qPCR Reunion

Part 1:

Where are we now?

What have we learned?

Plan for the session

Time	Activity	Presenter
10:00-10:15	Overview of project	Erin Dreelin, Michigan State University
	Preview of qPCR validation study results	Rich Haugland, EPA
10:15-10:45	Troubleshooting 1: Lessons learned from running draft Method C	Brian Scull, Grand Valley State University
10:45-11:15	Troubleshooting 2: Reagents, supplies, and tracking contamination: What can we substitute? What is acceptable?	David Szlag, Oakland University
11:15-11:50	Panel discussion and open Q&A	
11:50-noon	Next steps, wrap up	Shannon Briggs, Michigan DEQ

What's going on with qPCR in Michigan?

- State of Michigan provided $500,000 for:
  - Equipment for 11 new qPCR labs
  - Training for new labs

- Goal is to build capacity for using qPCR methods for monitoring recreational water quality
  - Also potential for source tracking

Building qPCR capacity in MI

2014

Michigan funds new labs

2015

Train-the-trainer

Beach samples frozen for later analysis

2016

Lab training

qPCR validation study

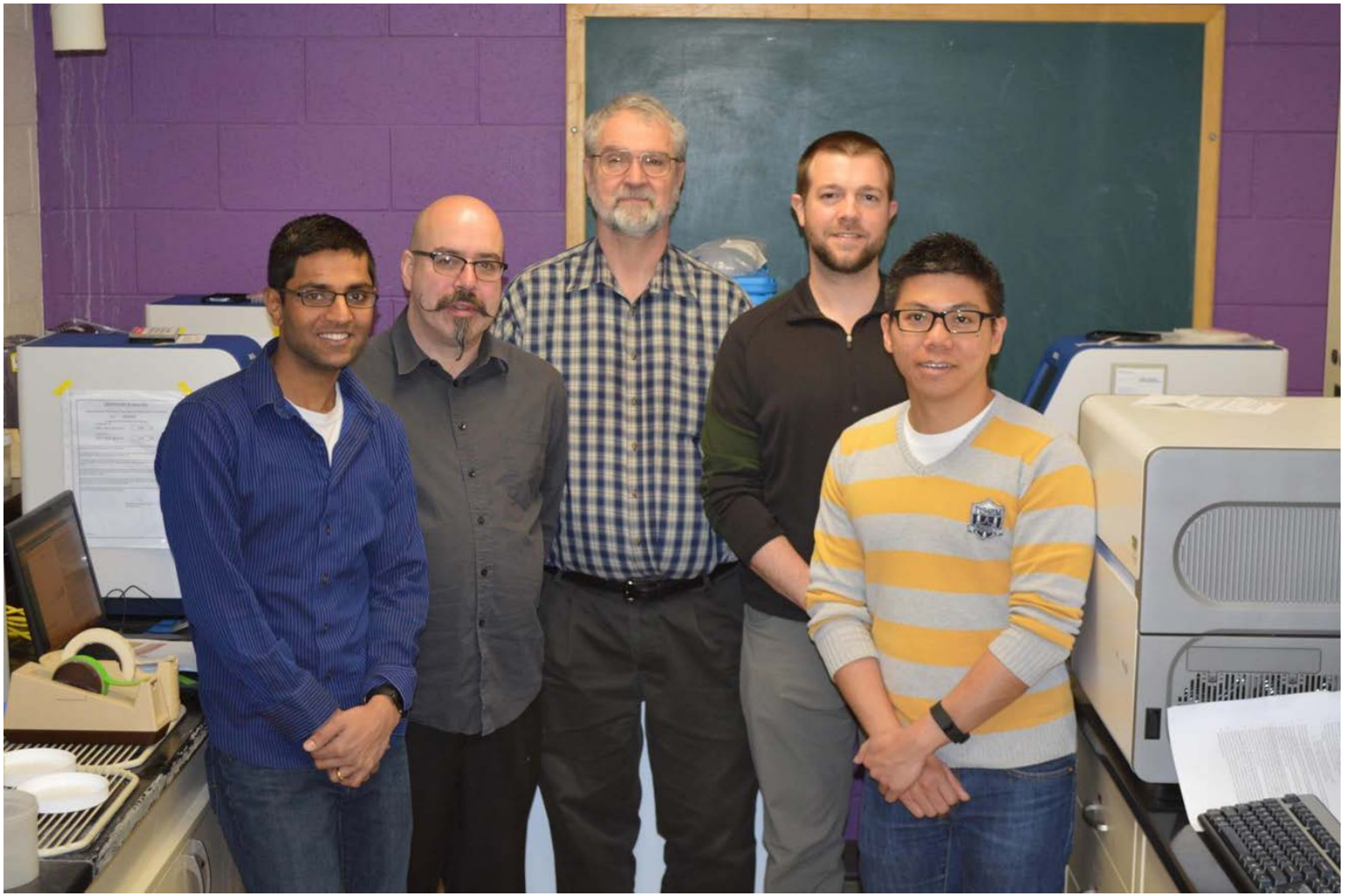
2017

Colilert + qPCR analysis during beach season

Our Network of Michigan qPCR Labs

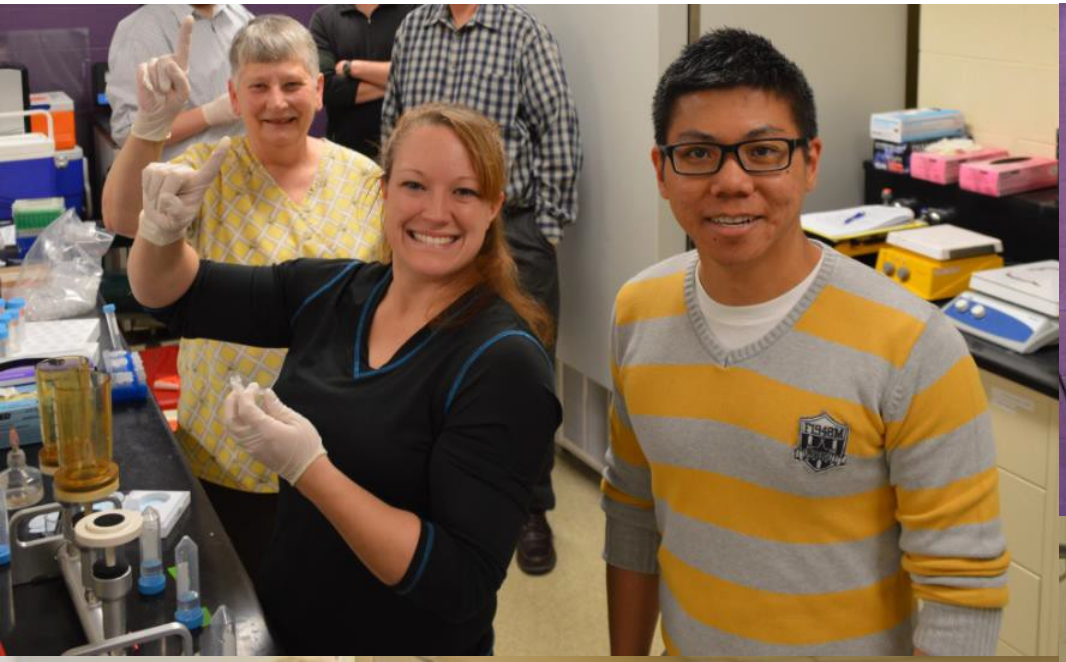
Marquette Area Wastewater Treatment Plant
Lake Superior State University
Northwest Michigan Regional Lab
NPS- Sleeping Bear Dunes
Central Michigan Health District
Ferris State University
Saginaw County Dept of Public Health
Saginaw Valley State University
Grand Valley State University
Hope College
Kalamazoo County Health & Community Services
Michigan State University
USGS- Lansing
Oakland County Health Department
Oakland University

