STEC Molecular Serotyping Protocol

Luminex-based Suspension Array to Identify STEC O serogroups O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, and O157

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1.0 Background

1.1 STEC Background

Identification and serotyping of Shiga toxin-producing *Escherichia coli* (STEC) during foodborne outbreaks can aid in matching clinical, food, and environmental isolates when trying to identify the sources of illness and ultimately food contamination. Furthermore, identifying O serogroups of STECs can help to differentiate pathogenic STECs from STECs that are not associated with human illness. STECs are a significant public health concern causing approximately 170,000 illnesses in the United States each year. Although O157 is the most common STEC serogroup in the U.S., more than 170 different O serogroups have been identified, many of which produce Shiga toxin and have been associated with human illness. Non-O157 STECs are responsible for over 60% of STEC infections or an estimated 112,000 illnesses in the U.S. each year. Over 74.2% of non-O157 STEC infections in the U.S. are caused by serogroups O26 (23.9%), O45 (7.8%), O91 (2.3%), O103 (16.7%), O111 (12.6%), O113 (unknown %), O121 (7.5%), O128 (unknown %), and O145 (3.4%). Of these, O26, O103, O111, O121, and O145 are known to cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), and O45 is associated with HC. Other serogroups that may cause HC and HUS, but are less commonly isolated, are O91, O113, and O128. In addition, in 2011 STEC O104:H4 was identified as the causative agent in a multi-national outbreak involving 850 cases of HUS and 32 deaths in Germany, France and the U.S.

1.2 Assay Information

A Bio-plex assay based on Luminex xMAP technology has been developed to identify the O serogroup of pure culture isolates of the eleven most clinically relevant STECs: O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, and O157. The STEC Bio-plex assay correlates with the traditional serotyping method of testing agglutination with O serogroup specific anti-sera, but can be done in one multiplex reaction.

The STEC Molecular Serotyping Protocol first describes basic operation of the Bio-plex 200 instrument section 2.0. The protocol is performed in 5 stages; section 3.0 describes template preparation, section 4.0 PCR, section 5.0 Bead Hybridization, Section 6.0 Bio-plex Analysis, and Section 7.0 Data Analysis. Stages 4-6 should be completed on the same day. If necessary, PCR reactions can be stored overnight at 4°C, but longer storage may result in failure of the assay. The supplies needed are listed for each section, and a complete list for the entire protocol is listed in Appendix B with recipes for reagents in Appendix C.

1.3 Supporting Documents

Bio-plex Manager Software 6.0 User Manual
Bio-plex 200 System Hardware Instruction Manual

Bio-plex Care and Maintenance Quick Guide
http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_3076.pdf

96 well template sheet
http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_039767.pdf

Luminex Corporation (2007). Sample Protocol for Direct DNA Hybridization- Washed Assay Format Using Magnetic Microspheres. http://www.luminexcorp.com/prod/groups/public/documents/lmnxcorp/washed-direct-dna-magnetic.pdf

Andrew Lin, Lam Nguyen, Teresa Lee, Laurie M. Clotilde, Julie A. Kase, Insook Son, J. Mark Carter, Carol R. Lauzon. Rapid O serogroup Identification of the Ten Most Clinically Relevant STECs by Luminex Microbead-Based Suspension Array. *Journal of Microbiological Methods*. 2011; **87**:105-10.

Andrew Lin, Julie A. Kase, Michelle M. Moore, Insook Son, Nelly Tran, Laurie M. Clotilde, Karen Jarvis, Kelly Jones, Kuppuswamy Kasturi, Khamphet Nabe, Melissa Nucci, Gail S. Wagley, Fei Wang, Beilei Ge, Thomas S. Hammack. Multi-laboratory Validation of a Luminex Microbead-Based Suspension Array for the Identification of the Eleven Most Clinically Relevant STEC O serogroups. *Journal of Food Protection*. Accepted January 2013.

Screen captures in this document were taken from Bio-plex Manager™ 6.0 (Bio-Rad Laboratories, Inc., Hercules, CA) and Microsoft Excel version 14.0.6129.5000 part of Microsoft Office Professional Plus 2010 (Microsoft Corporation, Redmond, WA).

1.4 Safety Information

Tetramethyl ammonium chloride is acutely toxic by ingestion, skin contact, and inhalation. Please refer to the MSDS for safe handling.

2.0 Bio-plex 200 Operation

Supplies Needed:

- Sterile DI water
- 70% Isopropanol
- 10% Bleach
- Bio-plex Calibration Kit
- Bio-plex Validation Kit
- Bio-plex Sheath Fluid
- Bio-plex MCV Plate IV
- Vortex
- Sonicator (optional for needle cleaning)

The Bio-Plex instrument should be maintained and operated according to procedures in the Bio-Plex Manager 6.0 User Guide, and Bio-Plex Care and Maintenance Guide. All maintenance should be documented using the Bio-Plex Maintenance log (Appendix D) or similar.

2.1 Instrument Set-up

Bio-plex 200 should be placed in a room separate from the sample set-up laboratory. All manipulations of PCR reactions should be done in a designated post-PCR room. PCR products, reagents or other supplies should NOT go back into the sample set-up laboratory once they have been in the post-PCR room, unless they are treated to remove DNA contamination.

The array reader contains sensitive optics that can be forced out of alignment through improper handling and unnecessary moving. It is recommended that an authorized service representative move your system. Following any system moves, it is necessary to validate the optical alignment and report any changes. Refer to the Bio-plex validation kit manual for validation of the optical alignment.

The ambient temperature should be stable and within the range of 15–30°C (21°C is optimal), and the relative humidity should not exceed 80%, non-condensing. It is preferable to place the instrument in a location where the temperature does not deviate by more than ± 2°C. Avoid drafty locations as this may contribute to excessive temperature fluctuation.

Place on a clean, flat, and stable surface free of excessive dust or moisture. This surface must be free of other instrumentation that may cause vibration (e.g., vortex).

The Bio-plex High Throughput Fluidics (HTF) should be next to the array reader with the Sheath fluid bottle 3-4 feet below the HTF with the cap tightened securely.

The waste container should be on the bench next to the instrument or not more than 3-4ft below the instrument and should have a vented cap.

Do not obstruct the area below the array reader, and allow at least 2" of clearance around the machine.

Do not place any items on top of the array reader. The cover is not designed to support objects and thus the optics could be damaged.

2.2 Bio-plex Maintenance- Daily:

Check sheath fluid and waste container levels. Replace sheath fluid and empty waste container as necessary. If sheath fluid is changed, prime HTF and perform remove bubbles function 3x before performing any analyses.

2.2.1 Start Up

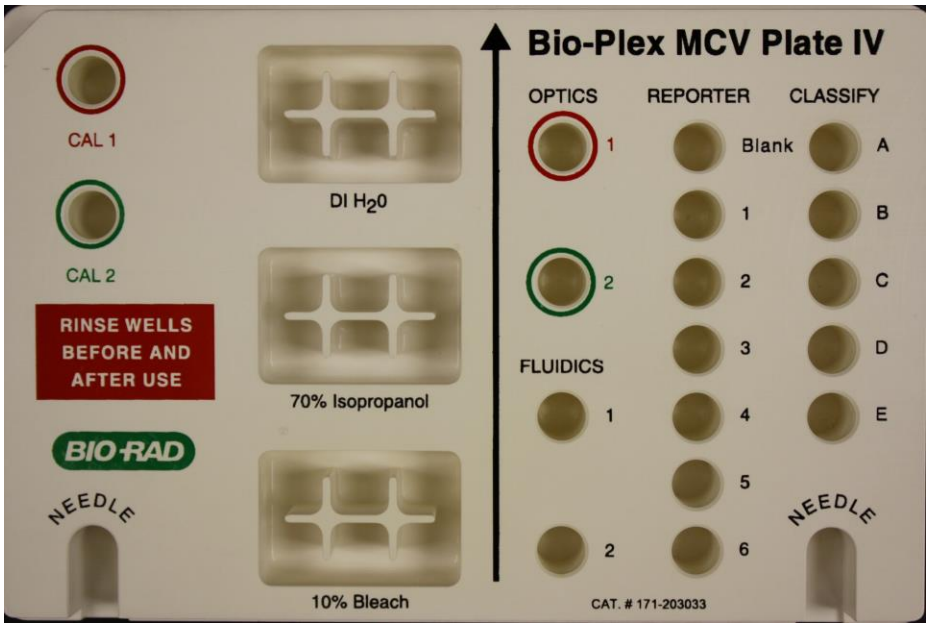
Turn on Bio-Plex instrument components (Do this about 90 minutes into the PCR run)

A. Turn on system reader
B. Turn on microplate platform (XY platform)
C. Turn on power on high-throughput fluidics (HTF)
D. Turn on computer

Note: Power switches are in back above power cords on the right when facing the instruments.

Open Bio-Plex Manager (BM) 6.0 software from the short-cut icon  located on the desktop or start menu of the computer. An error message will occur if BM 6.0 is opened before the Bio-Plex instruments have been turned on.

In BM 6.0, run the start-up procedure (3 minutes) by clicking the  button.
Follow instructions from BM6.0 software, filling the appropriate reservoirs of the MCV Plate IV.

