Protocol: Quantifying species abundance using 16S Sanger Sequencing and CASEU

Jack Reddan, Kapil Amarnath

May 25, 2022

Contents

1 Culture Collection

1.1 Overview

This section describes collecting cultures for which you wish to quantify species abundances. In addition to any experimental mixtures, pure cultures of community members are needed for the analysis.

1.2 Materials

- Pure and mixed liquid cultures
- 2-mL microcentrifuge tubes

1.3 Method

1. In a 2-mL microcentrifuge tube, Collect 1 ODmL of liquid culture for all mixtures and pure cultures. Spin-down and remove supernatant.
2. Either store pellets at $-20°C$, or continue to gDNA Isolation.

2 gDNA Isolation

2.1 Overview

This section describes how to isolate gDNA to be used for 16S rDNA amplification. These steps are for the QIAGEN DNeasy Blood & Tissue Kit. If you happen use a different kit, please follow its instructions, and then proceed to 16S rDNA Amplification.

2.2 Materials

- QIAGEN DNeasy Blood & Tissue kit
- 100% ethanol
- Cell pellets from cultures collected in Culture Collection

2.3 Method

Follow the subsequent instruction in parallel for all samples to be processed.

1. Resuspend pellet in 180 μL of buffer ATL, and add 20 μL of Proteinase K. Mix thoroughly by vortexing. Incubate at 56°C for 1 hour, and vortex every 15 minutes.
2. Vortex for 15s, then add 200 μL of buffer AL and mix thoroughly by vortexing. Immediately add 200 μL of 100% ethanol and, again, mix thoroughly by vortexing.
3. Pipet the mixture from step 2 into a DNeasy Mini spin column placed in a 2-mL collection tube. Centrifuge at $\geq 6,000$ x g for 1 min. Afterwards, discard flow-through and collection tube.
4. Place DNeasy Mini spin column into a new 2-mL collection tube and add 500 μL of buffer AW1. Centrifuge at $\geq 6,000$ x g for 1 min. Afterwards, discard flow-through and collection tube.
5. Place DNeasy Mini spin column into a new 2-mL collection tube and add 500 μL of buffer AW2. Centrifuge at $20,000$ x g for 3 min. Afterwards, discard flow-through and collection tube.
6. Place DNeasy Mini spin column into a clean 1.5-mL microcentrifuge tube. Add 200 μL of buffer AE and incubate at room tempurature for 1 min. Centrifuge at $\geq 6,000$ x g for 1 min to elute.
7. Check gDNA concentration on the nano spectrophotometer. Then store at -20°C or continue to 16S rDNA Amplification.

3 16S rDNA Amplification

3.1 Overview

This section describes how to amplify the 16S rDNA using Phusion polymerase for Sanger sequencing. If you are using a different polymerase, please follow its specific instructions and proceed to Sanger Sequencing.

3.2 Materials

- Phusion polymerase
- 5X High Fidelity (HF) reaction buffer
- dNTPs
- 27F forward and 1492R reverse primers
- gDNA template from gDNA Isolation

3.3 Method

Follow the subsequent instruction in parallel for all gDNA samples collected in gDNA Isolation.

1. Mix the ddH_2O, dNTPs, forward/reverse primers, 5X HF buffer, and polymerase according to Appendix A for $N+1$ reactions, where N is the number of gDNA samples to be processed.
2. Pipet 38 μL of the PCR master mix into 200-μL PCR tubes. Then add 2 μL of template gDNA, at a concentration of 100 ng/μL, to the mix for each sample.
3. Place reactions into the thermocycler and run the PCR program specified in Appendix A.

4. Once the run is complete, check for synthesis of the desired product using gel electrophoresis. If the proper product was amplified, either store the reaction at $-20°$ C or proceed to 16S rDNA Purification

4 16S rDNA Purification

4.1 Overview

This section describes how to purify the 16S rDNA amplicons from 16S rDNA Amplification using the QIAGEN QIAquick PCR Purification Kit. If you are using a different purification kit, please follow its instructions and continue to Sanger Sequencing.

