SOP for amplifying full length and internal region of 16S

FILE NAME: SOP_for_amplifying_full_length_and_internal_region_of_16S

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SECTION 1 INTRODUCTION

1.1 Purpose: The purpose of this protocol is to amplify a ~1500 base pair portion of the 16s rDNA gene by PCR for the purpose of sequencing and subsequent organism identification.

Primer Set:

Forward:	16sfD1nolinker	AGA GTT TGA TCC TGG CTC AG
Reverse:	16srD1nolinker	AAG GAG GTG ATC CAG CC

1.2 Scope: This procedure is used for amplifying 16s rDNA for use in sequencing and identification of unknown organisms by the Wiedmann-Boor Lab in the department of Food Science at Cornell University.

1.3 Definitions:

16s rDNA: ribosomal RNA gene that is highly conserved amongst bacteria and archaea

Bp: base pair

dNTPs: deoxyribonucleotide monomers, which are the building blocks of DNA. There are four dNTPs, cytosine, adenine, guanine and thymine

PCR: polymerase chain reaction, used to amplify a specific region within a DNA sequence

pGEM: acts as a reference to estimate the size of unknown DNA molecules and to approximate the mass of a band

Taq Gold: thermostable DNA polymerase responsible for DNA amplification during PCR

TBE Buffer: buffer solution containing a mixture of Tris base, boric acid and EDTA

Thermocycler: laboratory apparatus used to amplify segments of DNA via the polymerase chain reaction (PCR) process

Transilluminator: UV light source used to view DNA that has been separated by gel electrophoresis

1.4 Safety:

Ethidium bromide is an extremely strong mutagen. It should only be used in the designated room, Room 350C, and nitrile gloves should always be worn while there.

The full 16srRNA PCR is used in the sequencing and identification of unknown organisms. Many of these organisms, such as L. monocytogenes, are BSL-2 pathogens. Appropriate protective measures need to be taken when working with these species. All waste from these experiments needs to be treated as BSL-2 waste.

1.5 Alternative Primers:

If you wish to generate fragments of ~700 bp there are alternative sets of primers that may be utilized. The most success has been obtained using PEU7 and DG74 as the forward and reverse primers, respectively, for both PCR and sequencing. Please see the

Partial 16s protocol posted in the MQIP protocol section of the Food Safety Wiki, as this primer set uses a different Master mix and different thermocycling conditions.

SECTION 2 MATERIALS

- **Microwave.** In Room 350.
- **Plate with organism culture**
- **dH_2O.** Lab stock in Room 350A.
- **10X Amplitaq PCR Buffer.** Lab stock in Room 355.
- **Amplitaq $MgCl_2$ 25mM.** Lab stock in Room 355.
- **Taq Gold.** Lab stock in Room 355.
- **dNTP 10mM.** To prepare a solution of 10 mM dNTP pipet 60 mL of dH_2O and 10 mL from each of the nucleotide stock solutions into the 1.5 mL Eppendorf tube.
- **16sfD1nolinker 10µM**
- **16srD1nolinker 10µM**
- **Agarose.** Lab stock in Room 352B.
- **0.5X TBE Buffer.** Lab stock in Room 350.
- **pGEM.** Lab stock in Room 354.
- **Loading Dye.** Lab stock in Room 350.
- **0.2 mL Eppendorf tubes.** Lab stock in Room 350A.
- **1.5 mL Eppendorf tubes.** Lab stock in Room 350A.
- **Ethanol.** Lab stock in Room 350A.
- **Parafilm.** In Room 350
- **Gel Running Box.** In Room 350.
- **Ethidium Bromide.** In Room 350C.
- **Trays.** In Room 350C.
- **Spatula.** In Room 350C.
- **Transilluminator.** In Room 350C.
- **Face Shield.** In Room 350C.
- **Pipets**
- **Pipet tips**
- **Sterile wooden toothpicks.** Lab stock in Room 350A.
- **Bunsen burner**
- **Thermocycler.** In Room 356.
- **PCR trays**
- **Gel trays.** In Room 350.
- **Gel combs.** In Room 350.
- **Vortex**

