EMP 18S Illumina Amplicon Protocol

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Abstract

The 18S protocol detailed here is designed to amplify eukaryotes broadly with a focus on microbial eukaryotic lineages. The primers target the 18S SSU rRNA and are based on those of Amaral-Zettler et al. (2009). The constructs are designed to be used with the Illumina platform.

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.

The outlines of the protocol are the same as the 16S protocol, but different primers, PCR conditions, and sequencing primers are used. In addition, we have designed a blocking primer that reduces the amplification of vertebrate host DNA to be used on host-associated samples, especially those that have a low eukaryotic biomass. Blocking primer strategy is based on Vestheim et al. (2008).

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Guidelines

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. Primer constructs were designed by Laura Wegener Parfrey.

- Illumina 18S Primer Constructs (Euk1391f-EukBr)

Illumina_Euk_1391f forward primer:

Field descriptions (space-delimited):

1. 5′ Illumina adapter
2. Forward primer pad
3. Forward primer linker
4. Forward primer (1391f)

```
AATGATACGGCGACCACCGAGATCTACAC TATCGCCGTT CG GTACACACCGCCCGTC
```

Illumina_EukBr reverse primer, barcoded:

1. Reverse complement of 3′ Illumina adapter
2. Golay barcode
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (EukBr)

```
CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG CA TGATCCTTCTGCAGGTTCACCTAC
```

Mammal_block_I-short_1391f mammal blocking primer:

The mammal blocking primer is to be used when there is a high probability of picking up host genomic DNA. The C3 spacer (/3SpC3/) is a chemical modification that prevents extension during the PCR. Please note that the use of blocking primer reduces the number of host sequences detected but does not completely eliminate them. Thus remaining host sequences should also be filtered out during the analysis phase. We have found blocking primers to be particularly useful for host-associated samples with a low biomass of eukaryotic DNA. Note: Sequence is formatted for ordering from IDT.

```
GCCCGTCGCTACTACCGATTGG/ideoxyI//ideoxyI//ideoxyI//ideoxyI//ideoxyI/TTAGTGAGGC
CCT/3SpC3/
```

PCR reaction mixture

No blocking primer

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

With blocking primer

Reagent	Volume
PCR-grade water	9.0 µL
PCR master mix (2x)	10.0 µL
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
Blocking primer (10 µM)	4.0 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
- Final blocking primer concentration in 1x reaction: 1.6 µM

Thermocycler conditions

No blocking primer:

Temperature	Time	Repeat
94 °C	3 min	
94 °C	45 s	x35
57 °C	60 s	x35
72 °C	90 s	x35
72 °C	10 min	
4 °C	hold	

With blocking primer:

Temperature	Time	Repeat
94 °C	3 min	
94 °C	45 s	x35
65 °C	15 s	x35
57 °C	30 s	x35
72 °C	90 s	x35
72 °C	10 min	
4 °C	hold	

18S sequencing primers

Euk_illumina_read_seq_primer:

Field descriptions (space-delimited):

1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer (EukBr)

AGTCAGTCAG CA TGATCCTTCTGCAGGTTCACCTAC

Euk_illumina_index_seq_primer:

Field description (space-delimited):

1. Reverse complement of reverse primer (EukBr)
2. Reverse complement of reverse primer linker
3. Reverse complement of reverse primer pad

```
GTAGGTGAACCTGCAGAAGGATCA TG CTGACTGACT
```

References

- Amaral-Zettler, L. A., McCliment, E. A., Ducklow, H. W. & Huse, S. M. A method for studying protistan diversity using massively parallel sequencing of V9 hyper variable regions of small-subunit ribosomal RNA Genes. PLoS ONE 4, e6372 (2009).
- Caporaso, J. G. et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J (2012). doi:10.1038/ismej.2012.8
- Vestheim, H. & Jarman, S. N. Blocking primers to enhance PCR amplification of rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs. Frontiers in Zoology 2008 5:1 5, 12 (2008).

Materials

✓ Please see Guidelines section for required materials. by Contributed by users

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 1391f-Eukbr is ~260 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 (ThermoFisher/Invitrogen cat. no. P11496 P11496 by Invitrogen - Thermo Fisher

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 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.