Train-the-trainers: EPA-ORD 2015

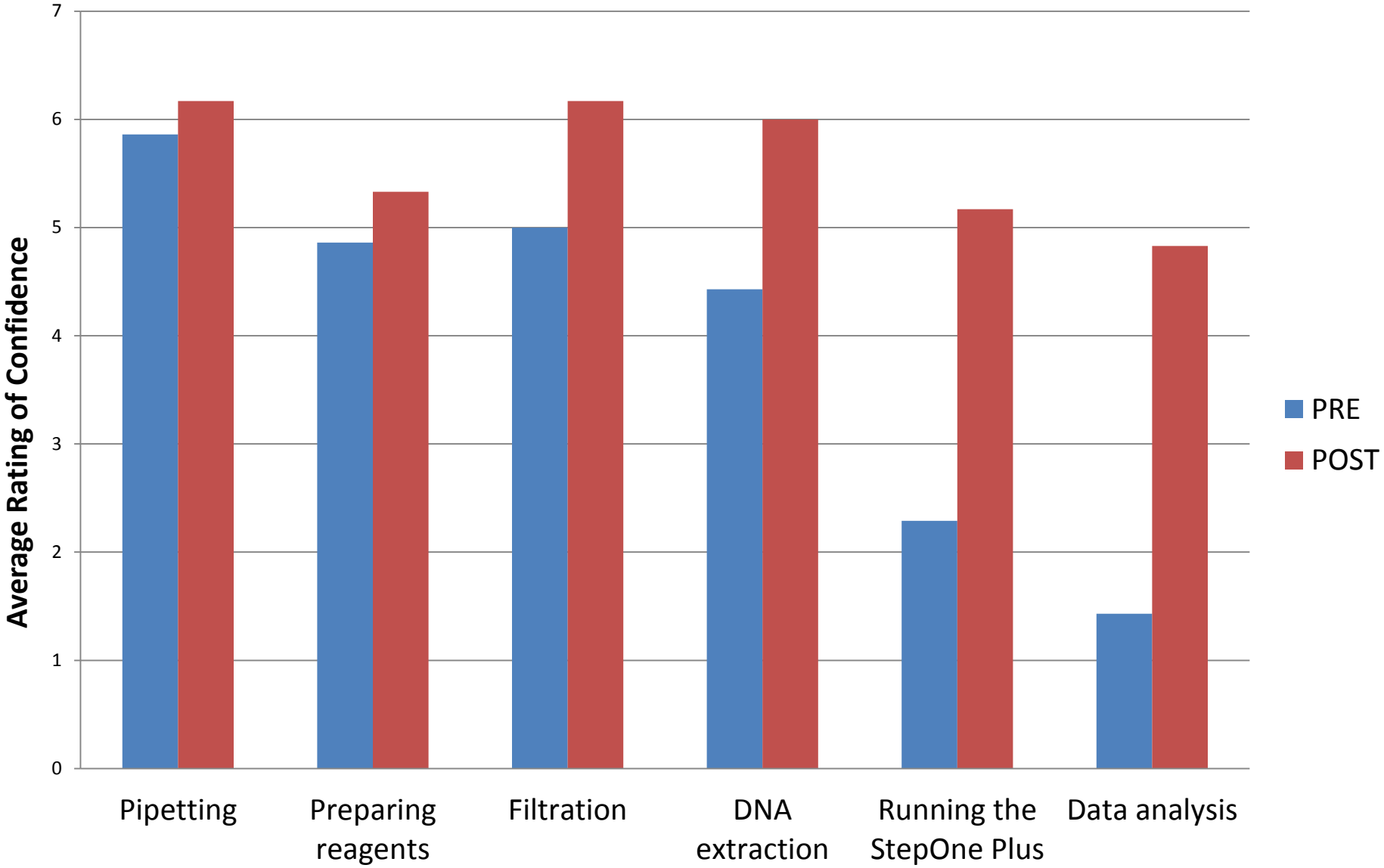


Our Trainers: Nilay Sheth, Brian Scull, Rich Haugland, Chris Otto and Tiong Aw

Training Labs: Feb 2016 @ MSU



How would you rate your confidence in your skills for completing each step of qPCR?



What's happening now

- Multi-lab validation study using Draft Method C
  - Michigan labs plus:
    - UW- Osh Kosh
    - City of Racine, WI
    - EPA-ORD
    - North East Ohio Sewer District
    - University of Illinois-Chicago
    - Georgia Southern University
    - University of North Carolina