4.2 Materials

- 16S rDNA PCR amplicon samples
- QIAquick PCR Purification Kit

4.3 Method

Follow the subsequent instructions in parallel for all PCR amplicons synthesized in 16S rDNA Amplification.

1. Add 5 volumes of buffer PB to 1 volume of PCR sample and mix by pipet.
2. Place a QIAquick spin column in a provided 2-mL collection tube. Then apply the sample to the column and centrifuge at $\geq 17,900$ x g for 60 s. Discard the flow-through.
3. Add 750 μL of buffer PE to the column and centrifuge at $\geq 17,900$ x g for 60 s. Discard the flow-through.
4. Centrifuge the column at $\geq 17,900$ x g for 60 s, then transfer the column to a clean 1.5-mL microcentrifuge tube.
5. To elute, add 30 μL of buffer EB to the center of the column. Let stand for 1 min 60 s, and then centrifuge at $\geq 17,900$ x g for 60 s.
6. Check the concentration of the purified product on the nano spectrophotometer. Then either store at $-20°$C or proceed to Sanger Sequencing.

5 Sanger Sequencing

5.1 Overview

This section describes how to prepare the purified 16S rDNA samples obtained from 16S rDNA Purification for Sanger sequencing by Genewiz. If you are using a different sequencing platform or provider, please follow the relevant instructions, as they most certainly will differ from what is listed here, and continue to Species Abundances with CASEU

5.2 Materials

- Purified PCR 16S rDNA amplicons
- 27F forward primer
- 8-tube PCR strip

5.3 Method

Follow the subsequent instructions in parallel for all PCR amplicons purified in 16S rDNA Purification.

1. Dilute an aliquot of the 16S rDNA amplicon to a concentration of 3 ng/μL.
2. Add the amplicon dilution, 27F forward primer, and ddH_2O to a PCR tube following the recipe in Appendix B.
3. Label tubes using the tube number with the prefix *HL* (e.g. HL1, HL2, …HLN), noting what each tube contains.
4. Submit a Genewiz Sanger sequencing order online and print the order form. Place both the PCR tubes and order form in a clear, sealable plastic bag and deposit in the Genewiz drop-box.

6 Species Abundances with CASEU

6.1 Overview

This section describes how to quantify species abundances with the R package **CASEU** (**C**ommunity **A**nalysis via **S**anger **E**lectropherogram **U**nmixing) [1] using the sequencing results obtained from Sanger Sequencing. If you choose to use different software to quantify abundances, I do not know why you are reading this protocol.

6.2 Materials

- .ab1 sequencing files for pure and mixed cultures
- Computer with an installation of R

6.3 Method

1. Refer to the vignette found on the CASEU Repository for instructions on installation and a full introduction to CASEU. Also, you can see Jack's Repository for an example script.

A PCR

A.1 Master Mix Recipe

Below is a recipe for a single, 40-μL 16S rDNA PCR using Phusion Polymerase. To generate a master mix for N samples, simply multiply the volumes by $N*1.1$ to account for pipetting errors.

Component	Volume (μL)
ddH_2O	20.8
5X HF reaction buffer	8.0
dNTPs	0.8
3 μM 27F forward primer	4.0
3 μM 1492R reverse primer	4.0
Phusion polymerase	0.4
template gDNA	2.0
Total	40.0

A.2 Thermocycler Program

The following program can be found under Kapil's folder in the Hwa-lab thermocycler (`NSB2106\kapil\16s phusion`).

Step	Temperature (°C)	Time (mm:ss)
1	98.0	00:30
2	98.0	00:10
3	54.0	00:30
4	72.0	01:30
Steps 2-4 x24		
5	72.0	10:00
6	10.0	INFINITE

B Genewiz

B.1 Sample Mix Recipe

References

[1] N. Cermak, M. S. Datta, and A. Conwill, "Rapid, inexpensive measurement of synthetic bacterial community composition by sanger sequencing of amplicon mixtures," *iScience*, vol. 23, p. 100915, mar 2020.