Identification of Unknown # 3 by PCR and sequence analysis of 16S ribosomal DNA (rDNA)

Day 1:

A. Prepare DNA template from your unknown bacteria

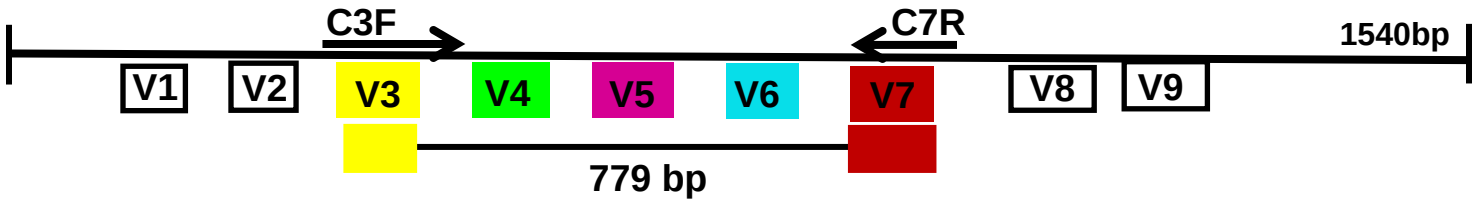
B. Amplify a 779 bp region from 16S Ribosomal DNA (1540 bp) by Polymerase Chain Reaction (PCR)

Day 2:

C. Analyze PCR product by Agarose gel electrophoresis. Your PCR product will be sequenced. Nucleotide sequence will be emailed to you.

Day 3:

D. BLAST your sequence data to identify your unknown.



C3F

```
401  GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGA
451  AGGGAGTAAAGTTAATACCTTTGCTCATTCGACGTTACCCGCAGAAGAAGC
501  ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT
551  TAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAG
601  ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGC
651  TTGAGTGCAGAAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT
701  AGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACT
751  GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
801  AGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTG
851  GCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAA
901  GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
951  TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCAC
1001 GGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGC
1051 TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCA
1101 ACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAA
1151 AGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA
```

C7R

(DAY 1):

A. PREPARING TEMPLATE DNA FOR PCR

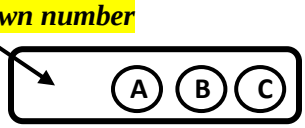
You will work in groups

1. Obtain one plate with two unknown microorganisms from your Professor. Label the two unknowns as A and B, e.g., ***unknown number 49, label as 49A and 49B***
2. Label two ***perforated*** 1.5 ml microfuge tubes and add 100µl of distilled water to each tube.
3. Using a sterile inoculating loop, pick half a loopful of each unknown and resuspend in 100µl of water. Close the top and use vortex to break any visible clumps.
4. Place the microfuge tubes in the hotplate for 10 minutes to lyse the cells. Bring the microfuge tubes to your Professor for centrifugation (for 10 min @ maximum speed).

Note: You will be dispensing volumes ranging from 1-10 µl into PCR tubes. It is very critical that you dispense the exact volume of each reagent.

5. ***Clear supernatant,*** obtained after centrifugation, will have the template DNA for unknowns A and B. Set up master mix for PCR using the following table (section B).

B. SETTING UP PCR TO AMPLIFY rDNA:



1. You will find a ***three well*** PCR strip, strip caps and a rack for PCR tubes on your table. Label the PCR strip with your unknown number and label the wells A-C.

2. PCR reagents (4 tubes/pk, P-R), stored at -20^{0}C, will be given to you by your instructor. Take out tube **labeled as P** and add **water (from the packet), F primer and R primer into P tube** according to the following table. This is your ***Master mix*** for setting up the PCR reaction. **Use a fresh tip each time**.

Label	Reagents/pk. for Master mix	Volume to be added to tube P
P	**2X PCR mix (includes buffer, dNTPs and polymerase)**	**40µl *already added to the tube***
W	**Sterile water**	34 µl
F	**F primer**	1.0 µl
R	**R primer**	1.0 µl
TOTAL VOLUME OF MASTER MIX IN TUBE P		**76.0 µl**
Template DNA		**1.0 µl to be added per well according to step 5.**

3. **Spin tube P**, using the benchtop spinner on your table, to mix all the reagents you added according to the table above.
4. Transfer **19.0** µl of the ***master mix*** **(from tube P)** to wells # A, B & C of your PCR strip.
5. **Add 1.0 µl of each DNA template to PCR strip wells A & B** .
6. **Add 1.0 µl of dH_20 to well #C**. This will serve as your **N**o **T**emplate **C**ontrol or NT**C**.
7. **Use the benchtop spinner for PCR strips to mix everything before bringing the 3-well PCR strip to your professor for the Thermocycler.**

