

Walk Lab

Montana State University

Fluorescent PCR Ribotyping for *Clostridium difficile*

December 1, 2014

Amplification

PCR reagents:

-Promega MasterMix – Promega (#M7502)

-Primers from Bidet et al. 1999. *FEMS Microbiol Lett.* 175:261-6.

>CD_Ribo_F

GTGCGGCTGGATCACCTCCT

>CD_RiboFAM_R

56-FAM/CCCTGCACCCTTAATAACTTGACC

PCR set-up (25 uL total volume):

12.5 uL of Promega MasterMix

0.5 uL CD_Ribo_F (10 pmol/uL)

0.5 uL CD_RiboFAM_R (10 pmol/uL)

10.5 uL ddH₂O

1 uL template*

*For high-throughput screening of clinical isolates, use a 1:10 dilution of overnight *C. difficile* culture (i.e. grown anaerobically at 37°C in BHIS broth). If you plan to store culture for later use as template, heat a 1:10 dilution of overnight culture to 95°C for 15 minutes and store at 4°C.

Thermocycling conditions:

Pre-soak – 95°C for 10 min.

35 cycles of:

Denaturation – 95°C for 0.5 min.

Annealing – 55°C for 0.5 min.

Elongation – 72°C for 1.5 min.

Final elongation – 72°C for 10 min.

Confirmation of amplification:

Amplicons can be electrophoresed in a 1% agarose gel to confirm the PCR worked. Multiple bands should be visible for most *C. difficile* isolates. After technical mastering of this protocol, confirmatory electrophoresis may not be necessary (i.e. we do not routinely run amplicons in gels for high-throughput analysis).

Storage of amplicons:

Fluorescence of PCR amplicons is sensitive to light and freeze/thaw. Store covered (foil) amplicons at -20°C until ready to submit for fragment analysis.

Submission to the University of Michigan DNA Sequencing Core

NOTE – You must set up an account at the Sequencing Core prior to submitting plates. This requires some paperwork and account information. Contact Robert H. Lyons (boblyons@umich.edu) for details.

Fragment analysis reagents:

- High Deionized (Hi-Di) Formamide – Life Technologies (#4311320)
- Size Standard – Bioventure Inc. (mm-ROX1000 Marker Rhodamine dye)
- CE loading plates – Bioexpress Inc. (Gene Mate #T-3107-IBCCS, 100/case semi-skirted, raised rim, bar coded) OR Life Technologies (#4306737, MicroAmp Optical 96-Well Reaction Plate with Barcode)
- Strip caps – VWR (#83009-684)

Sample preparation (per plate):

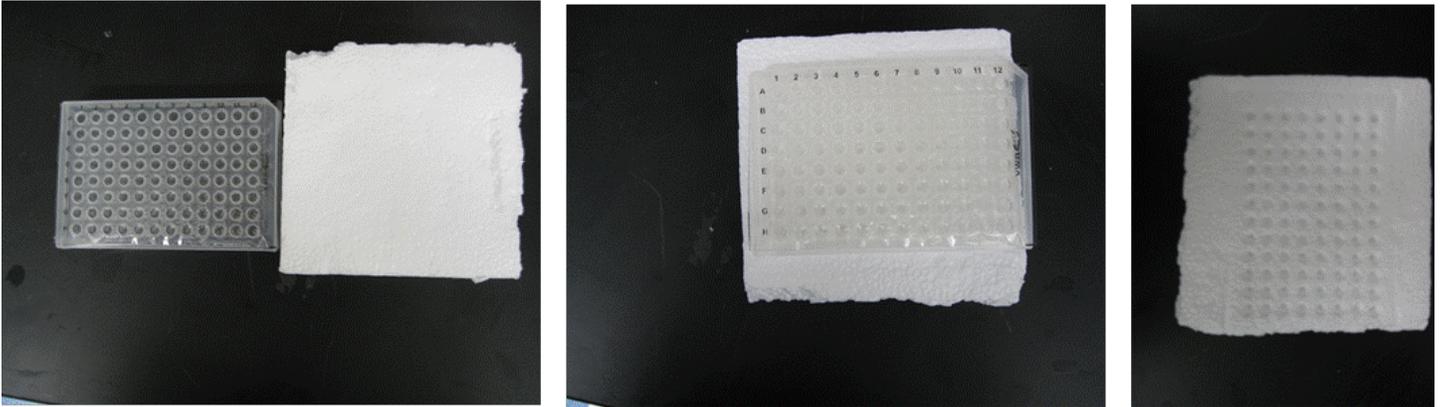
- 1) Dilute fluorescent PCR amplicons 1:1000 using sterile DNase/RNase free water
- 2) Make a solution of Hi-Di Formamide and ROX1000 size standard in a 1.5 ml Eppendorf tube as follows:
1.2 mL of Hi-Di Formamide + 5 uL of ROX 1000 size standard/plate
- 3) Add 12 uL of this solution to each well of the bar coded CE loading plate.
- 4) Add 5 uL of diluted (1:1000) fluorescent PCR amplicons to each well. Do not vortex or pipet to mix, as this may degrade chromatogram peaks.
- 5) If fewer than 96 samples are being submitted, fill empty wells with water.
- 6) Centrifuge the plate at low speed to ensure liquid is at the bottom of the wells.
- 7) Seal plate with strip lids.
- 8) Label the plate with the order number from the University of Michigan DNA Sequencing Core web site.
- 9) To ensure that plates and samples arrive intact, embed the plate in a sandwich of styrofoam and tape (see attached figures). Use an old plate to make an impression in styrofoam and place CE loading plate in this impression. Attach the plate using tape.
- 10) Ship the plate overnight on wet ice to:

UM DNA Sequencing Core
NCRC Building 14 Rm 122
University of Michigan
2800 Plymouth Rd
Ann Arbor, MI 48109-2800
Phone: 734-764-1461

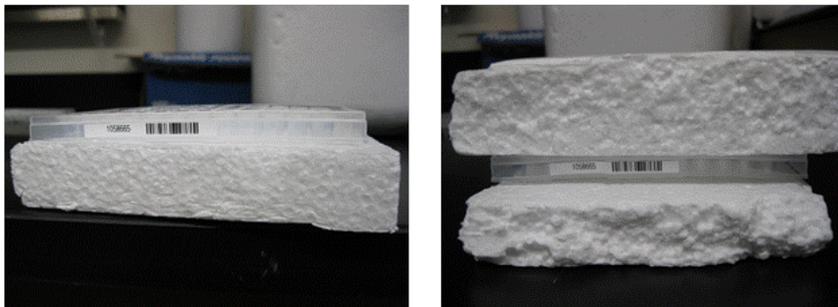
Figure. Shipment of CE loading plates.

We found that caps were less likely to leak than adhesive foil or other plate sealers (i.e. plastic). To prevent plates from breaking, twisting or leaking in transit:

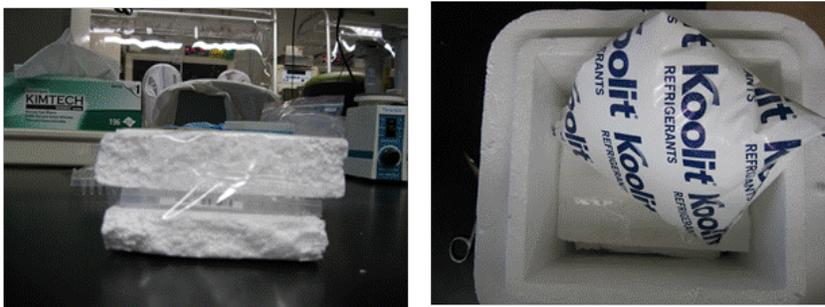
Using an old PCR plate, make holes in a piece of low density Styrofoam



Embed the optical plate with the diluted sample and the formamide/ROX in the hole in the Styrofoam. Using a second piece of Styrofoam, make a sandwich with the plate.



Tape the plate sandwich, put it in a Styrofoam shipping container with ice packs and send overnight.



Analysis

Required software:

PeakScanner Software (Applied Biosystems) – Currently, this free software can only be run on a PC. Go to the following website for a free download:

<http://download.sharewarecentral.com/6/271833/peak-scanner-software.html>

NOTE – We are scripting code to replace the PeakScanner step. This will make the analysis pipeline universal across computing platforms. We anticipate release of an updated pipeline in early 2015.

