Molecular Analysis of *E. coli* - Amplification of *E. coli* ITS (23-5 ITS of rDNA)

Colony PCR:

1) Obtain fresh streak plate with single colonies of *E. coli* (no more than 2 weeks old if stored at 4C or 4 days old if at room temp) grown on TSA or LB.
2) Prepare mastermix for PCR such that each reaction contains a total volume of 25µl. For each reaction, add the following to the mastermix tube:
   a) 11.5µl water
   b) 0.5µl 23-5ITS-F primer (10µM stock) ***OR 0.5ul 16-23 ITS-F (original) primer (10uM stock)***
   c) 0.5µl 23-5ITS-R-biotin primer (10µM stock) ***OR 0.5ul 16-23 ITS-R-biotin primer (10uM stock)***
   d) 12.5µl of NEB 2x quick-load Taq Mastermix (preloaded with green dye)
      i) *Add 1 extra reaction volume to be used for a negative control tube*.
      ii) Add volumes for 1 extra reaction for every 10 reactions prepared to compensate for pipetting error.
3) Aliquot 25µl of mastermix into each labeled PCR tube (do this in PCR room to decrease possibility of contamination). Use the strip tubes with individual attached caps located in the PCR room.
4) Take tubes out of PCR room to add *E. coli* sample. (Before adding cells to PCR mix, you may want to turn on the PCR machine as it takes a while to heat up). Load pipet with new sterile, *filtered* tip and carefully touch one colony to pick up a very small amount of cell paste from one of the samples.
   a) Only a very small amount is required for PCR - should not be clearly visible on tip.
   b) Do not pick up any of the agar from the plate - if you dig into the agar, discard tip and start over.
5) Resuspend the cell material in the appropriate reaction tube by pipetting up and down several times.
6) After all of the samples are inoculated with cells, place the tubes in the thermocycler block *that is already preheated to 95˚C* (this is a "hot start"). Close hot lid and for the 23-5 region cycle through the following parameters: (On PCR machine in room 33-387, User: "pyrotk", Program: "2x-taq-pcr")
   a) 95˚C for 2 minutes
   b) 95˚C for 30 seconds
   c) 56˚C for 30 seconds*****
   d) 68˚C for 1 minute (68˚C is set up for NEB mastermix)
   e) Return to step "b" and cycle another 44x (total 45 cycles)*****
   f) 68˚C for 5 minutes
   g) 4˚C hold

**** for 16-23 region, anneal at 68˚C for 30 seconds, and extend at 68˚C for 1 seconds. Return to step "b" for a total of 40 cycles. This is also a set protocol on the PCR machines in the EBI.***

7) After run is complete, load 5µl onto a 2.5% gel set up with 12-well combs (at least 2 per gel) and run at 100volts for 30 minutes. If only half of the gel is used, cut and save the remaining lanes at 4˚C in plastic wrap.
8) Save image by date in Molecular Forensics folder on Gel Doc computer in UBL.
9) Note date of PCR in the freezer stock log sheet and affix gel image (with lanes labeled) in pyroprint notebook.
10) Store remaining 20µl of the PCR products in -20˚C freezer for pyrosequence analysis.

Primers (100µM stocks in Black lab -20C freezer):

23-5ITS-F: 5'-ATG AAC CGT GAG GCT TAA CCT T-3'
23-5ITS-R-biotin: 5'-biotin-CTA CGG CGT TTC ACT TCT GAG T-3'

16-23ITS-F: 5'GGA ACC TGC GGT TGG ATC AC-3'
16-23ITS-R-biotin: 5'CTT CAT CGC CTC TGA CTG CC-3'

Gel #1 Date _____

Lane	Initials	Sample	Lane	Initials	Sample
1			**13**		
2			**14**		
3			**15**		
4			**16**		
5			**17**		
6			**18**		
7			**19**		
8			**20**		
9			**21**		
10			**22**		
11			**23**		
12			**24**		

Gel #1	Gel #2
Paste image here	Paste image here

Gel #2 Date _____

Lane	Initials	Sample	Lane	Initials	Sample
1			**13**		
2			**14**		
3			**15**		
4			**16**		
5			**17**		
6			**18**		
7			**19**		
8			**20**		
9			**21**		
10			**22**		
11			**23**		
12			**24**		

Pyrosequence Analysis of *E. coli* ITS (23-5S)

