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16S and GyrB bacterial amplification

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We use this protocol and it's working

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Abstract

This protocol is used for the amplification of the bacterial *gyrB* gene and the 16S gene for both PacBio Sequel II and Illumina MiSeq sequencing. This protocol is used in the paper titled *Long-read Sequencing Increases the Accuracy and Specificity of the gyrB Phylogenetic Marker Gene*.

Materials

- Isolated bacterial DNA
- Nuclease-free water (VWR Cat # 103307-278)
- Invitrogen Platinum SuperFi PCR Master Mix (ThermoFisher Scientific, Cat # **12368250**)
- 1 × TAE (Tris base [Millipore Sigma, Cat # 648311], acetic acid [Millipore Sigma, Cat # 695092], and EDTA [Millipore Sigma, Cat # E9884]) buffer
- Omnipur agarose (VWR, Cat # EM-2070)
- GelRed dye (VWR, Cat# 10098-684)
- 6× Gel loading dye, no SDS (Biolabs, Cat# B7025S)
- 100-bp DNA ladder (VWR, Cat# PAG2101)
- Ice bath
- NanoDrop UV-Vis Spectrophotometer Lite (Thermo-Scientific)
- Sterile 0.2-ml thin-wall PCR Tubes, strips of 8 tubes (Denville)
- Sterile 0.5- to 10-µl pipettes (Denville)
- Sterile 10- to 200-µl pipettes (Denville)
- Sterile 1000-µl pipettes (Denville)
- T100 Thermal cycler (Bio rad)
- Gel electrophoresis box (Labnet)
- ChemiDoc XRS+ (BioRad)

Primer info

	A	B	C	D
	Primer Name	Primer Descriptioon	Primer Sequence	Platform
	V4_16S_F	Forward primer for V4 16S sequencing	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA	MiSeq
	V4_16S_R	Reverse primer for V4 16S sequencing	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT	MiSeq
	FL_16S_F	Forward primer for full length 16S sequencing	/5AmMC6/GCAGTCGAACATGTAGCTGACTCAGGTCACAGRGTTYGATYMTGGCTCA	PacBio
	FL_16S_R	Reverse primer for V4 full length sequencing	/5AmMC6/TGGATCACTTGTGCAAGCATCACATCGTAGR	PacBio

	A	B	C	D
			GYTACCTTGT TACGACTT	
	SR_GyrB_Bac_F	Orginal short read forward primer to amplify the gyrB gene from Bacteroidaceae	TCGTCGGCA GCGTCAGATG TGTATAAGAG ACAGCGGAG GTAARTTCGA YAAAGG	MiSeq
	SR_GyrB_Bac_R	Orginal short read reverse primer to amplify the gyrB gene from Bacteroidaceae	GTCTCGTGG GCTCGGAGAT GTGTATAAGA GACAGGCRTA TTTYTTCARH GTACGG	MiSeq
	SR_GyrB_Bif_F	Orginal short read forward primer to amplify the gyrB gene from Bifidobacteriaceae	TCGTCGGCA GCGTCAGATG TGTATAAGAG ACAGGACRA CGGNCGNGG CATYCC	MiSeq
	SR_GyrB_Bif_R	Orginal short read reverse primer to amplify the gyrB gene from Bifidobacteriaceae	GTCTCGTGG GCTCGGAGAT GTGTATAAGA GACAGAGNC CCTTGTTNAG GAAVGCC	MiSeq
	SR_GyrB_Lac_F	Orginal short read forward primer to amplify the gyrB gene from Lachnospiraceae	TCGTCGGCA GCGTCAGATG TGTATAAGAG ACAGGGHGG AGGATAYAAG GTATCC	MiSeq
	SR_GyrB_Lac_R	Orginal short read reverse primer to amplify the gyrB gene from Lachnospiraceae	GTCTCGTGG GCTCGGAGAT GTGTATAAGA GACAGTRTAN GAATCRTTRT GCTGC	MiSeq
	LR_GyrB_Bac_F	Long read forward primer to amplify the gyrB gene from Bacteroidaceae	/5AmMC6/GC AGTCGAACAT GTAGCTGACT CAGGTCAC T GTAYATYGGT GACATYAGYR	PacBio
	LR_GyrB_Bac_R	Long read reverse primer to amplify t he gyrB gene from Bacteroidaceae	/5AmMC6/TG GATCACTTGT GCAAGCATC ACATCGTAG CCCATYARCA TRGARAAGAT R	PacBio
	LR_GyrB_Bif_F	Long read forward primer to amplify the gyrB gene from Bifidobacteriaceae	/5AmMC6/GC AGTCGAACAT GTAGCTGACT CAGGTCAC A	PacBio