PCR (20.0 µL) PROGRAM TO BE USED: Initial denaturation @ 95^{0}C 10 mins

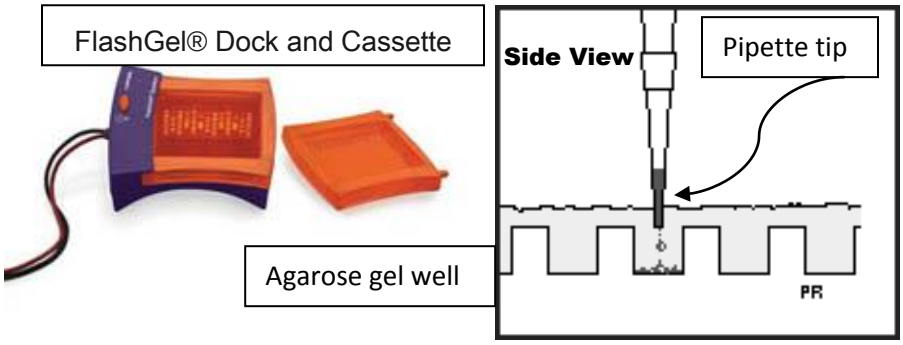
Denaturation @ 95^{0}C 15 sec.
Annealing @ 58 ^{0}C for 1 min.
Primer extension @ 72 ^{0}C for 30 secs.
} **30 cycles**

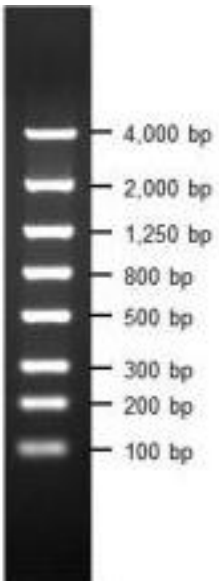
Final extension @ 72 ^{0}C for 10 min. and hold @ 4 ^{0}C

(DAY 2): C. ANALYZING THE PCR PRODUCT BY GEL ELECTROPHORESIS

1. Obtain your PCR strip from your professor and **add 4.0 µl of loading dye to each well**, mix the reagents by pipetting up and down.

2. Load 5 µl of each PCR sample to run on Lonza fast gel along with a DNA ladder and observe. You will be able to visualize PCR product within in 2-3 mins! We will be running 2 gels/class, each gel has 13 wells.





3. **Expected result: Samples A and B should show a band of 779 bp and sample C (NTC) should not have any bands.**

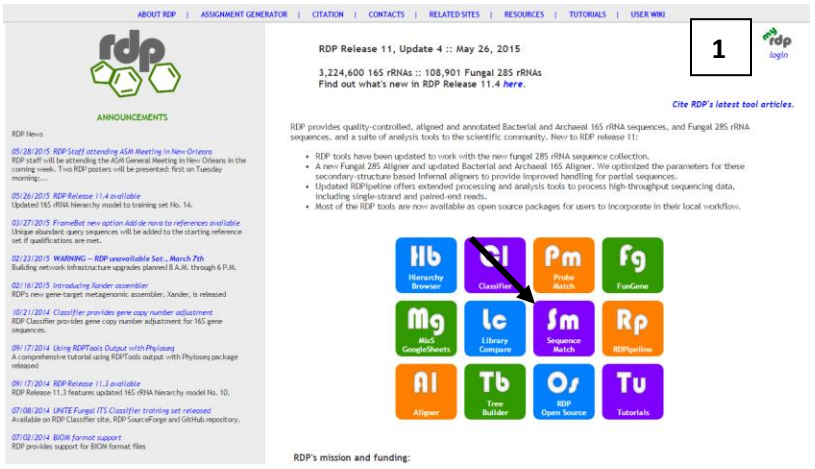
   If you see band(s) of expected size, your pcr products are ready to be sequenced.

4. Bring your PCR reaction strip to your professor and your professor will use 5-10 µl of each PCR product for sequencing reactions per group.

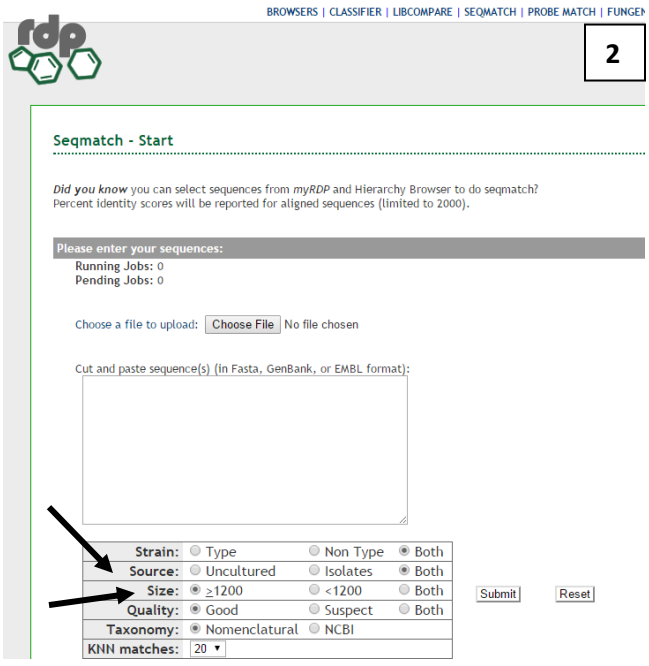
(DAY 3): D. ANALYZING SEQUENCE DATA TO IDENTIFY YOUR UNKNOWN

Ribsomal data base: http://rdp.cme.msu.edu/

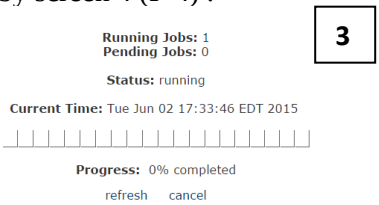
1) Select **SEQ MATCH** (pointed by the arrow in screen 1).