SECTION 3 Procedure for 16s rDNA PCR

A. Lysis and DNA Preparation:

3.1.1 Label the appropriate number of 0.2 mL Eppendorf tubes. Pipet 100 µL of dH_2O into each 0.2 mL Eppendorf tube.

3.1.2 Sterilize a wooden toothpick by briefly passing it over a Bunsen burner.
3.1.3 Using the sterilized toothpick remove a single colony from a pure plated culture.
3.1.4 Insert the toothpick into the 0.2 mL Eppendorf tube containing 100 µL of dH_2O and swirl colony into the bottom of tube.
3.1.5 Repeat this for each sample as well as the positive and the negative.
3.1.6 Make sure each Eppendorf tube is clearly labeled and place in microwave for 1-5 minutes.

B. PCR Preparation & Master-mix:

3.2.1 In PCR room, label the appropriate number if 0.2 mL Eppendorf tubes and aseptically prepare Master Mix using Taq Gold and its accompanying 10X PCR buffer and 25 mM $MgCl_2$. The Taq Gold polymerase should be added last to the Master Mix made as shown in the Master Mix set-up below.

Master Mix Set-up:	1X (50 µL rxn volume)
dH_2O	33.75 µL
10X Amplitaq PCR Buffer	5 µL
Amplitaq $MgCl_2$ 25mM	4 µL
dNTP 10mM	2 µL
16sfD1nolinker 10µM	2 µL
16srD1nolinker 10µM	2 µL
Taq Gold	0.25 µL

3.2.2 Briefly vortex Master Mix to mix.
3.2.3 Mix thoroughly and aliquot 49 µL of Master Mix solution to each PCR tube. Put the rack on ice.
3.2.4 At lab bench briefly vortex dirt lysates and add 1 µL of dirty lysate to corresponding tubes of Master Mix. Remember to change tips between each DNA template to prevent cross contamination.

C. PCR Conditions

3.3.1 Place the Eppendorf tubes into the thermocycler.
3.3.2 Enter the program below into the PCR or select the program <16sFull> under User <Steve> on thermocycler <Sneezy>. After selecting or entering program verify that thermocylcing conditions match protocol.

Thermocycling Conditions:

Time	Temperature ($^{\circ}C$)	Cycles	
10 minutes	95	20	
30 seconds	95	20	-0.3$^{\circ}C$ per cycle
30 seconds	57	20	
2 minutes	72	20	
30 seconds	95	20	
30 seconds	51	20	
2 minutes	72	20	

7 minutes	72	20
∞	4	-

3.3.3 Place Eppendorf tubes into thermocycler making sure tubes are closed and pushed all the way down.
3.3.4 Verify that final reaction volume reads <50 µL>.
3.3.5 The thermocycler will run approximately three hours and will hold the Eppendorf tubes at $4^{\circ C}$ until stopped.
3.3.6 To stop PCR select <Stop> twice. Select <History>, it should say <No Exceptions>. Select <Return>. Select <Exit>. Open lid and remove tubes.

D. Preparation of 1.5% Agarose Solution

3.4.1 Weigh 1.5 g Agarose into 250 mL Erlenmeyer flask and add 100 mL of 0.5x BE buffer.
3.4.2 Place two kimwipes in the top of the flask and swirl to mix.
3.4.3 Microwave for 1 min 35 sec. Stop every 30 seconds to swirl flask once solution starts to bubble. Ensure gel is clear and be free of flakes.
3.4.4 Let flask cool five minutes.
3.4.5 Set up gel tray and comb. Make sure combs are either pushed to very right or very left to ensure even DNA distribution.
3.4.6 Pour agar slowly into the bottom right corner of the tray. Use a pipette tip to burst any bubbles that form in the gel.
3.4.7 Let sit 30 minutes to an hour and remove combs from gel by gently wiggling in a back and forth motion.
3.4.8 Store in 0.5X TBE Buffer until ready for use.