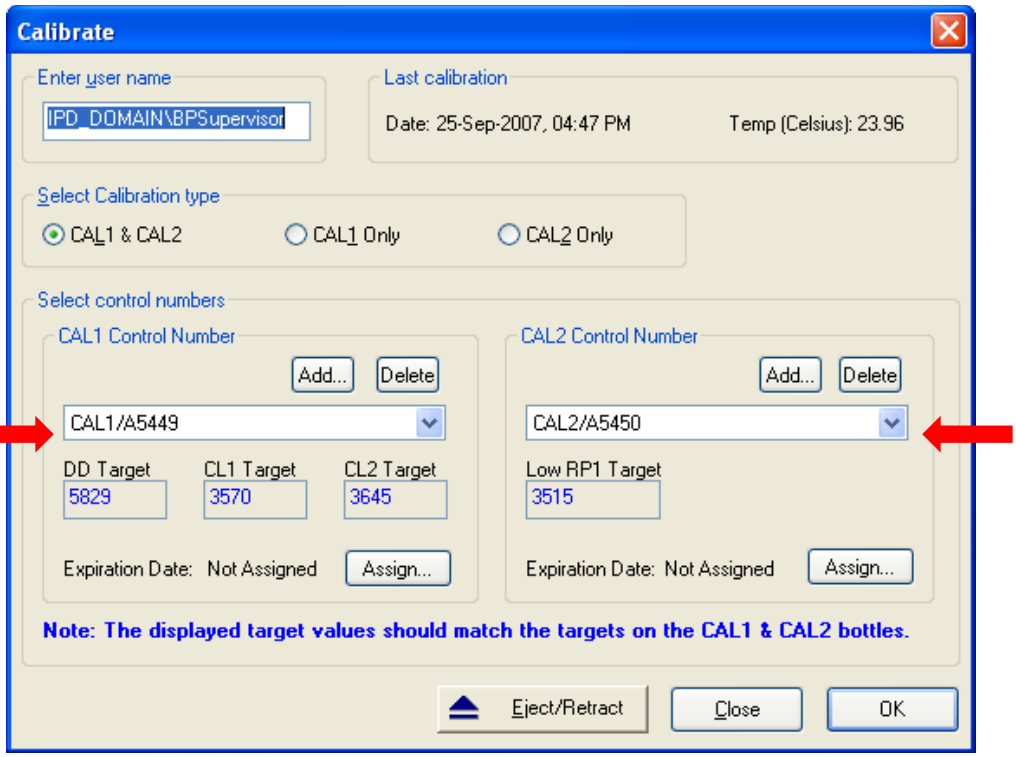


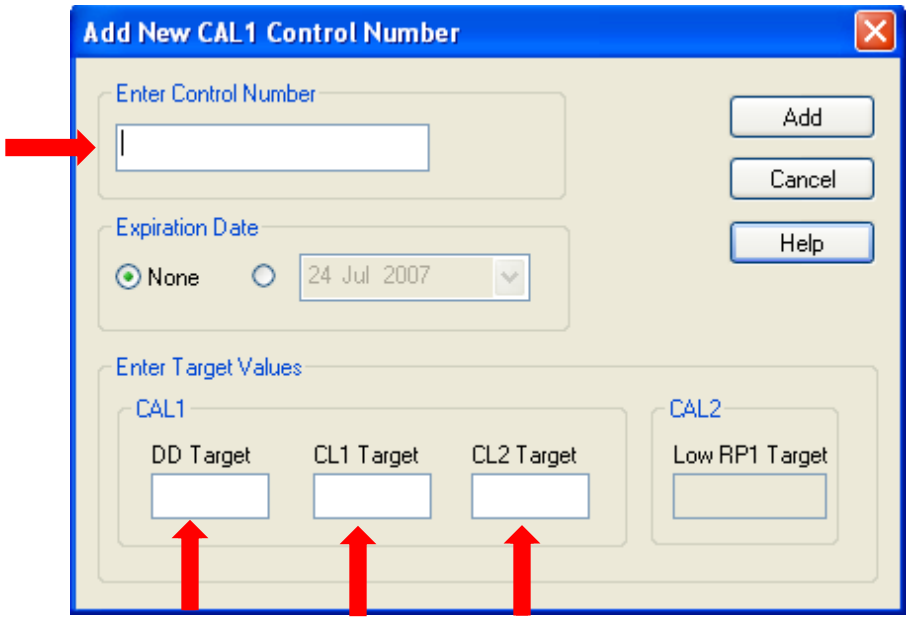
2.2.2 Warm up

Click warm up button to allow optics to warm-up for 30 minutes. The optics must be warmed up before calibration, validation, or sample analysis is done. This is **different** than the plate heater. BM 6.0 will alert you if you try to calibrate before the warm-up is complete, and it will count down time remaining if you select "Yes".

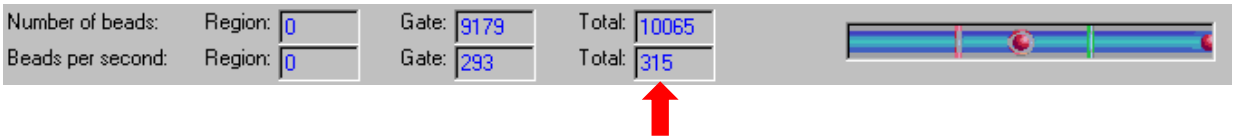
2.2.3 Calibration

After the start up and warm up procedures are completed, perform instrument calibration . Calibration reagents should be taken from the refrigerator just before use, vortexed 20 seconds and loaded in the appropriate wells of the MCV IV plate at 6 drops per well. Select the correct CAL1 and CAL2 control numbers in the Calibration field or if a new Calibration Kit is used click "Add" under control number and add the correct control numbers and target numbers in the Add New Cal Control Number dialog box. Return reagents to the refrigerator immediately after use.


Calibrate
Enter user name
IPD_DOMAIN\BPSupervisor
Last calibration
Date: 25-Sep-2007, 04:47 PM
Temp (Celsius): 23.96
Select Calibration type
CAL1 & CAL2
CAL1 Only
CAL2 Only
Select control numbers
CAL1 Control Number
Add...
Delete
CAL1/A5449
DD Target
5829
CL1 Target
3570
CL2 Target
3645
Expiration Date: Not Assigned
Assign...
CAL2 Control Number
Add...
Delete
CAL2/A5450
Low RP1 Target
3515
Expiration Date: Not Assigned
Assign...
Note: The displayed target values should match the targets on the CAL1 & CAL2 bottles.
Eject/Retract
Close
OK


Add New CAL1 Control Number
Enter Control Number
Add
Cancel
Help
Expiration Date
None
24 Jul 2007
Enter Target Values
CAL1
DD Target
CL1 Target
CL2 Target
CAL2
Low RP1 Target

While performing calibration, observe status bar:



The number of beads per second should be 100 or higher. If beads per second is < 100, perform unclog and recalibrate. If problem persists, remove and sonicate needle.

2.2.4 Instrument Settings

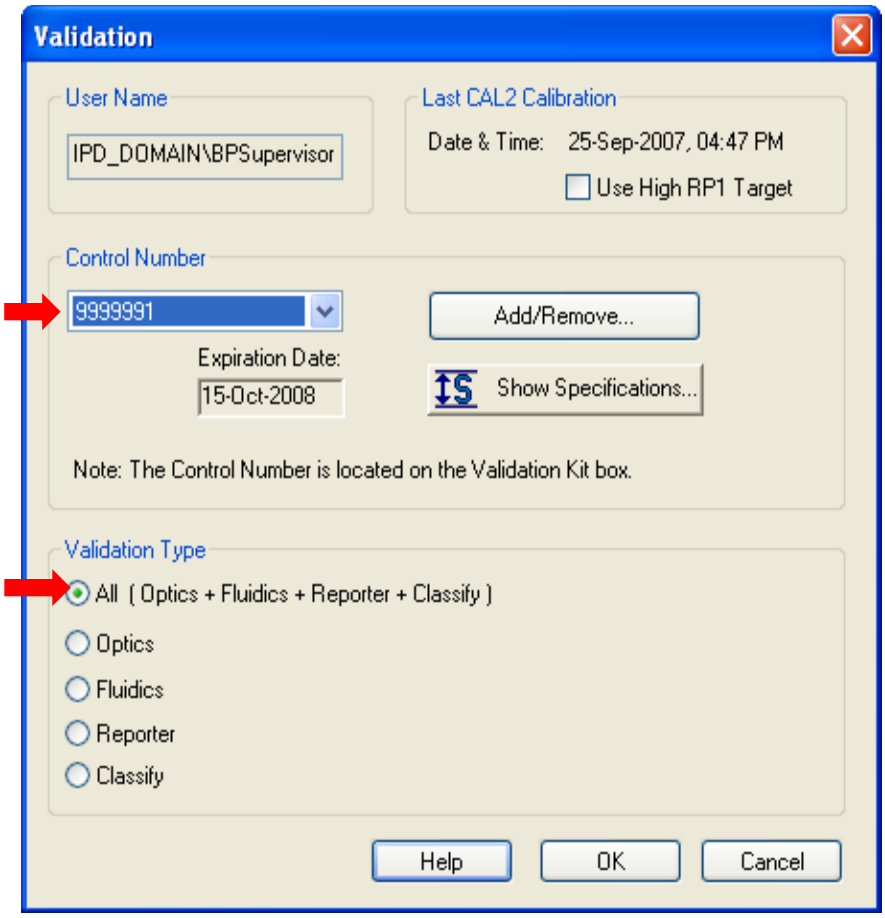
Under Instrument, Setup, choose “adjust needle”. Select “PCR Plate”.