PREVIEW OF QPCR VALIDATION STUDY RESULTS

Rich Haugland, USEPA

2016 METHOD C (*E.coli by qPCR*) MULTI-LABORATORY STUDY

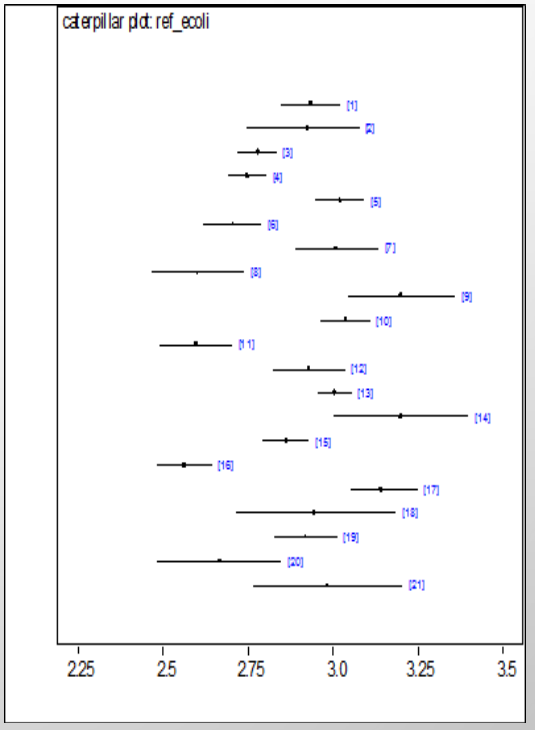
OVERVIEW

FUN FACTS

- 22 PARTICIPATING LABORATORIES
  - 11 PUBLIC HEALTH, MUNICIPAL, REGIONAL AND GOVERNMENTAL LABORATORIES
  - 11 COLLEGE AND UNIVERSITY LABORATORIES
  - VARYING LEVELS OF TRAINING AND EXPERIENCE
- TWO PHASES IN THE STUDY
  - PHASE 1: STANDARDS AND CALIBRATOR SAMPLE ANALYSES, PROFICIENCY DEMONSTRATION
  - PHASE 2: UNKNOWN WATER SAMPLE ANALYSES AND *E. COLI* TARGET SEQUENCE QUANTIFICATION
- TOTAL OF 54 BLINDED WATER SAMPLES ANALYZED IN COMMON BY EACH LAB IN PHASE 2
  - WATER SAMPLES COLLECTED FROM 6 SITES (2 INLAND AND 4 GREAT LAKES)
  - EACH COLLECTED SAMPLE DIVIDED INTO 3 SUBSAMPLES (1AMBIENT AND 2 *E. COLI* SPIKE LEVELS OR AMBIENT AND 2 LAKE WATER DILUTIONS)
  - 3 REPLICATES OF EACH SUBSAMPLE ANALYZED IN DUPLICATE BY EACH LAB
- 21 LABORATORIES COMPLETED THE STUDY (PHASE 1 AND PHASE 2)

CONCLUSIONS

- PRELIMINARY RESULTS LOOK PRETTY GOOD!
- COME LEARN MORE AT THIS AFTERNOON'S PRESENTATION ☺



TROUBLESHOOTING 1: LESSONS LEARNED FROM RUNNING DRAFT METHOD C

Brian Scull, Grand Valley State University

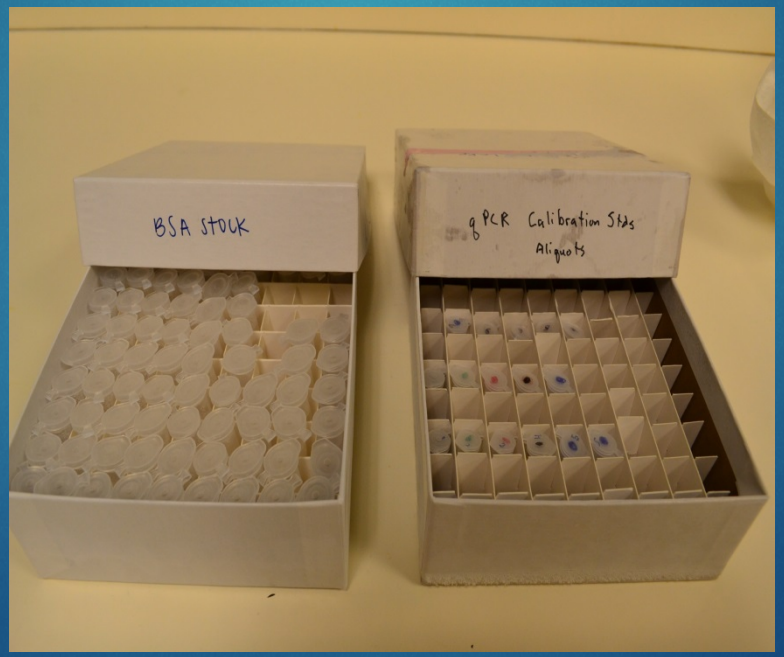
qPCR Method C

Lessons Learned

Brian T. Scull
Annis Water Resources Institute

Importance of aliquoting

- Minimizing # of freeze thaw cycles
- Minimizing chances of contaminating master stocks—less waste
- Convenience/Time Saving



Condensing Method C {Cheat Sheets}

Condensed Lab Protocol for Standard Curves, Controls, and Calibrator Samples

Primers:

Dissolve lyophilized primers in AE buffer to make 500 µM stock solutions (yield in nmol on tube x 2 = volume in µL to add to tube). ABI probes come already dissolved in 100 uM solution.

EC23S (E. coli) and Sketa22 (Salmon) Primer/Probe Working Stocks

1. **Ratio of Primer/Probe mix components is:**
   a. 10 uL forward primer 500 uM stock solution
   b. 10 uL reverse primer 500 uM stock Solution
   c. 4 uL probe 100 uM stock solution
   d. 576 uL PCR grade water

Reaction Mixes:

1. **Ratio of reaction mix components is:**
   a. 2 parts water
   b. 2.5 parts BSA
   c. 3 parts primer/probe mix
   d. 12.5 parts EMM per reaction as per Method C; always make a few extra reactions; same procedure is used for making EC23S reaction mix.

2. **Sketa22 (Salmon) reaction mix**

   Example: 24 reactions + 6 extra.
   a. 60 µL of distilled water
   b. 75 µL of BSA
   c. 90 µL of the primer/probe working stock
   d. 375 µL of Environmental Master Mix

3. **EC23S (E. coli) reaction mix**

   Example: 72 reactions + 8 extra
   a. 160 µL distilled water
   b. 200 µL BSA
   c. 240 µL primer/probe working stock
   d. 1000 µL Environmental Master Mix (EMM)

Transfer 20 ul aliquots of EC23S master mix and Sketa22 master mix to reaction plates as shown in **(Table 1).** Cover plate with aluminum foil and put in fridge. **Note:** Volumes of reagents required will change depending on the number of samples you are running on a well plate.