PeakScanner Protocol:

- 1) Open PeakScanner and select the “Start New Project” button. For first-time users, you will need to create a new size standard. Select “New” and “New Size Standard”. Name the standard appropriately (i.e. something like “Rox-1000”), select the “Red” dye color, and copy/paste the following size definitions into the left box.
50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000
Click the “Add Size(s)” button and then “Save”.
- 2) Click the “Add files” button, browse to find your .fsa fragment analysis files, click “Add Selected Files”, and then “OK”. This will populate the Sample Explorer window with the appropriate files, where each row represents one .fsa file.
- 3) Under the “Size Standard” menu for the first row, find and select your size standard (“Rox-1000”).
- 4) Highlight the entire column by clicking “Size Standard” and press and hold the “Ctrl” and then “d” buttons on your keyboard. This will auto-populate the same size standard for all rows.
- 5) Under the “Analysis Method” menu for the first row, find and select either of the two default analysis methods (i.e. it doesn’t matter which).
- 6) Highlight the entire column and auto-populate for all rows as above. This should change the “Status” of the rows and an icon showing a figure over a play button should appear. The “Analyze” button on the left menu should also become active.
- 7) Click the “Analyze” button. After the analysis completes, you should look at the columns “Offscale” and “Quality”. Successfully analyzed rows will have a green box with a white check. Rows that may be offscale or of poor quality will have an orange box with a white question mark. Rows that failed the analysis will either have a red box with a white “X” or be blank (no boxes).
- 8) Remove all rows that failed the analysis by highlighting the row numbers and clicking “Remove Files” and “OK”. If you removed rows, reselect the size standard for the remaining files (Steps 3 and 4) and reanalyze the remaining files by selecting “Analyze”. After this analysis, there should be four sections of the screen as follows: a vertical left-hand menu, a top “Samples View”, a middle “Plot View”, and a bottom “Sizing Table View”.
- 9) **IMPORTANT** – Highlight all rows. The following commands will only be performed on highlighted rows.
- 10) Select the Plot Settings icon. This icon is sometime difficult to find for first-time users. It is on the middle section “Plot View” on the right-hand side and looks like a horizontal hand that is pointing to the left. It should be just to the left of a blue circle with a white question mark. Sometimes this window will appear, but sometimes it is minimized as soon as it appears, so check the bottom of your computer screen to see if it is behind the peakscanner icon. The Plot Settings window has three tabs as follows: “Display”, “Labels”, and “Scaling and Zooming”.
- 11) Select only blue peaks by clicking the rainbow square in the “Display Dyes” section of the “Display” tab of Plot Settings and then selecting the blue square.
- 12) Under the “Labels” tab of the Plot Settings, select “Height” from the dropdown menu next to the “Label Peaks” button. Change the range as follows: “From:” = 50 and “To:” = 100000000. The value in “To:” is arbitrary and simply needs to be large (>1 million). Click the “Label Peaks” button. Select “Size” from the dropdown menu

next to the “Clear Labels” button and change the range as follows: “From:” = 0 and “To:” = 200. Click the “Clear Labels” button. Minimize the Plot Settings window (do not close it).

- 13) Select the Sizing Table View window by clicking where it says “Sizing Table View”. Be sure to not click anywhere else, as this may de-select rows in the Sample View window.
- 14) From the “Show” dropdown menu on the Sizing Table View window, select “Show Labeled Peaks”. Wait for this to run (the dropdown menu will return to normal after analysis is complete).
- 15) Select “Export” from the top menu and click “Export”. Browse and save the text file somewhere on your computer. The naming of this file is important for downstream analysis, but there is only one rule. The file must end in “peaks.txt”. Feel free to add a plate or project identifier to the file name, but do so before the “peaks.txt” and separate these two by an underscore (“_”). Click “Save”.
NOTE – Do not include spaces in the file names. Use underscores.
- 16) Log onto the Walk Lab website (<http://walklab.rcg.montana.edu/ribo.html>) and follow instructions on the website for submitting the peaks.txt file for analysis. Your results will be emailed to the address that you supply.