2.2.5 Shut Down

Perform system shut down by clicking button and following BM 6.0 instructions. Fill appropriate reservoirs on MCV plate with DI water and 10% bleach. This shutdown procedure is designed to clean the fluidics lines and prevent a buildup of debris in the system. Remove MCV plate from Bio-plex and rinse all wells.

2.3 Bio-plex Maintenance- Monthly Validation

Validation is performed monthly to evaluate the optics, fluidics, reporter and classification components of the instrument to ensure that it is fit for its intended use. To perform validation, click on validation icon. Select the correct control numbers for the validation kit in the dialog box. For validation type, select all.



For new validation kits follow the instructions in BM 6.0 software manual and use CD included in the kit to install new control numbers.

Vortex standards for 20 seconds and fill appropriate wells on MCV Plate IV with 5 drops of each optic, fluidics, reporter, and classify standards, and fill DI water and 70% Isopropyl alcohol reservoirs.

2.4 Bio-plex Maintenance- To be performed as needed

Wash between plates: Perform wash between plates if multiple plate readings are done on the same day.

Remove air bubbles: Perform as indicated and after changing sheath fluid or after disconnecting any tubing.

Unclog: Perform as indicated or if beads/second during calibration is less than 100.

Sonicate needle: Perform if beads/second during calibration is less than 100 and unclog is unsuccessful. Perform “Adjust Needle” and “Wash between plates” after replacing needle. Refer to the Bio-plex Care and Maintenance Quick Guide for detailed instructions.

Adjust Needle: Perform after replacing needle. Set to PCR plate and Save.

3.0 DNA template preparation

Supplies Needed:

- Sterile microcentrifuge tube or sterile 96 well PCR plate
- Sterile Water
- Sterile loop or needle
- TSA or TSA-YE plates
- 35°C incubator
- Boiling Water Bath or thermocycler
- Microcentrifuge or tabletop centrifuge

Only pure culture isolates should be tested for O serotype by the STEC Bio-plex assay. DNA is extracted by boiling as follows:

For Microcentrifuge tubes:

1. Cultures are streaked for isolation on TSA or TSA-YE plates and incubated overnight at 35±2°C.
2. Using a sterile loop or needle, pick a well isolated colony and resuspend in 100 µL of sterile water in a microcentrifuge tube.
3. Place microcentrifuge tube in boiling water bath for 10-15 minutes.
4. Centrifuge to pellet cell debris at 12000 x g for 3 minutes.
5. Transfer supernatant containing genomic DNA to new sterile tube.
6. Store DNA at -20°C until use.

For PCR strip or plate:

1. Cultures are streaked for isolation on TSA or TSA-YE plates and incubated overnight at 35±2°C.
2. Using a sterile loop or needle, pick a well isolated colony and resuspend in 100µL of sterile water in one well of PCR strip tube or 96 well PCR plate.
3. Place PCR strip tube or 96 well plate in thermocycler.
4. Program and run thermocycler for 99°C for 15 minutes.
5. Centrifuge to pellet cell debris in table top centrifuge at 3000 x g for 10 minutes
6. Transfer supernatant containing genomic DNA to new sterile tube or plate.
7. Store DNA at -20°C until use.

4.0 PCR

Supplies Needed:

PCR clean Microcentrifuge tube
Hotstar Taq Master Mix
Nuclease Free Water (not DEPC treated)
STEC Primer Mix
Positive control DNA from *E. coli* O157 ATCC 43894 or 43895
Negative control DNA from *E. coli* ATCC 25922
DNA extracts from isolates
PCR tubes or PCR plate
PCR caps
Thermocycler for conventional PCR

Hotstar Taq Master Mix, STEC Primer Mix, positive and negative controls should be stored at -20°C until use and kept on ice when thawed. STEC Primer Mix contains forward and biotinylated reverse primers for all 11 targets as described in Appendix C. Positive control DNA should be positive for O157 and negative for all other O serogroups. Negative control DNA should be negative for all O serogroups. **Positive control, Negative control and three No Template controls must be run concurrently with each analysis. Control DNA from each of the 11 target O serogroups and one negative control (see Attachment B) should be run with each new lot of STEC Primer Mix or STEC Bead Pool 100x for reagent quality control.**

1. Program thermocycler with STEC PCR conditions
   - 95°C for 15 minutes
   - 40 cycles of 94°C for 30sec
     52°C for 30sec
     72°C for 1 minute
   - 72°C extension for 7 minutes
   - 4°C hold

2. Calculate # of reactions to make reagents for, a minimum of: (number of DNA extracts to be tested + 1 positive control + 1 negative control + 3 no template control) * 1.1 (for extra). See Appendix A for examples of calculations for PCR Master Mix Set up. **Do Not Use Qiagen Master Mix Kit Water**.

   PCR Master Mix/reaction
   1.62µL STEC Primer Mix

12.5µL Qiagen Hot StarTaq Master Mix
8.88µL Nuclease free water
23µL total

3. Follow your labs procedure for PCR setup (use of BSC or PCR hood, cleaning hood, decontaminating pipettes and racks, PCR workflow. . .). After thawing reagents, vortex and centrifuge all reagents before opening tubes. Set up PCR master mix as previously calculated. Aliquot 23µL of PCR master mix to appropriate number of PCR tubes.

4. After thawing, briefly centrifuge all DNA extract and control microcentrifuge tubes before opening. Add 2µL of Nuclease free water to three PCR tubes for no template controls which will serve as negative control for PCR and blank in the Bio-plex. Add 2µL of each DNA extract, and positive and negative controls to PCR tubes. ***Be careful not to cross contaminate PCR reactions. Be careful in adding DNA templates and controls to correct wells. Use 96 well template sheet if necessary.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	B		X1	X9		25922						
B	B		X2	X10								
C	B		X3	X11		43894						
D			X4	X12								
E			X5	X13								
F			X6	X14								
G			X7	X15								
H			X8	X16								

Example of 96 well plate format. B= no template control, X#=samples, 25922=negative control, 43894=positive control. To prevent cross contamination, spaces can be skipped on 96 well plates.

5. Place PCR tubes in thermocycler and start run.

6. PCR product can be stored at 2-8°C for up to 48 hours or used in Bio-plex assay immediately. ***Do not freeze PCR product**

5.0 Bead hybridization

Supplies Needed:

Low profile 96 well microplate
Microseal A adhesive film
STEC Bead pool 100x
1.5 X TMAC Hybridization Solution
1.0 X TMAC Hybridization Solution
Streptavidin Phycoerythrin (SAPE) 1mg/mL
1 X TE
96 well Life Sep magnetic separator
96 well heat block
Vortex
Sonicator

STEC Bead pool contains all 11 even probe oligonucleotides conjugated to specific MagPlex-C beads. Store STEC Bead pool and SAPE at 2-8°C protected from light. 1.5X TMAC Hybridization solution, 1.0X TMAC Hybridization Solution, and 1x TE should be prepared ahead of time according to Appendix D, and stored at room temperature.

1. Perform Bio-plex start-up, warm up, and calibration as described in the Bio-plex Manager User guide and in the next section before bead hybridization.
2. Insert heated brass plate into Bio-plex and set Bio-plex incubation temperature to 52°C. Set heat block with 96 well magnetic separator, and 96 well heat block to 52°C.
3. Program thermocycler with the following Bio-plex Hybridization run protocol:
    - 15 minutes 94°C
    - 52°C hold (hybridization temperature)
4. Make STEC Bead Working Solution fresh on day of use. Sonicate and vortex STEC Bead pool stock (100x) for 20sec. Dilute STEC Bead pool stock 1:100 in 1.5X TMAC Hybridization Solution to make enough for all reactions. Mix by vortexing 20 seconds. Add 12µL/reaction of TE to Bead solution. See Appendix A for examples of calculations of Bead Hybridization Reaction mixes.

    Bead hybridization/ reaction:
    - 0.33 µL STEC Bead Pool 100x
    - 32.67µL 1.5X TMAC
    - 12µL 1x TE

Add 45µL Bead Hybridization Reaction mix to each well

Add 5µL of each PCR reaction to wells.

*Careful to not cross contaminate. Multi-channel pipet can be useful for large numbers of samples.