Salmon DNA Buffer

1. Salmon DNA stock to 10 ug/mL working stock from 1 mg/mL stock solution, and prepare 0.2 µg/mL salmon DNA extraction buffer from 10 µg/mL working stock by diluting with AE buffer.

   10.0 ug/ml Working Stock = 2.4 ml of 1.0 mg/l DNA Stock added to 240 ml AE Buffer
   0.2 ug/ml Working Stock = 1.0 ml of 10.0 ug/ml DNA Stock added to 49 ml AE Buffer

***E. coli* DNA for Calibrator Samples**

1. Checklist for making 5.1 ml of ~10E4 cells/per mL cell suspension. Cell suspensions were made using BioBalls.

   a. Add 0.9 ml PBS to thawed 100 uL 5x10E4 *E. coli* cell tube. Vortex.
   b. Transfer entire 1 mL volume to a 15 mL tube.
   c. Add 4.1 mL PBS to 15 mL tube and vortex gently.

Filtration for Filter Blanks (FB) and Calibrator Samples (Cal).

1. Checklist for filter blanks:
   a. Filtration units with polycarbonate filters.
   b. Rinse filter with 20 mL of PBS to make filter blank samples (FB 1, FB 2, and FB 3).
   c. Transfer filter to a labeled bead tube with 0.3g ~300um glass beads.

2. Checklist for Calibrator samples:
   a. Filtration units with polycarbonate filters.
   b. Filter a 1 mL aliquot of a 5.1 mL E. coli cell suspension through the filter to make calibrator samples (Cal 1, Cal 2, and Cal 3).
   c. Transferring the filters to a labeled bead tube with 0.3g ~300um glass beads.

3. Add 600uL of salmon DNA buffer to "FB" and "Cal" sample filters in bead tubes.
4. Tubes are then:
   a. Tightly capped and bead milled for 1 min.
   b. Centrifuge for 1 min.
   c. Crude supernatants (as much as possible) are transferred to clean 1.7 mL low-retention tubes labeled with "FB" and "Cal".
   d. Centrifuge supernatants for 5 min.
   e. 100uL of clarified supernatants are transferred to clean 1.7 ml low-retention tubes labeled with "FB" and "Cal".

Load reaction plates with DNA standards and calibrator and blank sample extracts; load reaction plates in instruments and start thermal cycling runs.

1. DNA standards (**Table 4**). Prepare Standard 6 by diluting an extra tube of Standard 5, two fold.
2. Seal the reaction plate with optical adhesive tape and centrifuging the plate in preparation for loading it on qPCR instrument.
3. Set up plate to run on a StepOnePlus Instrument.

Controls and Unknown Samples

Reference Matrix Spike (RMS) and Matrix Spike (MS)

1. Add 1 mL of their 5.1 mL E coli cell suspension to 50 mL PBS for reference matrix spike (RMS) sample.
2. Filter a 50 mL ambient water sample.
3. Add 1 ml of E coli cell suspension to 50 mL of an ambient water sample for matrix spike (S1-MS) sample.

   Check list:

   a. Set up 3 filtration units with polycarbonate filters at manifold station.
   b. Filter 50 ml ambient water sample, RMS and MS samples.
   c. Rinse filters with 20 mL PBS.
   d. Transfer filters to bead tubes labeled "Sample", "RMS" and "S1-MS".

4. Extract water sample, filter blanks, RMS, S1-MS and calibrator sample filters.

   Check list:

   a. Add 600uL of salmon DNA extraction buffer to extraction tubes.
   b. Seal tubes tightly and bead mill 1 min.
   c. Centrifuge tubes for 1 min.
   d. Transfer crude supernatants to clean, labeled 1.7 mL tubes.
   e. Centrifuge crude supernatants for 5 min.
   f. Transfer 100 uL of clear supernatants to clean, labeled 1.7 mL tubes, or to 96 well plates as indicated in **Table 2 or 3**. **Table 3** is the setup for using Multichannel pipettors.

5. Load reaction plates with sample extracts; load reaction plates in instruments and start thermal cycling runs.

   a. Transfer 5 uL each of filter extracts to prepared reaction plates.
   b. Reaction plates are sealed with optical adhesive tape and centrifuged.
   c. Set up plate to be run on the StepOnePlus instrument.