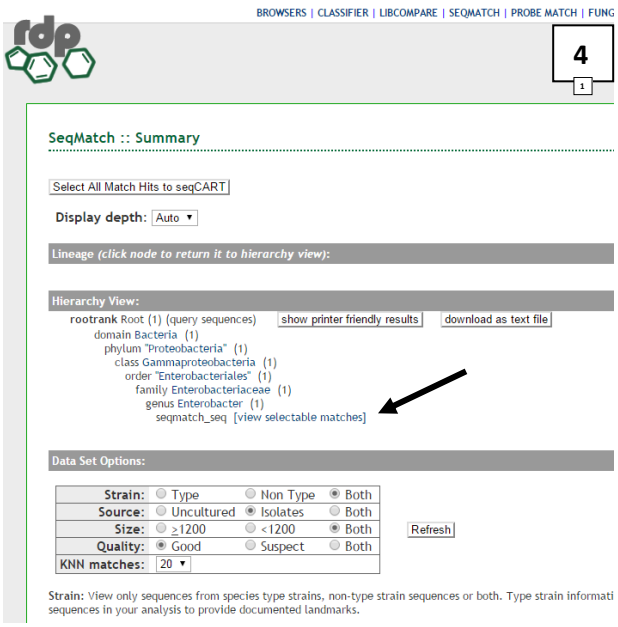
2) Copy and Paste the sequence (in FASTA format) into the box (screen 2).

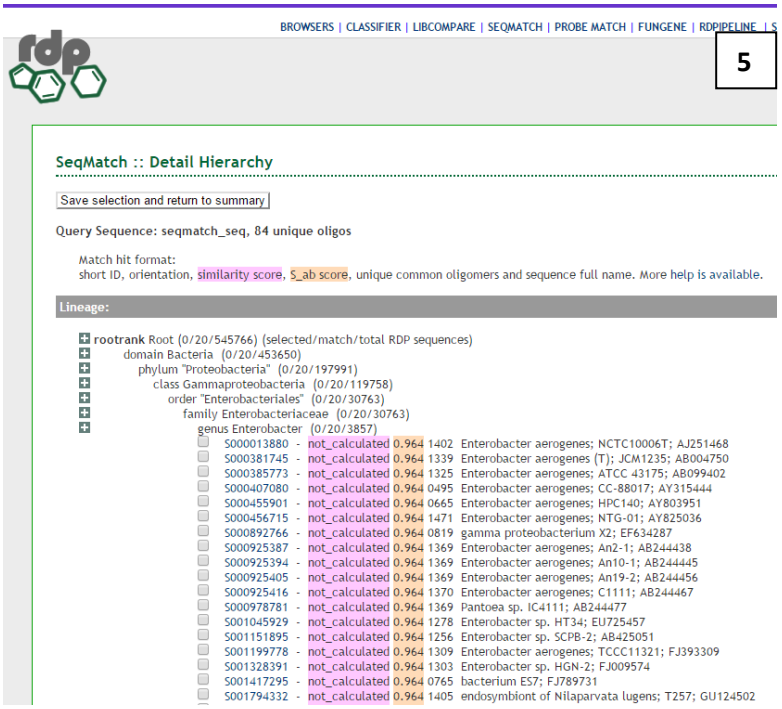


3) Use the radio buttons to change ***Source* to isolates, and *size* to both,** (pointed by the arrows in screen 2) . **Click on submit** and you will get to screen 3, followed by screen 4 (P-4) .



4) On this page, under **Hierarchy View**, next to '**seqmatch_seq**', click on **'[view selectable matches]'** and it will take you to screen number 5. Your organism should be listed on the top few lines.





FOR A TUTORIAL: http://rdp.cme.msu.edu/help/SM.jsp

Alternative approach:

BLASTing the sequence using NCBI database:

1. Copy the FASTA sequence data and paste it into the box labelled **Enter Query Sequence** @ this site http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome
2. Select **Nucleotide collection (nr/nt)** from the drop down menu in the following box where you need to choose the database in search set.
3. Select **Uncultured/environmental sample sequences** to exclude it from the data base.
4. Scroll all the way down and click on **BLAST** on the left hand side to begin. This can take a couple of minutes.
5. In the output screen, scroll down to where it says 'description', select the first five w/ the highest Max score and lowest E value. Click on the accession number hyperlink to get the name of the organism. For a tutorial go to http://www.youtube.com/watch?v=HXEpBnUbAMo for a tutorial on BLAST and how to interpret the output.