18S V4 tag sequencing PCR amplification and library prep (Illumina)

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Abstract

Protocol for PCR amplification of extracted cDNA or DNA and downstream library preparation for MiSeq sequencing.

See other related protocols for extraction of RNA and/or DNA from environmental samples

This protocol is specific to 18S rRNA gene tag sequencing targeting to V4 hypervariable region. We use V4 primers with Illumina adapters that then anneal to the P5 & P7 Illumina-specific indices. This barcoding step allows us to multiplex many samples for MiSeq sequencing. The V4 region is ~400bp, so we typically do MiSeq 250x250bp PE sequencing.

Citation: Lisa Mesrop, Sarah Hu 18S V4 tag sequencing PCR amplification and library prep (Illumina). **protocols.io** dx.doi.org/10.17504/protocols.io.hdmb246
Published: 24 May 2017

Before start

1. Make fresh 80% ethanol.
2. Remove Agencourt AMPure XP beads from storage and let sit out at room temperature for at least 30 minutes.
3. Ensure the thermocycler is preheated to Q5 activation temperature 98ºC.
4. Thaw appropriate materials on ice.

Materials

- Q5 High-Fidelity 2X Master Mix - 100 rxns M0492S by New England Biolabs
- 96-well Microtiter Plate Magnetic Separation Rack S1511S by New England Biolabs
- AMPure XP Beads by Contributed by users
- Fresh 80% Ethanol by Contributed by users
- Thermocycler by Contributed by users

- 25 ml Reservoir by Contributed by users
- Qubit 2.0 Fluorometer by Contributed by users
- Nuclease-Free Water by Contributed by users
- Mini Centrifuge by Contributed by users
- TE Buffer by Contributed by users
- V4 Illumina Adaptors by Contributed by users
- Illumina P5 & P7 Index Primers by Contributed by users

Protocol

Amplification step with V4 primers (optional Illumina Adaptors for in house barcoding)

Step 1.

We use Q5 High-Fidelity 2x Master Mix for our PCR reactions. The convenient 2x master mix formulation is easy to use and is suitable for PCR applications requiring greater accuracy and amplification of difficult or low genomic DNA concentrations. Depending on projects, we use anywhere between 1 ng to 10 ng input of DNA for a final concentration reaction mixture of .04 ng/ul to .4 ng/ul, respectively.

Reagent	Volume	Stock Concentration	Final Concentration
Q5 High Fidelity 2x Master Mix	12.5 ul	2x	1x
18S V4 Forward Illumina Adaptor	1.25 ul	10 uM	.5 uM
18S V4 Reverse Illumina Adaptor	1.25 ul	10 uM	.5 uM
DNA Template	Variable		
Nuclease-Free Water	To 25 ul		

REAGENTS

Q5 High-Fidelity 2X Master Mix - 100 rxns M0492S by New England Biolabs

Step 2.

Ensure the thermocycler is preheated to Q5 activation temperature 98ºC.

Note: Depending on template size, annealing and extension temperatures and times may vary. Please refer to BioLabs Protocol for Q5 High-Fidelity 2x Master Mix thermocycler conditions.

	Step	Temp	Time	Cycles
Step 1	Activation of Q5	98ºC	2 min	
Step 2	Denaturation	98ºC	10 sec	10x
Step 3	Annealing	53ºC	30 sec	
Step 4	Extension	72ºC	30 sec	
Repeat Steps 2 - 4 **10** times				
Step 5	Denaturation	98ºC	10 sec	15x
Step 6	Annealing	48ºC	30 sec	
Step 7	Extension	72ºC	30 sec	
Step 8	Final Extension	72ºC	2 min	
Repeat Steps 5 - 6 **15** times				
	Hold	4ºC		

PCR Clean Up

Step 3.

Remove Agencourt AMPure XP beads from storage and let sit out at room temperature for at least 30 minutes.

Steps 3-19 can also be found with [AgencourtsAMPure bead information.]

REAGENTS

- AMPure XP Beads by Contributed by users

Step 4.

Make fresh 80% Ethanol.

Step 5.

Vortex Agencourt AMPure XP beads for 1 minute to ensure the beads are evenly distributed prior to use.

Step 6.

Add 1:1 volume ratio of beads to PCR product (25 ul, if you're following this protocol) into each sample and pipette mix up and down 20-30x gently.