Table 2. Plate set ups

Plate Setup												
Plate Rows	1	2	3	4	5	6	7	8	9	10	11	12
A EC23S	Cal 1		Cal 2		Cal 3		FB 1		FB 2		FB 3	
B EC23S	RMS		sample 1 - MS		sample 1		sample 2		sample 3		sample 4	
C Sketa22	Cal 1		Cal 2		Cal 3		FB 1		FB 2		FB 3	
D Sketa22	RMS		sample 1 - MS		sample 1		sample 2		sample 3		sample 4	

Table 3. Multichannel Extract Plate set ups (Optional)

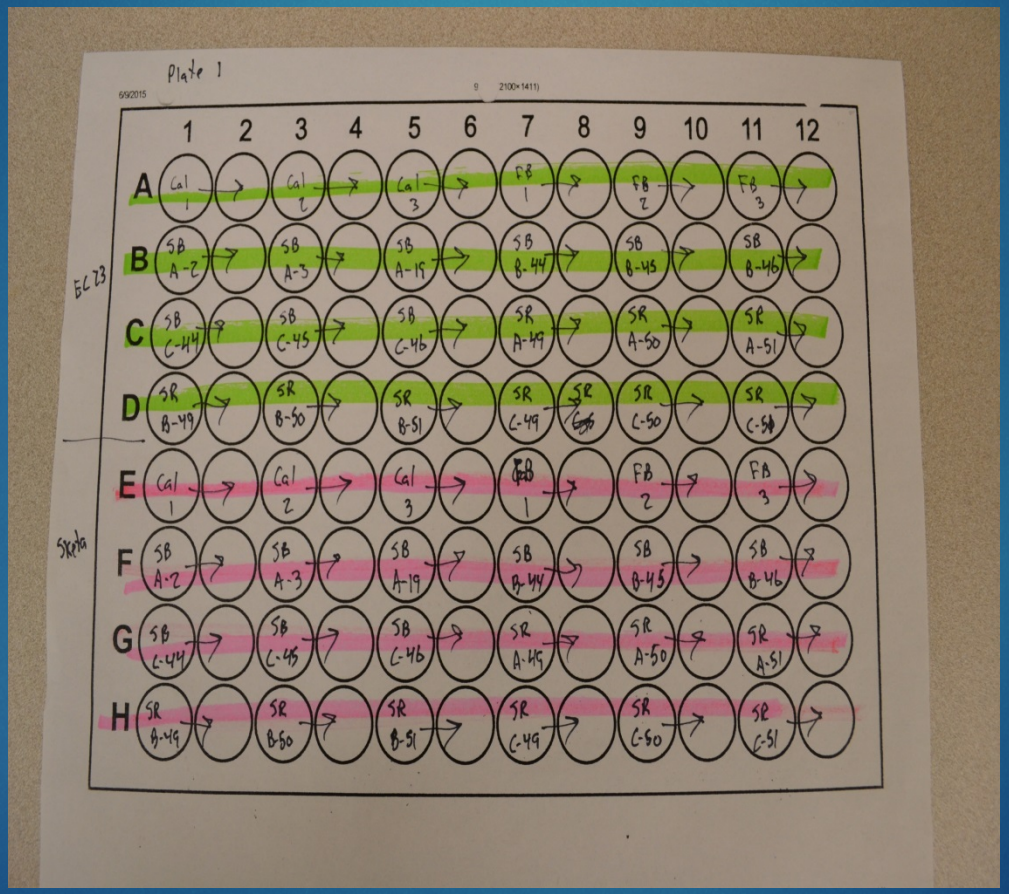
Plate Setup												
Plate Row	1	2	3	4	5	6	7	8	9	10	11	12
A EC23S	Cal 1	Cal 2	Cal 3	FB 1	FB 2	FB 3	RMS	S1-MS	Sample 1	Sample 2	Sample 3	Sample 4
B EC23S	Cal 1	Cal 2	Cal 3	FB 1	FB 2	FB 3	RMS	S1-MS	Sample 1	Sample 2	Sample 3	Sample 4
C Sketa 22	Cal 1	Cal 2	Cal 3	FB 1	FB 2	FB 3	RMS	S1-MS	Sample 1	Sample 2	Sample 3	Sample 4
D Sketa 22	Cal 1	Cal 2	Cal 3	FB 1	FB 2	FB 3	RMS	S1-MS	Sample 1	Sample 2	Sample 3	Sample 4

Table 4. EPA DNA Standards

Name	Concentration	Tube cap color
Standard 1	2.3x10^4 copies/5 µL	clear
Standard 2	2.4x10^3 copies/5 µL	green
Standard 3	2.4x10^2 copies/5 µL	red
Standard 4	4.4x10^1 copies/5 µL	black
Standard 5	1.2x10^1 copies/5 µL	blue
Standard 6*	6x10^0 copies/5 µL	

* Prepare by diluting the extra tube of standard 5, 1:1 with AE buffer

Cheat Sheets cont.



