Microbiology Experiment #7 – PCR amplification of rRNA gene fragment

Design of oligonucleotide primers to amplify and sequence ribosomal RNA genes.

the 16S rRNA gene is present in all Bacteria and Archaea. Certain sequences within the gene have not changed much in billions of years due to their essential nature for the function of the 16S rRNA gene product. These conserved sequences can be used as primer annealing sites to amplify the 16S rRNA gene by the Polymerase Chain Reaction (PCR). Many researchers around the world use the same common set of "Universal" oligonucleotide primers that we will use today.

27f - 5' - AGAGTTTGATCMTGGCTCAG

1492r - 5' - TACGGYTACCTTGTTACGACTT

Lane, D. J. (1991). 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics. E. Stackebrandt and M. Goodfellow, eds. New York, NY, John Wiley and Sons: 115-175.

The 16S rRNA gene is a little larger than 1500 bp, so these primers will amplify nearly the full length gene. Notice that there are some non-standard letters (M,Y) in the primer sequences. These correspond to "degenerate" positions, i.e. positions that are less highly conserved, so that more than one base must be included to be "Universal". Standard nucleotide naming conventions are listed below

IUPAC Nucleic acid codes

A = Adenine	C = Cytosine
G = Guanine	T = Thymine
	U = Uracil
R = Purine (A or G)	Y = Pyrimidine (C, T, or U)
M = C or A	K = T, U, or G
W = T, U, or A	S = C or G
B = C, T, U, or G (not A)	D = A, T, U, or G (not C)
H = A, T, U, or C (not G)	V = A, C, or G (not T, not U)
N = Any base (A, C, G, T, or U)	

Thus, half of the 27f primers have a C at position 12, and half have an A. Likewise, half of the 1492r primers have a C at position 6 and half have a T. During oligonucleotide synthesis, this is accomplished by adding a mixture of the desired nucleotides when adding the nucleotide to the specified position.

During the Polymerase Chain Reaction (PCR), heating of the double stranded template DNA to 94^{o}C separates the two strands. Upon cooling to 55^{o}C, the primers will hybridize (base pair) with their complementary sequences on the template DNA. Heating to 72^{o}C allows the thermal stable Taq DNA polymerase to add new nucleotides to end of the primer to produce double stranded DNA. This process is continued in a thermal cycler to produce in excess of 10^{9} copies of the DNA fragment defined by the two primers. This DNA will subsequently be used for gel electrophoresis and DNA sequencing.

A - Set up PCR:

1. **Prepare template**: Pipette 100 µL sterile dH_2O into two 0.5 mL microfuge tubes labeled with your initials and K or UK. Inoculate cells from a single, well isolated colony and vortex to suspend cells. Transfer resuspended cells into a -70°C block for 2 minutes, then place in the +70°C heating block until thawed. Pass cells through a second freeze-thaw cycle.

2. Set up 25µL PCRs in **clearly-labelled** 0.2 mL thin wall PCR tubes.

   12.5 µL 2x Taq PCR Premix (contains enzyme, buffer, dNTPs)
   12.5 µL 2x rRNA primer mix (1.5 µM each)
   - Primer 27f - 5' - AGAGTTTGATCMTGGCTCAG - 3'
   - Primer 1492r - 5' -TACGGYTACCTTGTTACGACTT – 3'

   1 µL twice frozen and thawed cells, mix by pipetting.

3. Close tubes tightly, store on ice until thermal cycler is ready to run. Store the frozen & thawed cells at -20°C.

4. Initiate thermal cycling program.

	rRNA.fl
Phase 1 (initial denaturation) - 1 cycle	
Initial denaturation	2 **min**. @ 94°C
Phase 2 - 35 cycles	
standard denaturation	30 sec. @ 94°C
Primer annealing	30 sec. @ 50°C
Primer extension[2]	1.5 min @ 72°C
Phase 3 (extra extension) - 1 cycle	
Primer extension	**9 min.** @ 72°C

B - Prepare 1% agarose gel (Thursday – will be done for you).

1. Securely tape the ends of a gel tray such that a small amount of tape is on the underside of tray. Place tray on a sheet of plastic wrap in case of spillage. Align comb in tray parallel with and 1-2 cm from the end of the tray.

2. Add an appropriate amount of agarose (0.4g) to 40 mL water in a 125 mL erlenmeyer flask, heat mixture in microwave on high setting for 1 - 1.5 min, or until mixture begins to boil.