E. Confirmation of PCR Product via Gel Electrophoresis:

3.5.1 Ethanol gel area and pipettor. Cut gel so that there are an appropriate number of wells.
3.5.2 Remove pGEM from fridge and place on ice.
3.5.3 Load 5 µL pGEM into first well.
3.5.4 Aliquot 100 µL of dye from container into a 1.5 mL Eppendorf tube.
3.5.5 Pipet 2 µL of loading dye onto a piece of parafilm. Pipet 10 µL of PCR product onto parafilm next to first dot of loading dye. Pipet loading dye and PCR product back and forth to mix. Load 5 µL of dye-PCR product mixture into well. Repeat for each sample being tested.
3.5.6 Load 5 µL of pGEM into last well.
3.5.7 When all PCR product and pGEM has been loaded make sure that the 0.5X TBE Buffer is covering the gel and that the gel is straight.
3.5.8 Insert lid into gel box and turn on electricity. Set voltage to 120-123 V. Never go above 130 V.
3.5.9 Verify that set up is running properly. Bubbles should be forming and DNA should be running from negative to positive.
3.5.10 Let run for 17-27 minutes. Check gel. If more time is needed, i.e. yellow loading dye has not reached the end of the gel, set timer for more time.
3.5.11 Turn off electricity box and wearing nitrile gloves remove gel from 0.5x TBE Buffer and carry to designated ethidium bromide room.

3.5.12 Check outer door of ethidium bromide room for last time stain was added. If it was added recently, the gel may be stained for 30 seconds. If it is older it may be more like 5 minutes.
3.5.13 Place gel into tin foil covered box labeled ethidium bromide (0.005%) and leave in stain for appropriate amount of time.
3.5.14 Remove from stain using spatula and place gel in DI water next to ethidium bromide. Let sit 30-60 minutes.
3.5.15 Once gel has been stained and destained, place gel on transilluminator platform and adjust the focus and light aperture of camera while viewing the picture on the monitor.
3.5.16 Put on protective face shield, turn off overhead light and turn on UV light.
3.5.17 Take a picture by:
3.5.17.1 Clicking on the mushroom cloud with the X to stop frame.
3.5.17.2 Clicking on < Camera > to capture the image.
3.5.17.3 Clicking on < File > < Save As >. Then go to the appropriate folder on the server to save your files. Within this folder save picture with date and PCR name (ej. 8-23-13 16s).

SECTION 4 TROUBLESHOOTING

4.1 Issues with $MgCl_2$
4.1.1 $MgCl_2$ forms a concentration gradient when frozen and needs to be vortexed prior to use.
4.1.2 Every PCR reaction has an optimal $MgCl_2$ concentration range, usually between 1-4 mM. Since Mg^{+2} forms complexes with dNTPs and acts as a cofactor for polymerase you may need to try several conditions to optimize your concentration.
4.2 Too Much Enzyme
4.2.1 Excess enzyme in your PCR can lead to smearing of PCR products.
4.3 Wrong Primer Concentration
4.3.1 If you have too little primer you won't see any product.
4.3.2 Too much primer and you get primer dimerization and not enough amplification. Primer concentration should be between 0.1-1.0 µM.
4.4 Wrong PCR Program
4.4.1 Check your program while its cycling to make sure it is the right program.
4.5 Excess or Insufficient Template
4.5.1 Too much template can inhibit PCR by binding all of the primers. Take a smaller sample from the culture.
4.5.2 Too little template and amplification may be undetectable. Make sure you correctly prepared your dirty lysate and that you took a sufficiently large sample from your culture.
4.6 Too Much dNTP or Degraded dNTP
4.6.1 Excess dNTP inhibits PCR. Between 40-200 µM is optimal.
4.6.2 dNTP is sensitive to repeated freeze-thaw cycles. Make small aliquots when you get a fresh batch and turn over your stock frequently.

SECTION 6 REFERENCES

1. Frame, Peter. (2010). Ten Things That Can Kill Your PCR. *Bio-Synthesis*. www.biosyn.com.