Sample & Reagent Storage

- Storage Temps.
- Freeze/Thaw Cycles

Sample and Reagent Storage				
	Room Temp	4°C	-20°C	-80°C
Water Samples		X		
Sample Filters			X	X
DNA Extraction Buffer (Extracted Sample Filters)		X		
PBS	X			
PCR Water	X			
BSA			X	
Primer/Probe Stocks			X	
EMM			X	
AE Buffer	X			
SAE Buffer		X		
SAE Buffer (Aliquots)			X	
BioBall Suspensions/Calibrators				X
Salmon dna Stocks			X	
E.coli Calibration Stds				X
Note:				
X	Short term Storage 6 months or less			
X	Long Term Storage > 6 months			

Laboratory Refrigerators & Freezers

- Dedicated storage for qPCR Project Materials
- Accucold Medical by Summit Appliance http://www.accucold.com/vacseries

Miscellaneous

- Run periodic checks on Stds., Calibrators, SAE Buffer, BSA etc.
- Limit freeze/thaw cycles
- Only filter calibrator and RMS samples right before extraction
- Make sure loaded 96-well plates are close to room temp before analysis
- Salmon DNA stocks made in AE Buffer, not PCR Water
- Don’t forget to use your Qubit
- Bleach residue in filter funnels

TROUBLESHOOTING 2: REAGENTS, SUPPLIES, AND TRACKING CONTAMINATION

David Szlag, Oakland University

Some observations on trouble shooting Method C

Reagents, supplies, and tracking contamination: What can we substitute? What is acceptable?

Dave Szlag , Oakland University

October 2016

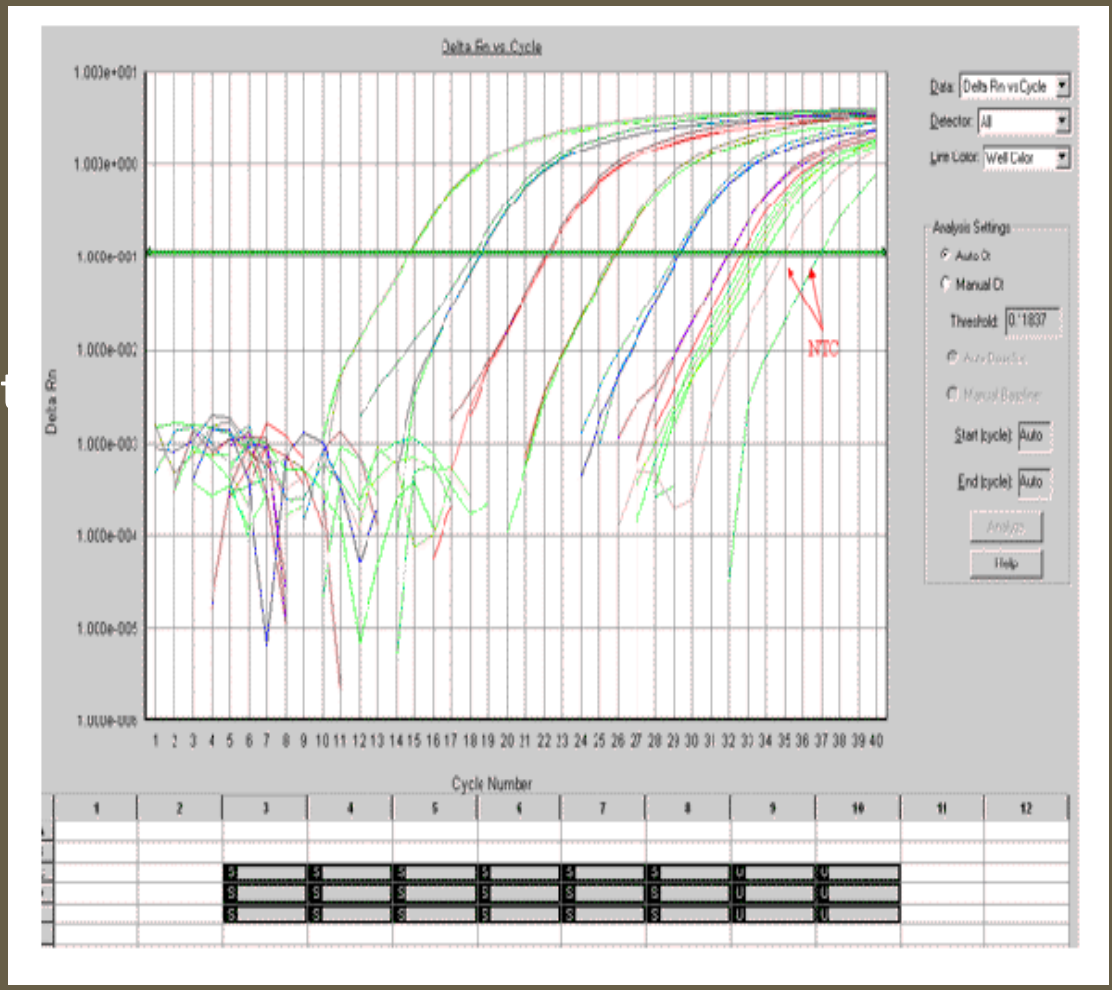
Contamination of Reactions by DNA

- Random
- Reagent
- NTCs can be your best friend!
- MBs can be your best friend!