5. Seal plates firmly with MicroSeal A Adhesive or equivalent plate sealer, place plate in thermocycler and start Bio-plex Hybridization protocol, noting time of start.
6. Prepare Reporter Mix by diluting 1mg/mL stock SAPE 1:250 in 1.0X TMAC Hybridization solution to a final concentration of 4µg/mL. Make enough reporter mix for all reactions. See Appendix A for example calculations.
7. After at least 30 minutes of hybridization temperature stop thermocycler.
8. Set PCR plate on heated 96 well magnetic separator for 1 minute to allow magnetic beads to collect on bottom.
9. Remove 45µL of supernatant without disturbing magnetic beads
10. Place 96 well plate in heated 96 well heat block to keep temperature at constant 52°C.
11. Add 75µL Reporter Mix to each well, mix by pipetting.
12. Insert 96 well PCR plate into brass plate of Bio-plex. Plate must be read within 30 minutes of adding Reporter Mix. ***Do Not Seal Plate for Bio-Plex Analysis.**

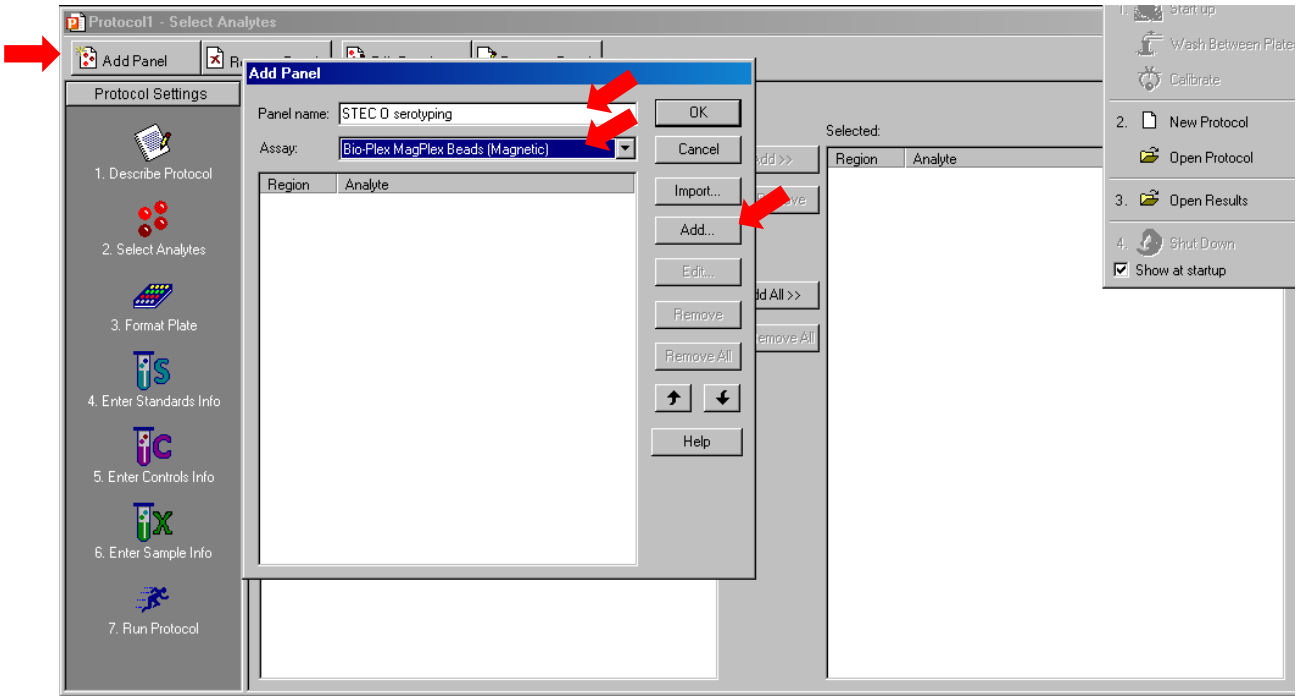
6.0 Bio-plex Analysis

Supplies Needed:
Bio-plex 200

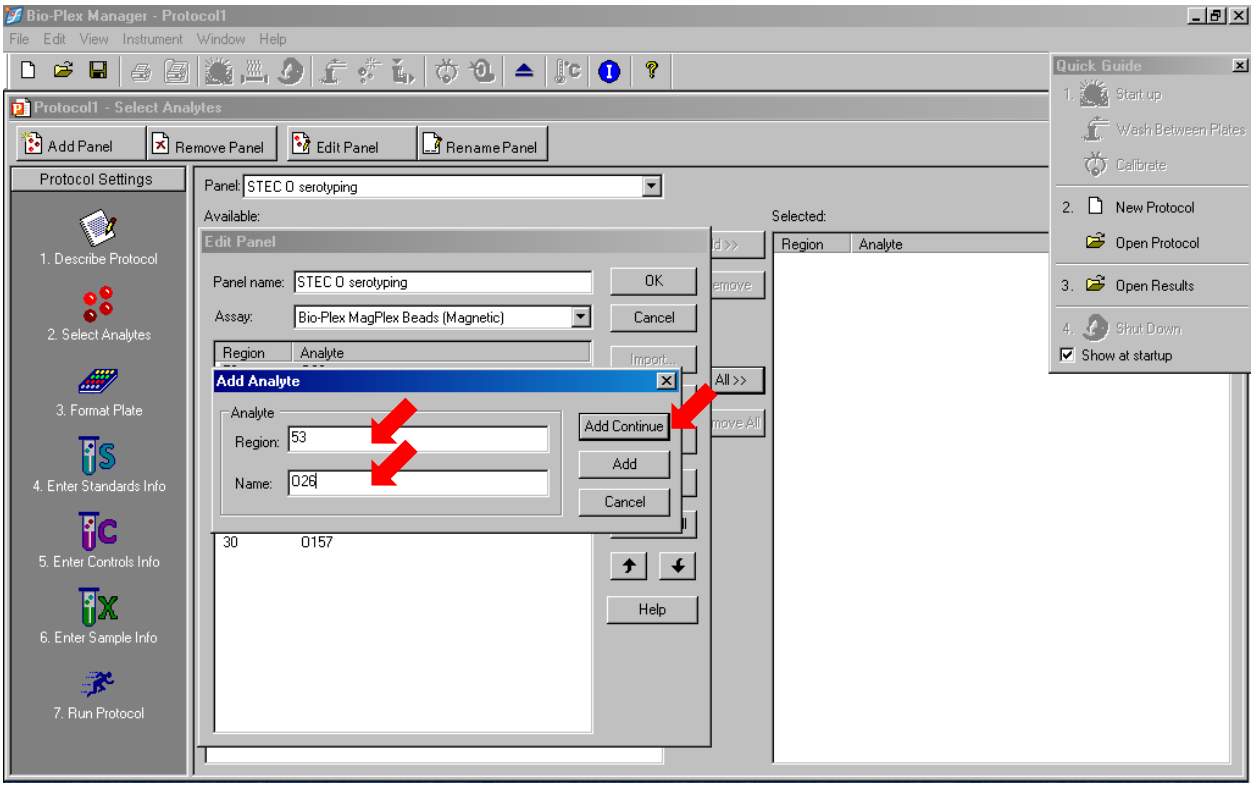
From the Bio-plex Quick Guide click “New Protocol”. Alternatively, from the File menu, select “New Protocol”. Once STEC protocol is programmed and saved, protocol can be opened by selecting open protocol. The protocol files have the orange icon and *.pbx extension.

6.1 Describe Protocol is the window the protocols open in. It can be used to add notes and comments to a protocol/run.

6.2 Select Analytes is used to identify the bead sets that will be used in the assay. From the <Select Analytes> window, click “Add Panel”



In the <Add Panel> window, fill in “STEC O serotyping” for panel name, and select “BioPlex MagPlex Beads (Magnetic) for Assay. Click <Add> to open the <Add Analyte> window.

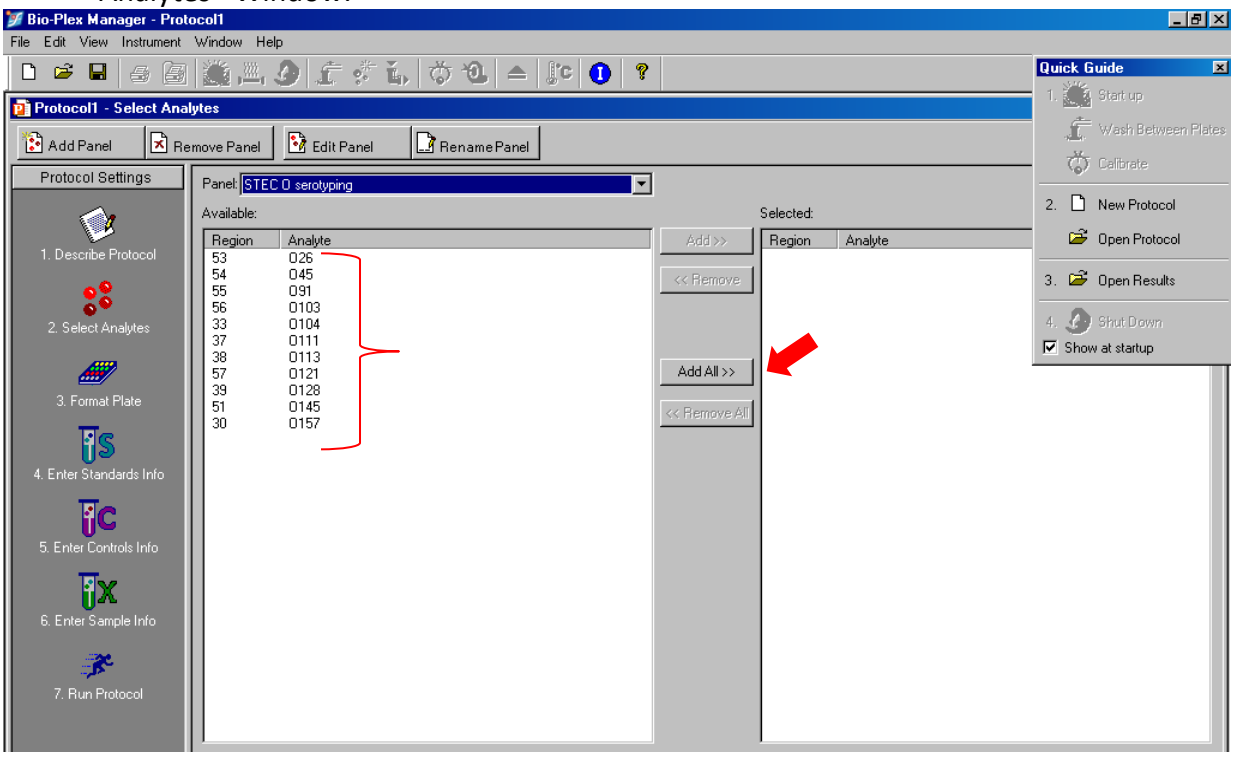


From the <Add Analyte> window, fill in correct bead region number with corresponding O serogroup. Click <Add Continue> until all 11 bead regions and O serogroups are entered. Fill out bead regions and O serogroup as follows:

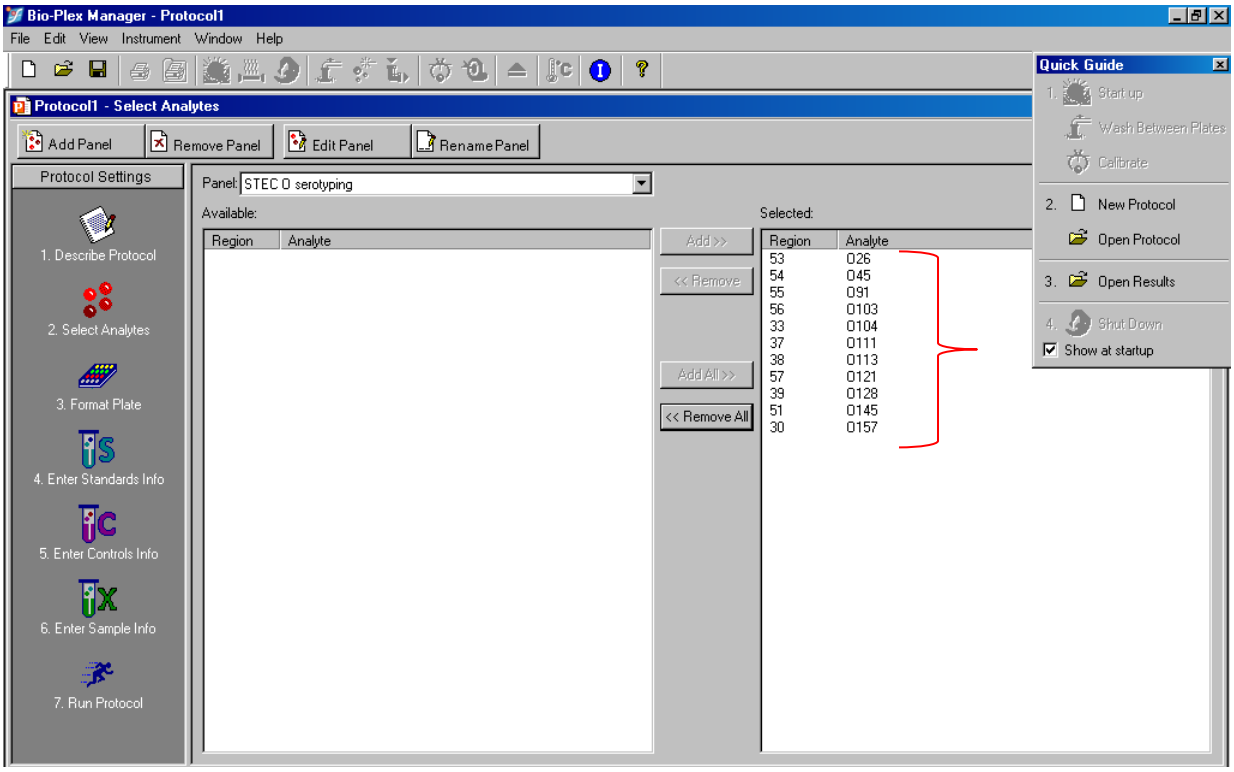
Bead Region	Target
53	O26
54	O45
55	O91
56	O103
33	O104
37	O111
38	O113
57	O121

39	O128
51	O145
30	O157

Eleven Bead Regions and Targets should appear in the Available menu of the <Select Analytes> Window:



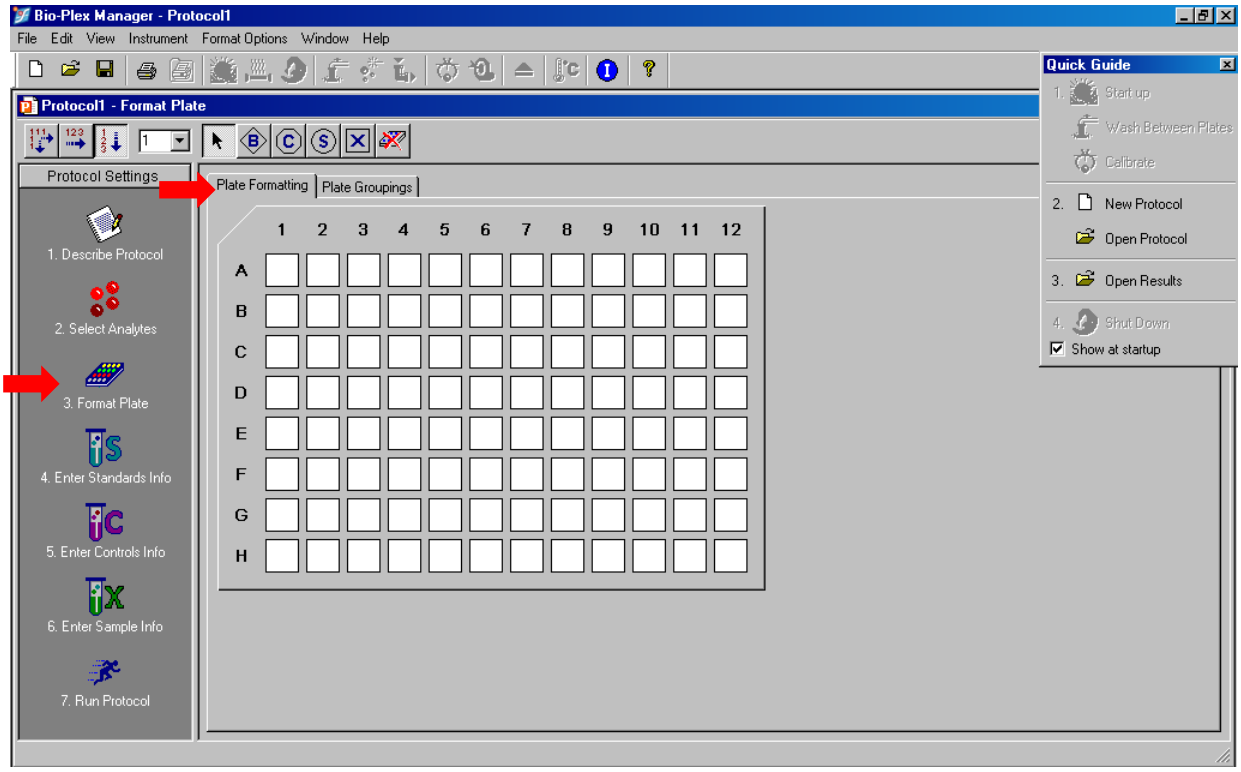
Click on <Add All> to move all eleven Regions and Analytes to Selected menu:



Add analytes by numeric order by Serogroup (O26, O45 . . . O157), or click edit panel and use up and down arrows to arrange analytes.