	A	B	C	D
			TCGARGTSAC GATTCTGCCG	
	LR_GyrB_Bif_ R	Long read reverse primer to amplify t he gyrB gene from Bifidobacteriacea e	/5AmMC6/TG GATCACTTGT GCAAGCATC ACATCGTAG GGATCCATGG TGGTYTCCCA C	PacBio
	LR_GyrB_Lac_ F	Long read forward primer to amplify the gyrB gene from Lachnospiraceae	/5AmMC6/GC AGTCGAACAT GTAGCTGACT CAGGTCAC S AGRGGWCTB CATCATYTRG T	PacBio
	LR_GyrB_Lac_ R	Long read reverse primer to amplify t he gyrB gene from Lachnospiraceae	/5AmMC6/TG GATCACTTGT GCAAGCATC ACATCGTAG TCMGGATCC ATDGTBGTCT CC	PacBio

This is a table for all primers used in this project. The first column is the primer name. The second column is the primer description. The third is the primer sequence with the added illumina or PacBio adaptor. The fourth column is what system the primer is used fro, either MiSeq or PacBio.

Before start

Before starting make sure to have isolated bacterial DNA and selected primers for amplification.

Prepare DNA for Amplification

1 **Thaw the isolated DNA**

2 **Measure DNA concentration on the Nanodrop**

This requires only 1 µL of isolated DNA. Concentration values typically range from [M] 100 ng/µl to [M] 400 ng/µl. In addition, the NanoDrop gives only an estimate of the total DNA concentration. For a more accurate result, submit samples for quantification on a Bioanalyzer.

3 **Create a 100 µl aliquot at [M] 10 ng/µl concentration.**

3.1 **Figure out how much DNA to add by dividing 1000 by the average DNA concentration.**

For example: if your average DNA concentration was [M] 254 ng/µl you would take 1000/254 which equals 3.94. So we would use 3.94 µL for the aliquot.

3.2 **Figure out how much nuclease-free water to add by subtracting the result of step 3.1 from 100.**

So to continue the example it would be 100 - 3.94 which equals 96.06. So we would add 3.94 µL of original DNA to 96.06 µL of nuclease-free water. This results in a [M] 10 ng/µl Bacterial DNA solution.

16S and *gyrB* PacBio and Illumina Amplicon PCR Protocol from Nichols et al.

4 **Prepare the following PCR mix.**

	A	B	C
	Reagent	Concentration	Volume to make 20 uL of product
	Forward primer	10 µM	0.4 µl

	A	B	C
	Reverse primer	10 µM	0.4 µl
	Platinum SuperFi Master Mix	N/A	10 µl
	Nuclease Free water	N/A	8.2 µl

This is for each well so multiply each volume by the number of samples plus one or two, to make sure enough master mix is available for all tubes. For example if I was running a 15 sample PCR I would multiply each volume product by 17 (15 samples + 2 extra)

5 **Fill an adequate number of wells with** 19 µL **of master mix each**

6 **Add** 1 µL **of** [M] 10 ng/µl **DNA directly into the master mix of each appropriate well**

It helps to watch the DNA go into the master mix to ensure that the DNA has been added.