Random

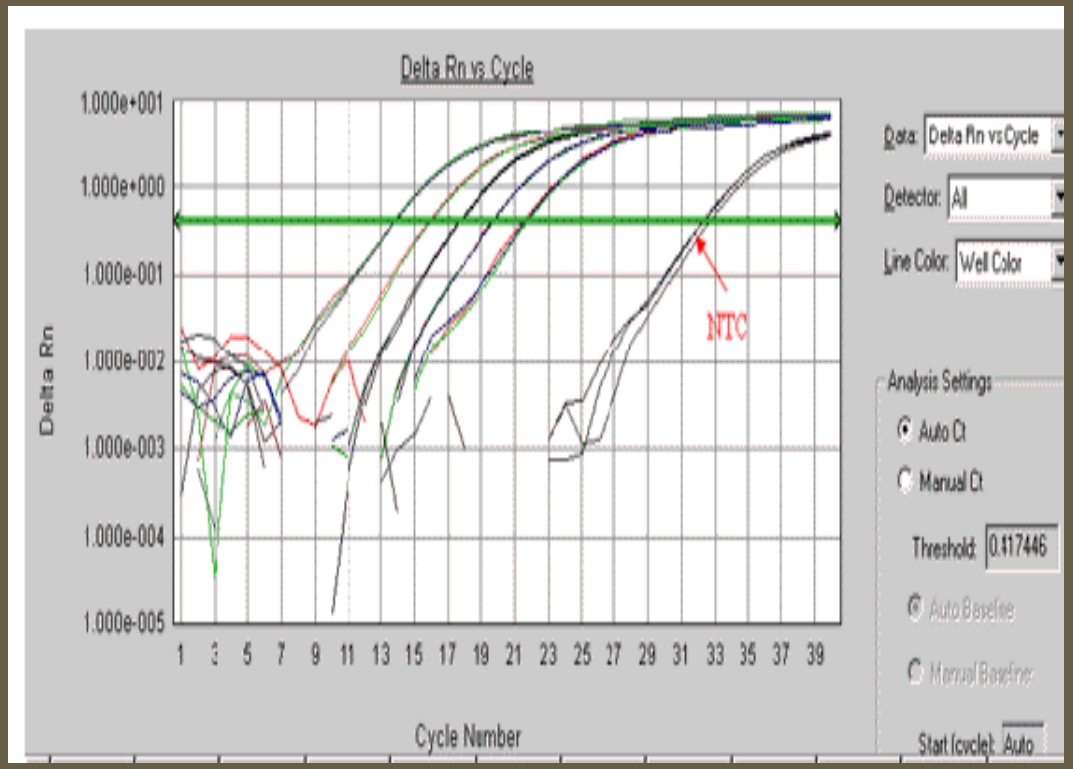
Problem with loading or areas

- NTC contamination occurs when loading
  - NTC will show loading in some or all of the NTCs at varying CT values
- The fix:
  - Cleanliness
  - Separate areas
    - PCR mix prep
    - Template addition
    - Performing reactions



Reagent Contamination

- All reagents used in qPCR can become contaminated with template
  - Mastermix, water, primer/probe
  - Amplification of the NTC replicates will be close; b/c same amount of template
- Solution
  - Clean Work Practices
  - Separate work areas
  - But what if…



The Mastermix is made in *E. coli*…

- How low will your NTCs be?
  - Undetermined
- What can we tolerate?

- Mastermix
- Toughmix
- Environmental Mastermix 2.0
  - Both had issues in 2015
  - Must Track Lot numbers!!!!

Other Supplies? Don't be penny wise and dollar foolish.

- Plates
  - Use Life Technologies
- Water
- Membrane filters
- Tubes
  - Match to your bead beater and make sure they fit your centrifuge
- Beads
  - UV sterilize

Filter blanks

- Sterilization
  - UV
    - How long?
  - Bleach
    - Sodium Thiosulfate
  - Rinsing
- How clean is clean enough?

Let’s look at some data

Open Q&A Session

- Is there anything giving you problems?
- What have you learned from your experience that you think could help others?
- What advice would you give someone just starting to use qPCR?

Continue the discussion…

- qPCR Reunion Part 2 at 1:30
  - Validation study preliminary results
  - Technical discussion: SOP, calculations, comparing reagents, etc.
  - More troubleshooting
- If you have any unanswered questions, write them on the flipcharts
- Join- and use!- the MIqPCR listserve
  - See Erin Dreelin to join