A. Preparation of sample file

1) Start up PyroMark Q24 program (not the PyroMark Assay Design program - that is for primer design).
2) Click on the small icon at the top left with a green circle and white arrowhead ("new run" should pop up when you place your cursor over the icon).
3) Use pull down menu in "Instrument Method" box to select the method. Unless told otherwise, our cartridges use the "*PyroMark Q24 Method 003*" method - select this one.
4) Plate ID, Barcode, and Reagent ID are optional - leave these boxes blank unless told otherwise.
5) Scroll to the plate set up at the bottom of the page. You will recognize it based upon the 24 large boxes that are each divided into three sections. The boxes are divided into 3 rows (A-C) and 8 columns (1-8).
6) Click in the top section of one of the boxes that represents the position in the tube rack that has one of your samples. This should cause the section to turn blue with a lavender background. Right-click in this section and select "load assay" from the menu that appears.
7) Go to the folder containing the PyroMark assays and select the one you need to use for your sample. The assay will define the dispensation order for your sequence run.
    a. One that is frequently used in this project is the "*ModGATC-2controls.pyrosetup*". This is in the pyrotechies folder under 2-0-0 MATERIALS AND METHODS\2-1-0 Assay Design Files.
8) If more than one reaction will use the same assay (dispensation order), then drag the bottom corner of the box to the other boxes while the assay section of the box is highlighted (in blue).
9) Enter the name of the sample in the 2nd section of each box.
10) The 3rd section of each box is optional and may be used for notes.
11) After entering all of the information, you may then save your file to a flash drive to be transported to the USB of the Q24 pyrosequencer. Please also save in Pyrotechies Folder\3-0-0 RAW DATA\3-0-2 Assay Run Files.
12) Before closing the file, go to the "Tools" menu and select "Pre Run Information". You can print out this page so you have the volumes that will be required for the E-mix, S-mix, and nucleotides A-T. These will be necessary for preparing the cartridge in Part F. This page can be later put into your notebook.
13) Safely remove the flash drive and plug it into the Q24. Do not start the run until you have completed Parts B-F.

B. Preparation block and reaction mix

1) Turn on heat block to 80˚C. Put the plate holders on the heat block. They must be up to temperature for the denaturation step.
2) Turn on the pyrosequencer to allow it to warm up prior to the run (~30 minutes). Take out all reagents to get them to room temperature.
3) Add the 70% ethanol (50 mL), denaturing solution (40 mL), and wash solution (50 mL) to the plastic troughs in the workstation. Also should be at room temperature.

4) Prepare the reaction master mix that will later be added to the reaction plate*, as described below:

	1 reaction	24 reactions
a. Annealing buffer……	25µl	600µl
b. Seq primer (23-5ITS-S**, 10µM)……	0.75µl	18µl
Final concentration of primer is 0.3µM.		

* The reaction master mix will be added to the 24-well reaction plate in part D, step 7a.
** Sequence primer (100µM stocks in EBI -20C freezer) 23-5ITS-S: 5'- CGT GAG GCT TAA CCT T-3'
***16-23 primer is at 25uM: The sequencing primer for this reaction is the 16-23 ITS-F.

C. Attach beads to biotin-labeled PCR amplicon

1) Prepare binding master mix by adding the following volumes:

	1 reaction	25 reactions
a. Binding buffer…….....	40µl	1000µl
b. Streptavidin sepharose beads*...…….	2µl	50µl
c. Nanopure water…….	18µl	450µl

* Be sure to thoroughly mix beads before pulling volume for master mix, as the beads will settle out.

2) Add 60µl of master mix to each tube in 24-tube plate cut out from a 96-tube plate, with the A-1 tube (mark with star) at top left and C-8 tube at bottom right.

3) Add ~20µL of PCR product to the binding buffer in the plate and mix by pipetting up and down. Cover with a strip of parafilm if desired.

4) Put on orbital shaker for 5 minutes at 1400 rpm and then start the denaturing procedure immediately after shaking.
    a. If the filtering is delayed, return the plate to the orbital shaker for 5 minutes at 1400 rpm

5) While PCR product is shaking, sonicate vacuum filter probe apparatus (VFPA) in water bath for 1'. Shake off excess water. Test sucking ability of probes by aliquoting 80ul of water into the 24-well "water test" plate, turn on vacuum pump, flip switch to "on" on the VFPA, and lower VFPA into 24-well plate. Make sure water is gone from all tubes. If they aren't, see Jennifer or Alice to change the offending probe.

D. Denaturing the PCR product

1) Using the handheld vacuum filter probe apparatus (VFPA), you will pull the double stranded biotin labeled PCR product using the following procedure:

2) Turn on vacuum pump and be sure the switch on the handheld vacuum filter probe apparatus (VFPA) is switched on.

3) Carefully place the VFPA in the PCR plate such that the line to the vacuum is coming out from the right side of the plate (closest to the C-8 tube).

4) Pull the double stranded biotin-labeled PCR product from the 24-tube plate into the VFPA membrane

5) Once all of the binding mix is pulled through, place the VFPA in the 70% ethanol trough. Pull the ethanol through the VFPA until the fluid line makes its way to the collection flask.
    a. This often takes a few minutes - you can also just drain the ethanol until no more of it is able to enter the VFPA.

6) Place the VFPA in the denaturing buffer. Pull the 40 mL of buffer through the VFPA until the fluid line makes its way to the collection flask.