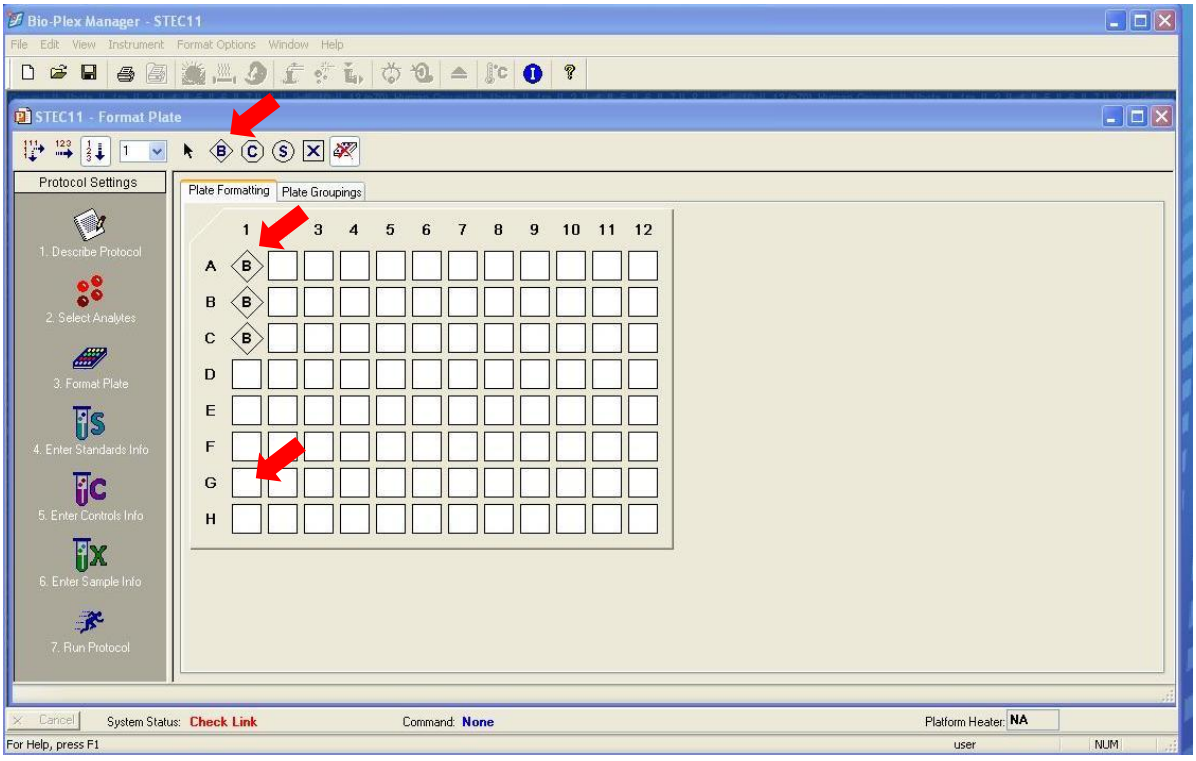
6.3 Format Plate tells the array reader which wells to read, and tells BM 6.0 how to analyze the different sample types in each well. Format Plate contains two views, the **Plate Formatting** view and the **Plate Groupings** view. **Plate Formatting** tools are used to define the types of wells in the plate (sample, control, standard, blank, etc.). **Plate Groupings** tools are used to organize the well types into groups, with one member of each group defined as the Reference member. The ratio of the fluorescent intensity of each well to the fluorescent intensity of the reference well is calculated. The ratio values are what determine if a sample is positive or negative.

Note: Plate formatting is required to perform a reading because the reader will only read formatted wells. Plate groupings can be defined before or after the reading.

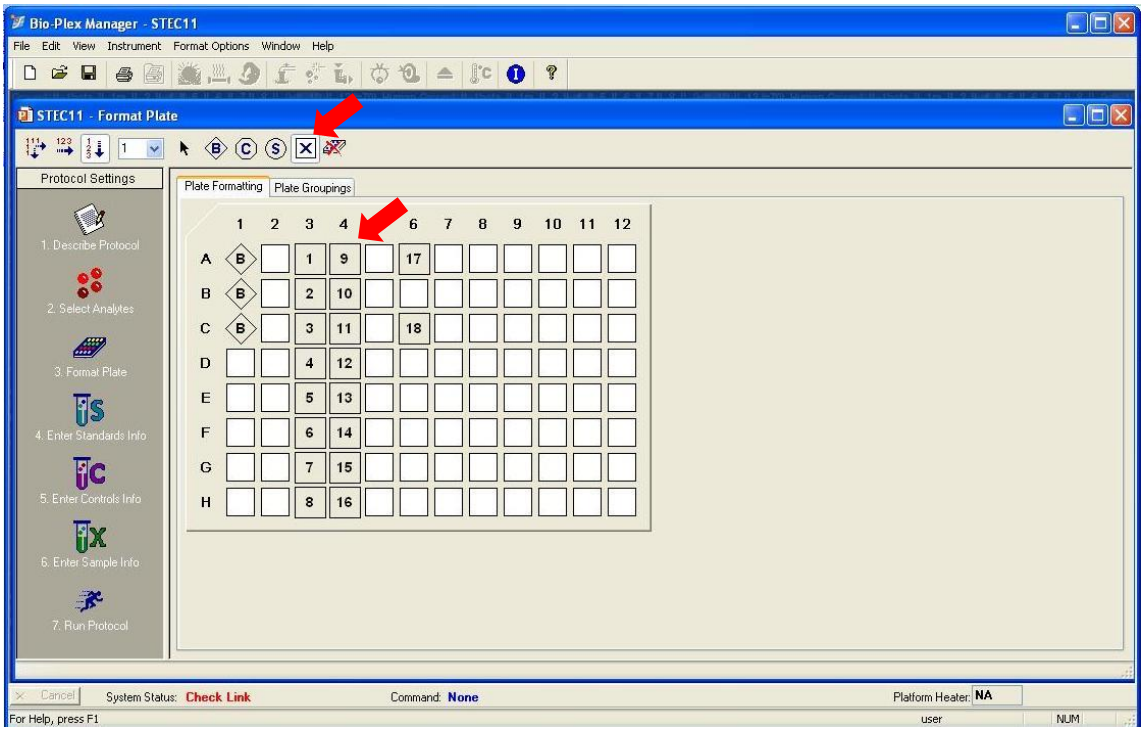


6.3.1 Plate Formatting

In the **Plate Formatting** tab, click the Blank button on the toolbar above the plate template, then drag cursor over the three wells where the no template controls will go.

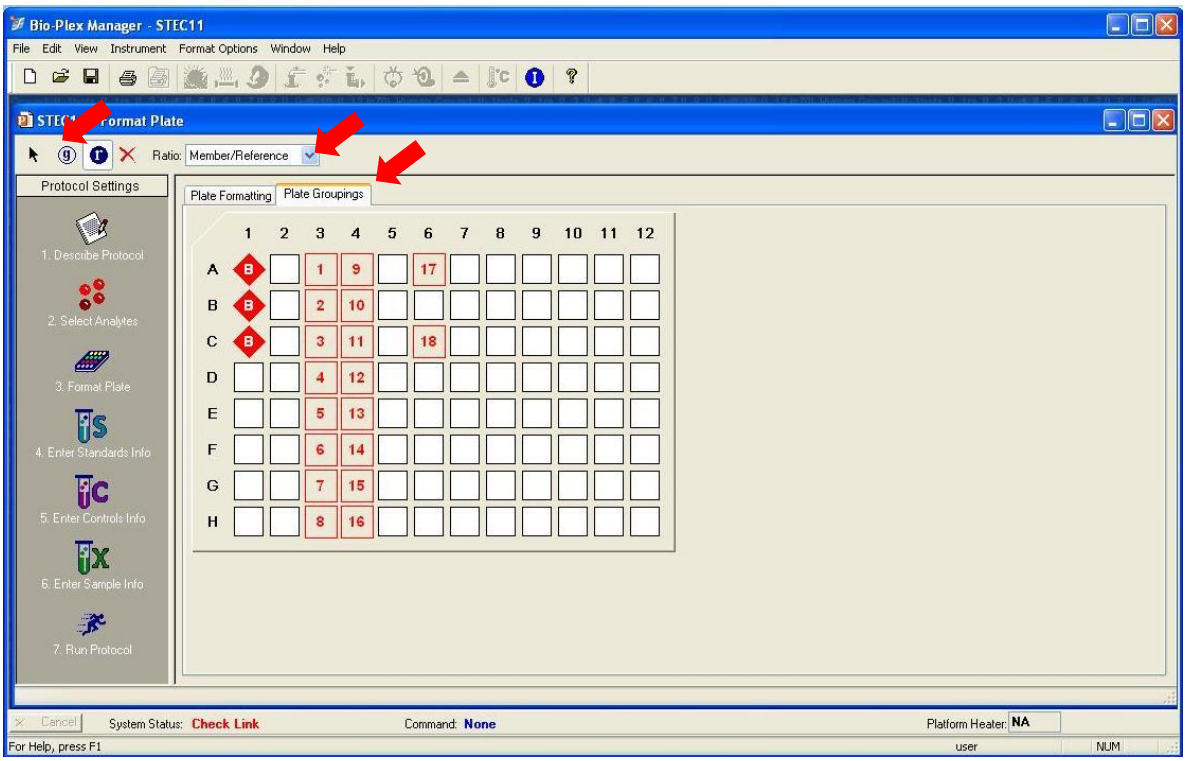


Click Unknown Sample button on the toolbar above the plate template, then click on the first well where samples will go and drag the cursor across the appropriate number of wells corresponding to samples in the run, including positive and negative controls. If you make a mistake, select the erase button and start again. The wells are read in columns from top to bottom (A1-H1; A2-H2 etc). Undefined wells will not be read by the sample reader.



6.3.2 Plate Groupings

1. In the **Plate Groupings** tab, click the **Group** button on the toolbar above the plate template, then click on the first formatted well and drag the cursor across the appropriate number of wells corresponding to samples in the run (including the no template control wells). The selected wells should all now be the same color. If you make a mistake, select the erase button and start again.



2. The desired reference member will always be the three no template controls ( ), which serve as blanks in the Bio-Plex assay. The reference wells will appear a solid color on the plate template. If you make a mistake, select the erase button and start again. If you place your no template control in a different well, you can select that well as the reference by clicking the **Reference** button , then click on the desired reference member in the group.