3. Using a folded paper towel to hold the neck of the erlenmeyer flask, swirl the gel mixture well, and return to microwave. Heat for an additional 30 - 45 sec, or until mixture begins to boil.

4. Carefully remove molten gel solution from microwave using a folded paper towel to hold the neck of the erlenmeyer flask.

5. Add 0.8 mL 50x TAE buffer, 10 µL of 0.3 mg/mL ***ethidium bromide*** solution, swirl to mix, pour into gel tray, allow to stand at room temp for 20 - 30 min to solidify.
   Caution, ethidium bromide is a carcinogenic, fluorescent DNA dye! Handle with gloves

6. Remove comb, slide gel off tray onto plastic wrap, wrap and label gel, store at 4°C.

C - Quantify PCR products

Our goal for today is to determine whether our PCRs were successful and estimate the concentration of DNA in our samples for the sequencing reactions to be conducted next week.

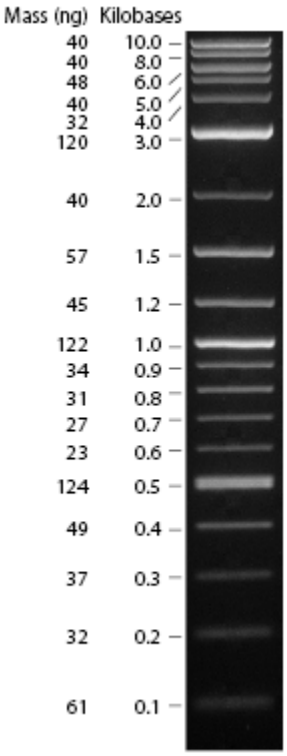
Gel electrophoresis

1. Fill gel chamber with 1x TAE buffer such that the level of liquid just covers center platform. Carefully unwrap the gel that was prepared at the last meeting and slide into a gel tray. Place gel in chamber with the wells near the negative electrode (black), add sufficient 1x TAE to just cover the gel.

2. Cut a small piece of parafilm, place on bench near gel, "spot" a 1-2 μL aliquot of loading dye onto parafilm for each sample to be loaded on gel.

3. Draw 5 μL of sample into a pipette tip, pipette up and down onto a spot of loading dye to mix, load sample into well of gel. Be careful not to poke pipette tip through bottom of well. Samples should be loaded in the following order (from left right):

   Lane 1 – 5 μL uncut λ DNA (10 ng/μL)
   Lane 2 – 5 μL uncut λ DNA (25 ng/μL)
   Lane 3 – 5 μL uncut λ DNA (60 ng/μL)
   Lane 4 – 5 μL NEB 2 log ladder
   Lane 5 – Student 1 UK-PCR
   Lane 6 – Student 1 K-PCR
   Lane 7 - Student 2 UK-PCR
   Lane 8 - Student 2 K-PCR



4. After fastest migrating blue dye (bromophenol blue) has migrated 2/3 the length of the gel, turn off power, carefully remove gel from chamber, drain, slide onto a piece of plastic wrap. Photograph the gel under UV light.

5. Compare the intensity/brightness of the bands to estimate the concentration of DNA. Record the concentration of PCR products here and on the log sheet on the front bench.

   _K=_____UK=_____ng/μL

D. "Clean-up" of PCR products in preparation for Sanger sequencing

Our samples will be sequenced using the Sanger dideoxy chain termination method by GeneWiz (https://www.genewiz.com/en). They request that for each sequencing reaction, we send 10 uL of "Clean" template at a concentration of 5 ng/µL and 5 µL of primer at 5 pmol/µL. The cleaning involves digestion with an exonuclease specific for single stranded DNA to degrade the primers, and alkaline phosphatase (ExoSap) to cleave phosphate from the resulting nucleotides. The enzymes are then inactivated by heat treatment and diluted to 5 ng/µL

1. In a 0.5 mL microfuge tube, add 2µL ExoSAP and 3µL dH20 for each reaction to be cleaned. Mix by pipetting up and down once or twice. (**this will be done for you**)

2. Distribute 5 µL diluted ExoSAP into a labelled 0.5 mL microfuge tube for each PCR to be cleaned.

3. Add 15µL of the PCR product to each tube, incubate at 37^{o}C for 30 min.

4. Heat to 85^{o}C for 15 min.

5. Add dH_2O to achieve a final [DNA] of 5 ng/µL. (derive an equation where X is the original DNA concentration in ng/µL and Y is the amount of water to be added in µL).
   You should get Y = 3x-20. If the amount is >450 µL, just add 450 µL dH_2O.

6. Place your clearly labelled tube on the front bench.