duplicate, and if amount of DNA in samples is unknown or varys widely, it is also best to run both the high and low DNA standards.

Pico Green dsDNA Assay

Step 4.4.

Determine amount of sample to assay (eg, 2µl sample in total of 100µl TE buffer). Add correct amount of TE buffer to all wells. Add standards to wells. Then add samples to wells.

NOTES

Bonnie Poulos 12 Oct 2015

See Guidelines for amount of DNA standards to add to standard wells.

Pico Green dsDNA Assay

Step 4.5.

Dilute PicoGreen 1:200 in TE buffer and protect from light until ready to add to plate.

NOTES

Bonnie Poulos 12 Oct 2015

A 1:200 dilution of PicoGreen reagent is prepared by adding 10 µl of PicoGreen per 2 mL of 1X TE buffer. You will need 100 ul diluted PicoGreen per well containing 100 ul sample.

Pico Green dsDNA Assay

Step 4.6.

Add equivalent volume (100 µl) of diluted PicoGreen to every well (keeping plate in the dark as much as possible).

Pico Green dsDNA Assay

Step 4.7.

Tap plate to mix.

Pico Green dsDNA Assay

Step 4.8.

Incubate 5 minutes at room temperature keeping plate in the dark.

DURATION

00:05:00

Pico Green dsDNA Assay

Step 4.9.

Take fluorescent readings using 485nm excitation and 535nm emission filters.

Pico Green dsDNA Assay

Step 4.10.

Determine standard curve and calculate concentration of DNA in samples (see table in the guidelines).

Step 5.

Combine an equal amount of amplicon from each sample (240 ng) into a single, sterile tube. Higher amounts can be used if the final pool will be gel-isolated or when working with low-biomass samples.

NOTES

Luke Thompson 15 Mar 2018

When working with multiple plates of samples, it is typical to produce a single tube of amplicons for each plate of samples.

Step 6.

Clean amplicon pool using MoBio UltraClean PCR Clean-Up Kit (follow manufacturer's instructions).

REAGENTS

UltraClean PCR Clean-Up Kit (follow manufacturer's instructions) 12500 by Mobio

NOTES

Luke Thompson 15 Mar 2018

If working with more than 96 samples, the pool may need to be split evenly for cleaning and then recombined. Optional: If spurious bands were present on gel (in step 3), one-half of the final pool can be run on a gel and then gel extracted to select only the target bands.

Step 7.

Measure concentration and A260/A280 ratio of final pool that has been cleaned.

NOTES

Luke Thompson 15 Mar 2018

For best results the A260/A280 ratio should be between 1.8-2.0.

Step 8.

Send an aliquot for sequencing along with sequencing primers listed in Guidelines.

Warnings

Please refer to the SDS (Safety Data Sheet) for hazard information.