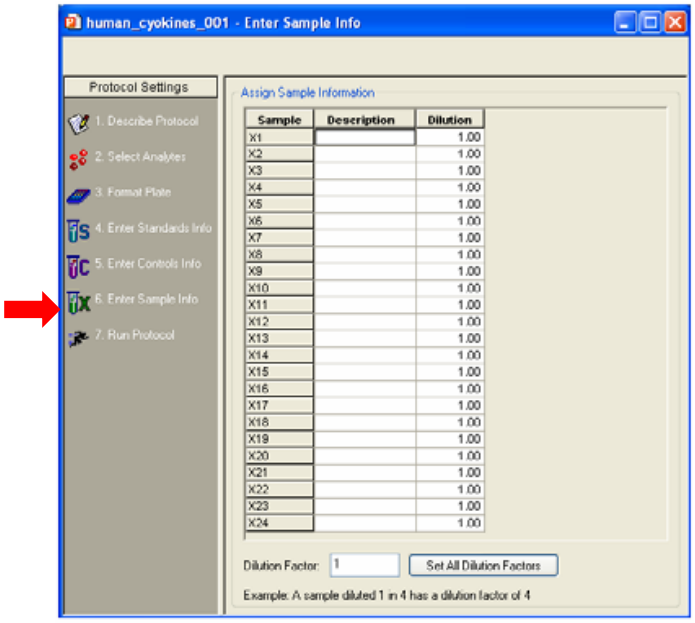
3. In the **Ratio** pull-down window, select **Member/Reference**.

6.4 Enter Standards is used for entering standards for titration curve analysis. This will **not** be used in the serotyping protocol.

6.5 Enter Controls is used for titration curve analysis. This will **not** be used in the serotyping protocol.

6.6 Enter Samples Info is used for sample identification and other identifying information. The number of samples that appear in this window will correlate with the

number of samples you input when you formatted the plate. The isolate identifiers will appear in the **Description** column of the data output.

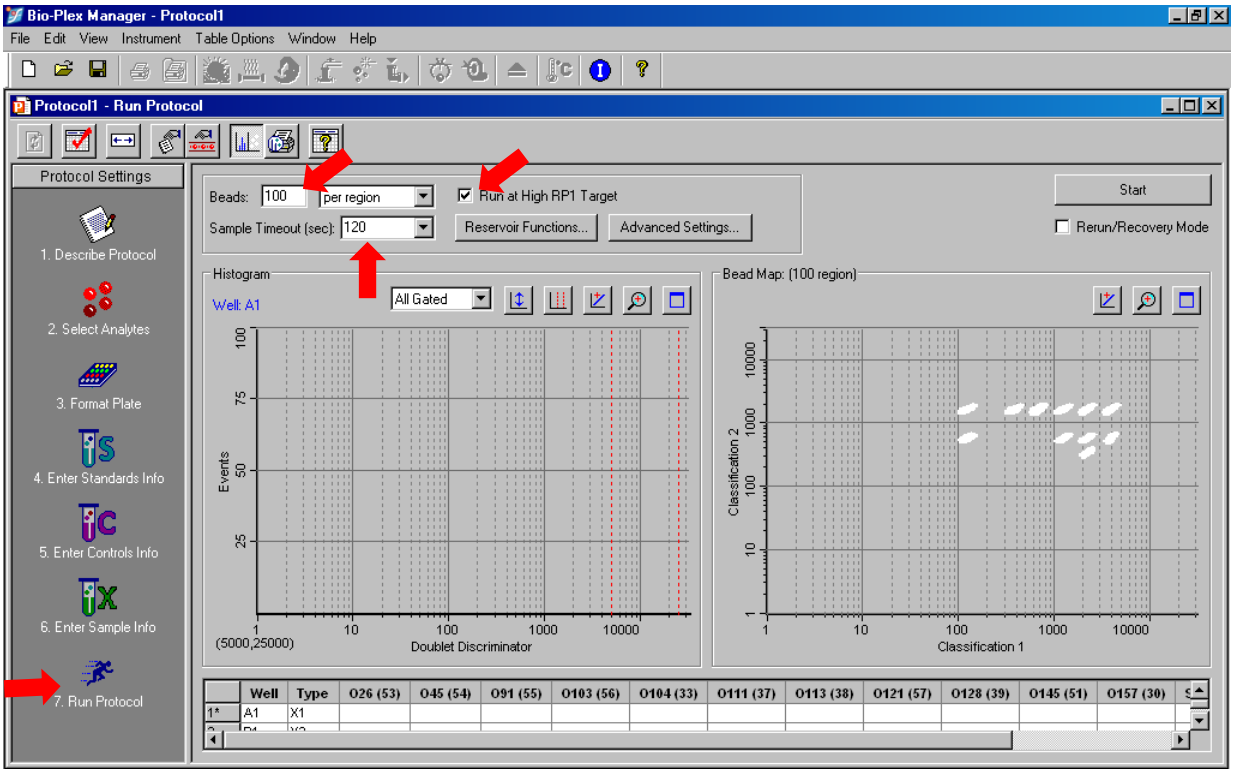


You can enter sample identification manually, or you can use the **Copy** and **Paste** commands on the **Edit** menu to copy the sample descriptions from an excel file.

6.7 Platform Heater Turn on Platform heater by clicking  button from the toolbar. Check boxes marked “Turn Heater On” and “Turn Heater off after run”. Set target temp to 52°C. When target temperature is reached, status bar will display temperature in green.

6.8 Run Protocol Settings Before the run is started, the **Run Protocol** settings must be set. The settings can be made at the time the protocol is set up, or just before the run is started using the **Run Protocol** button 

Set **Beads** to 100 beads per region.
Check the box **Run at High RP1 Target**.
Set **Sample Timeout** to 120 seconds.



The **Run Protocol** window has controls for performing the run, and two views for displaying the status of the run: the **Raw Data table** view and the **histogram/bead map** view. Click the **Show/Hide Histogram/Bead Map** button to display only the Raw Data table, or the histogram/bead map display with the Raw Data table below it (maximize the window to see both the table and the display).

After all of the parameters have been set, click the save icon to save the changed to the protocol.

Protocol Running Guidelines. Before starting the run, note the following guidelines and warnings:

- Protect the assay beads from light. Once photobleached, the beads are no longer usable.

- Make sure there is at least 75µL of sample in all the wells specified in the plate template before starting a reading. If the array reader attempts to draw sample from an empty well, air will enter the line, resulting in bubble formation and interference with analysis.

6.9 Starting a Bio-Plex Run

After all of the protocol options have been selected and reviewed, and the hybridization plate has warmed for 5 minutes at 52^{o}C in the Bio-Plex reader it will be time to start the run.

Select the **Start** button in the upper right corner of the **Run Protocol** view.

A **Save As** box will appear to name the assay.
The **Run Protocol** dialog box will appear.
Enter your name or initials in the User Name field. **Enter the plate ID**: DO NOT LEAVE THE PLATE ID BLANK. Click **OK**.

The array reader will begin to read the plate. Results for each sample will show in the raw data table as soon as it has been read.