7) Place the VFPA in the wash buffer. Pull the 50 mL of buffer through the VFPA until the fluid line makes its way to the collection flask.
    a. While the wash buffer is running through the VFPA, add 25µl of the reaction master mix to each well in the 24-well reaction plate (small flat plate with round shallow wells). Make sure the reagent covers the bottom of the well…do this by blowing air into the reagent opposite the "bubble".
8) Drain the tube by holding the VFPA upright and tilting it back and forth to allow the wash buffer to drain into the collection flask. You want the filter probes to be completely dry.
9) **Turn off the pump, and disconnect the tube from the VFPA**
    a. The single stranded biotin labeled PCR product is now held onto the VFPA by surface tension.
10) Make sure the vacuum pump is off and disconnected, then place the VFPA probes into the 24-well reaction plate that already contains the 25µl of reaction mix (see step 7a above).
    a. The vacuum connection should be facing the right side of the plate (closest to the C-8 tube).
11) Gently wiggle the probes to release the beads into the reaction mix for ~15 seconds. Go to Section E.

Continued ⟶

12) Perform the following VFPA clean-up while completing steps 1-3 in part E:
    a. Transfer VFPA to the sonicating water bath and resonicate for 1'.
    b. Transfer VFPA to the 2nd water trough containing 70ml of nano pure water and pull 70ml of water through.
    c. Drain the tube by holding the VFPA upright and tilting it back and forth to allow the water to drain into the collection flask. You want the filter probes to be completely dry.
    d. Park the VFPA in the last trough on the right side - it should be empty.

E. Preparation of the reaction

1) Place the 24 well PCR plate in the plate holder that has been heated up to 80˚C on the heat block and cover with the second plate holder on top of the 24 well PCR plate.
2) Allow the PCR product to denature at 80˚C for 2 minutes.
3) Remove from heat and allow the plate to cool in plate holder at room temperature for 5 minutes.
4) In the Q24 pyrosequencer, lift the plate holding frame and place reaction plate in the plate holder such that the alignment of the plate is the same (A-1 to the top left and C-8 at the bottom right).
    a. If the plate is inserted incorrectly, it will not be properly seated into the holder when you drop the plate holding frame.

F. Preparation of the cartridge

1) Add the S-mix (substrate), E-mix (enzyme) and individual dNTPs (do not use expired reagents) to the cartridge in the prescribed order. See picture. The label on the cartridge is always facing forward.

Mod GATC-2controls (24 samples)		
	1st run (µl):	Follow-up (µl):
E-mix =	119	69
S-mix =	119	69
ATP =	133	83
CTP =	125	75
GTP =	125	75
TTP =	116	66

2) The amounts of substrate, enzyme and dNTPs are defined in the pre-run instructions in the program software after you have prepared the file run (see Part A above).
3) Place cartridge in Q24 with the label facing forward.
4) You will have defined your run parameters and sample names and put the run file on a flash drive. Insert the flash drive into the Q24 and start your run.

G. Clean up - should be done within 30 minutes of the completion of the reaction

1) When the reaction has completed, pull the flash drive and shut down the machine. Once it is properly shut down, turn it off.
2) Remove the reaction plate and either:
    a. discard the tray if all 24 wells were used and you will not be reusing the template for another reaction.
    b. if not all of the wells were used, remove the samples from the used wells, mark the bottoms of the wells, and return to the cabinet for later use.
    c. if you want to rerun the templates at another time, transfer the reaction mix with the beads to a new tube and rinse the well with 60µl of nano pure water - add the rinse to the same tube as the reaction mix.
3) Remove the cartridge and dump the residual substrate, enzyme, and dNTPs into the sink.
4) Fill all of the chambers with nano pure water and, using gloves, hold the cartridge over the sink while pressing firmly on top of each compartment with a finger.
    a. If the needle is clear, a jet of water should come straight out of the tip of the needle.
5) After jets of water have come through each needle (only those compartments that had enzyme, substrate, and dNTPs), dump residual water.
6) Repeat steps 4 and 5 three more times to fully clean cartridge. Briefly rinse outside of needles. The cartridges are expensive, so please be careful and fully clean them after each run, unless a second run is immediately following. Store cartridge without the cap, on its side on a KimWipe in the cupboard.
7) Troubleshooting a blocked cartridge needle
    a. If the needle is blocked, (for example, if the reagent cartridge has been left overnight), fill the compartments with high-purity water, and immerse the PyroMark Q24 Cartridge in a beaker with enough high-purity water to cover the needles. Leave the reagent cartridge in the beaker for 1 hour, rinse it and repeat the cleaning
    b. If the jet of water comes out of the needle at an angle, (not parallel to the direction of the needle), repeat the cleaning.
    c. If the water still comes out at an angle, alert Alice Hamrick, Jennifer VanderKelen, or Dr. Black, as we may need to discard the PyroMark Q24 Cartridge.

Disposing of Vacuum Collection Jug Contents:

Contents of this jug are higher than pH 7. Add acetic acid to the buffer until it reaches pH7 and then dump contents down the drain.