7 **Make sure the reagents are mixed in the PCR tubes by gently flicking and then quickly spinning in a mini centrifuge.**

8 **Run PCR**

8.1 **If amplifying 16S samples for MiSeq use these PCR settings.**

	A	B	C	D
	Cycle Number	Time	Temperature	Description
	1 cycle	2 minutes	98°C	initial denaturation
	25 cycles	10 seconds	98°C	denaturation
		20 seconds	56.6°C	annealing
		15 seconds	72°C	extension
	1 cycle	5 minutes	72°C	final extension

It should be noted that the higher the number of cycles the greater chance for chimeric sequences. The user should optimize PCR cycles to their specifications

8.2 **If amplifying 16S samples for PacBio use these PCR settings.**

	A	B	C	D
	Cycle Number	Time	Temperature	Description
	1 cycle	30 seconds	95°C	initial denaturation
	25 cycles	30 seconds	95°C	denaturation
		30 seconds	57°C	annealing
		1 minute	72°C	extension
	1 cycle	5 minutes	72°C	final extension

It should be noted that the higher the number of cycles the greater chance for chimeric sequences. The user should optimize PCR cycles to their specifications

8.3 **If amplifying bacterial *gyrB* samples for MiSeq use these PCR settings.**

	A	B	C	D
	Cycle Number	Time	Temperature	Description
	1 cycle	2 minutes	98°C	initial denaturation
	30 cycles	10 seconds	98°C	denaturation
		20 seconds	56.6°C	annealing
		15 seconds	72°C	extension
	1 cycle	5 minutes	72°C	final extension

It should be noted that the higher the number of cycles the greater chance for chimeric sequences. The user should optimize PCR cycles to their specifications

8.4 **If amplifying bacterial *gyrB* samples for PacBio use these PCR settings.**

	A	B	C	D
	Cycle Number	Time	Temperature	Description
	1 cycle	30 seconds	95°C	initial denaturation
	30 cycles	30 seconds	95°C	denaturation
		30 seconds	57°C	annealing

	A	B	C	D
		1 minute	72°C	extension
	1 cycle	5 minutes	72°C	final extension

It should be noted that the higher the number of cycles the greater chance for chimeric sequences. The user should optimize PCR cycles to their specifications

Check for Amplification

9 **Create a 1x agarose gel**

This is made by combining 1 g of agarose and 100 mL of 1x TAE. Microwave this solution for 1 minute and 45 seconds. Pour into a mold with an appropriate comb. Add 10 µL of Gel Red dye (at 10,000x). Let this cool for 45 minutes to an hour.

10 **Prep the amplicons for gel electrophoresis**

First, prep the dye master mix as follows:

	A	B	C
	Reagent	Concentration	Volume
	Gel loading dye	6x	8 µl
	Nuclease-free water	NA	16 µl

Make sure to multiply the volumes by the number of samples

Then add in all 20 µL of amplified product to a well containing 24 µL of the dye water mixture.

11 **Run the gel to check for amplification**

Add in all of the amplicon sample + dye + water mixture the wells of the submerged gel. Run the gel at 80 volts for 1 hour.

Check the gel in a geldoc to see amplified bands.

Clean the amplicon samples with a gel clean up kit

12 **Cut out the appropriate bands from the gel from step 9**

It is easiest to use specialized pipette tips to punch out the appropriate bands. Also, this needs to be completed under UV light so be sure to wear propper PPE.

13 **Use the QIAquick Gel Extraction Kit to clean up gel** punch-outs

Briefly, dissolve the gel punch-outs in the provided buffer at 50°C for 10 minutes. Then add the dissolved punch-out mixture to the provided columns. Wash twice with the provided wash buffers and elute with either nuclease-free water or elution buffer (not provided).

14 **Submit samples for sequencing**

MiSeq samples were run on a 250x250 Illumina Miseq

PacBio samples were run on a PacBio Sequel II