Step 7.

Let mixture sit at room temperature for 10-15 minutes.

Step 8.

Place tubes onto magnetic stand for 2 to 5 minutes and let the beads clear from solution.

Step 9.

Step 10.

With the tubes on the magnetic stand, add 200 ul of freshly prepared 80% Ethanol to each sample opposite the aggregated beads being careful not to disturb them.

Step 11.

Incubate the tubes on the magnetic stand for 30 seconds and carefully remove the supernatant.

Step 12.

With the tubes on the magnetic stand, add 200 ul freshly prepared 80% Ethanol to each sample well opposite the aggregated beads being careful not to disturb them.

Step 13.

Incubate the tubes on the magnetic stand for 30 seconds and carefully remove the supernatant completely. Use a smaller volume pipette to remove any small amounts of ethanol collected at the bottom of the tubes.

Step 14.

Critical Step - With the tubes on the magnetic stand allow the beads to air-dry for 5 to 8 minutes. Make sure beads do not over dry and begin to crack.

Step 15.

Remove the tubes from the magnetic stand and add TE or nuclease-free water to each sample tube. Carefully pipette mix up and down for 20-30x.

Step 16.

Rehydrate sample tubes for 2-5 minutes.

Step 17.

Centrifuge sample tubes at 280 x g for 30 sec to collect solution in each tube.

Step 18.

Immediately place the tubes on the magnetic stand for 2-5 minutes to allow magnetic beads to collect.

Step 19.

Carefully transfer the supernatant to clean, labeled tubes. This is now cleaned up PCR product!

Step 20.

QC cleaned PCR products using Qubit fluorometer (concentration) High Specificity Dye & Buffer

- For the Qubit, load 2ul of product to 189 ul of High Specificity Dye & Buffer Solution for each sample

Indexing your PCR product with Illumina P5 & P7 indices

Step 21.

We have P5 & P7 Index Primers in 5 uM stocks.

Note: Make sure to normalize total input PCR product (total ng) for amplification Step 22.

Reagent	Volume	Stock Concetration	Final Concentration
Q5 High Fidelity 2x Master Mix	12.5 ul	2x	1x
P5 Indexed Primer	2.5 ul	5 uM	.5 uM
P7 Indexed Primer	2.5 ul	5 uM	.5 uM
DNA Template	Variable		
Nuclease - Free Water	Up to 25 ul		

REAGENTS

Q5 High-Fidelity 2X Master Mix - 100 rxns M0492S by New England Biolabs

PCR Thermal Profile for Step 22

Step 22.

Ensure the thermocycler is preheated to Q5 activation temperature 98ºC.

Note: Depending on template size, annealing and extension temperature and time may vary. Please refer to BioLabs Protocol for Q5 High-Fidelity 2x Master Mix thermocycler conditions.

	Step	Temp	Time	Cycles
Step 1	Activation of Q5	98°C	2 min	
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	54°C	30 sec	8x
Step 4	Extension	72°C	30 sec	
Repeat Steps 2-3 **8** times				
Step 5	Final Extension	72°C	2 min	
	Hold	4°C		

PCR Cleanup

Step 23.

Follow PCR Clean up Steps 3 to 19 (using Agencourt AMPure beads)

Quantify cleaned up PCR product with bar

Step 24.

QC cleaned PCR products using Qubit fluorometer (concentration) High Specificity Dye & Buffer

- For the Qubit, load 2ul of product to 189 ul of High Specificity Dye & Buffer Solution for each sample

Manual Library Normalization & Pooling

Step 25.

After determining the DNA concentration in ng/ul, convert this value from ng/ul to nM using the average library size of your known template (including adaptor length).

Calculate the conversion of ng/ul to nM using the following equation:

(ng/ul)*10^6/(660*library size)

Library Normalization & Pooling

Step 26.

Determine the common concentration to dilute your samples in nM. For Illumina sequencing

platforms, we prefer to dilute our samples to 10 nM starting concentration. Check with your sequencing center on how they prefer to receive samples for sequencing.

Calculate the dilution of your libraries using the following equation:

$(C_1)(V_1)=(C_2)(V_2)$

Library Normalization & Pooling

Step 27.

Pipette 2 ul from each 10 nM sample into a single tube.