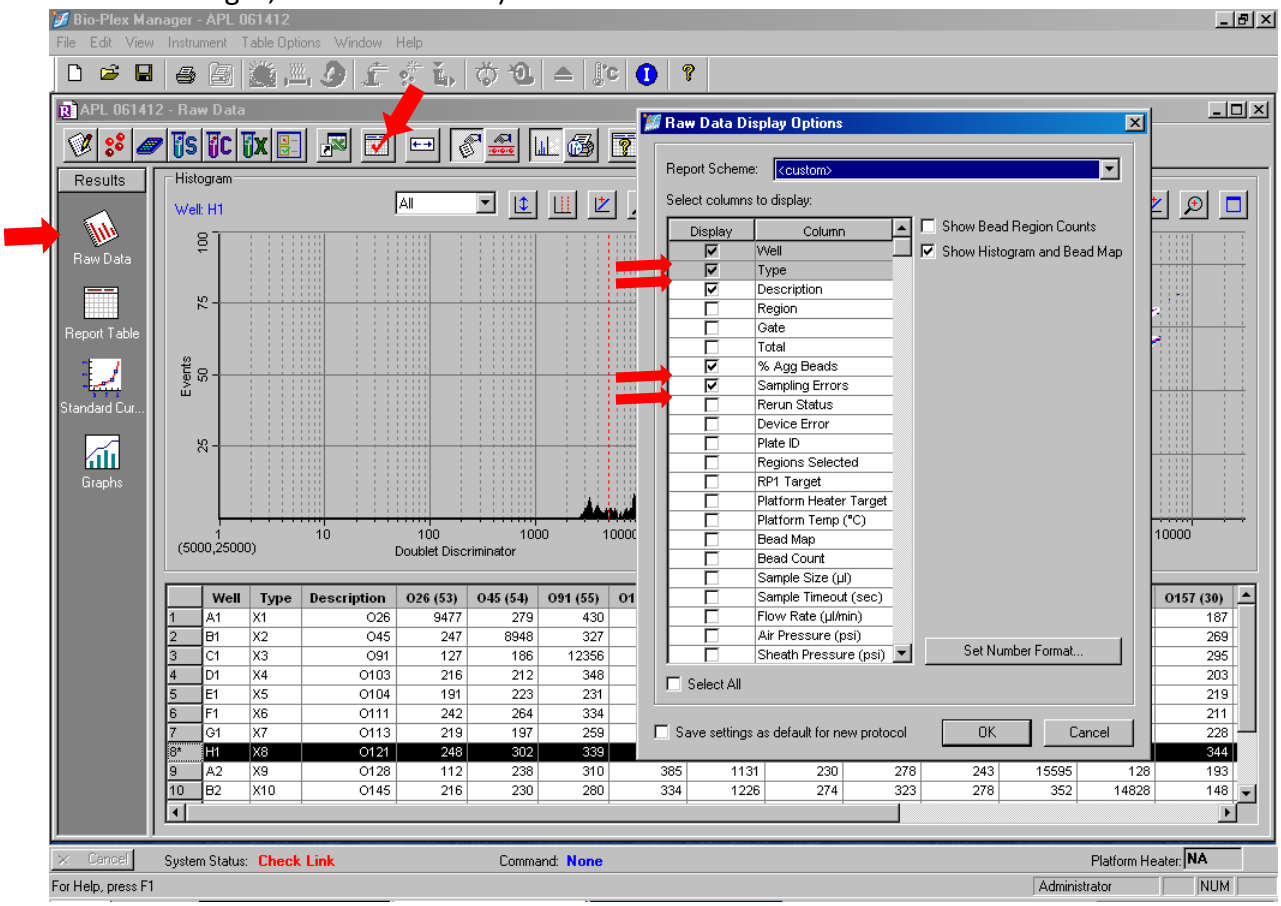
7.0 Data Analysis

Supplies Needed

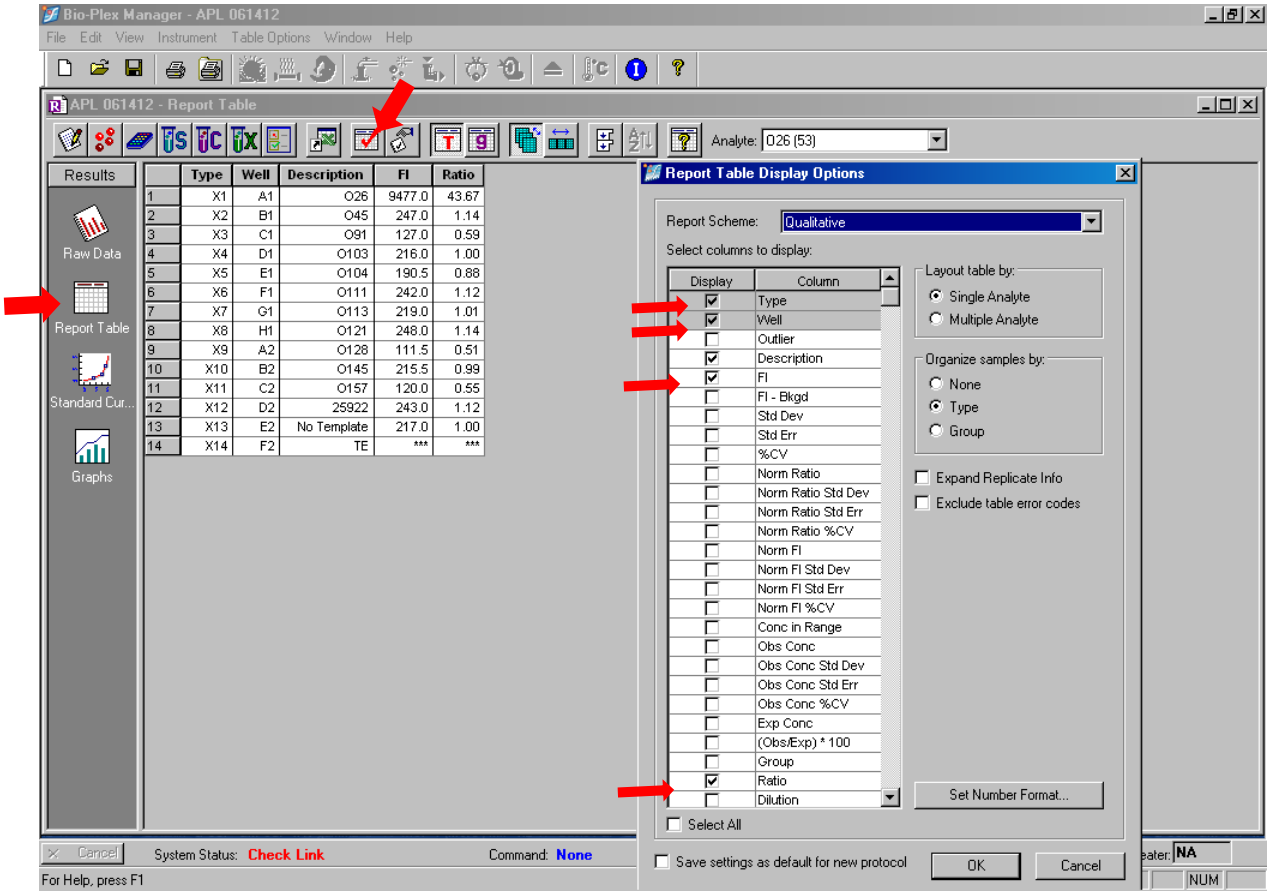
Bio-plex 200

Microsoft Excel

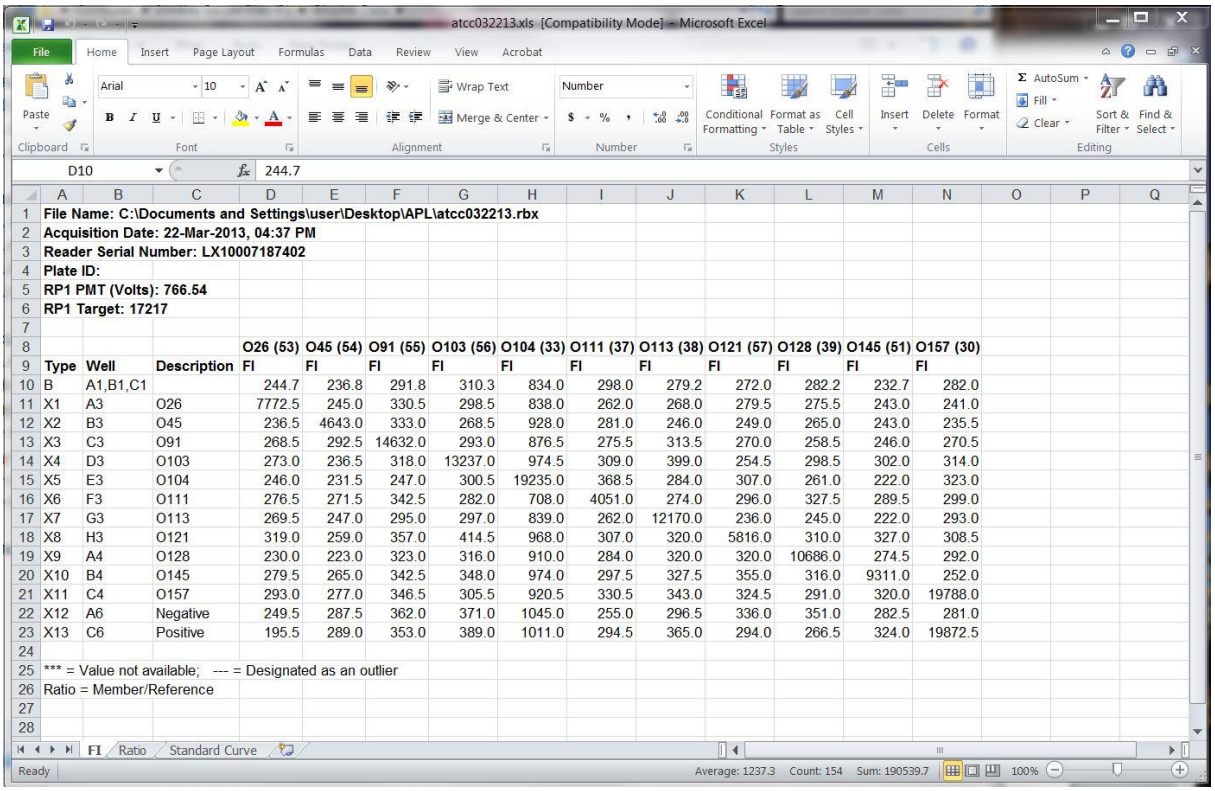
In the Raw Data display, click the Raw Data Display options from the toolbar. In the Report Scheme Box, select <Custom>. Under Display, check boxes for Well, Type, Description, % Agg Beads, Sampling Errors, and check “Show Bead Region Counts” on right side. Use scroll bar to view potential problems (% Agg Beads > 20; Sampling Error messages, low bead counts).



In the Report Table Display, click the Report Table Display Options button on the toolbar. From the Report Table Display Options Window, select Report Scheme <Qualitative>, under Display, check boxes for Well, Type, Description, F1, and Ratio. Uncheck all other boxes.



To Export Data, click Export to Excel button  on toolbar. Under "Table Export options" select <Multiple Analyte Layout>. Excel file will open displaying Median Fluorescent Intensities (MFI):



atcc032213.xls [Compatibility Mode] - Microsoft Excel

File Home Insert Page Layout Formulas Data Review View Acrobat

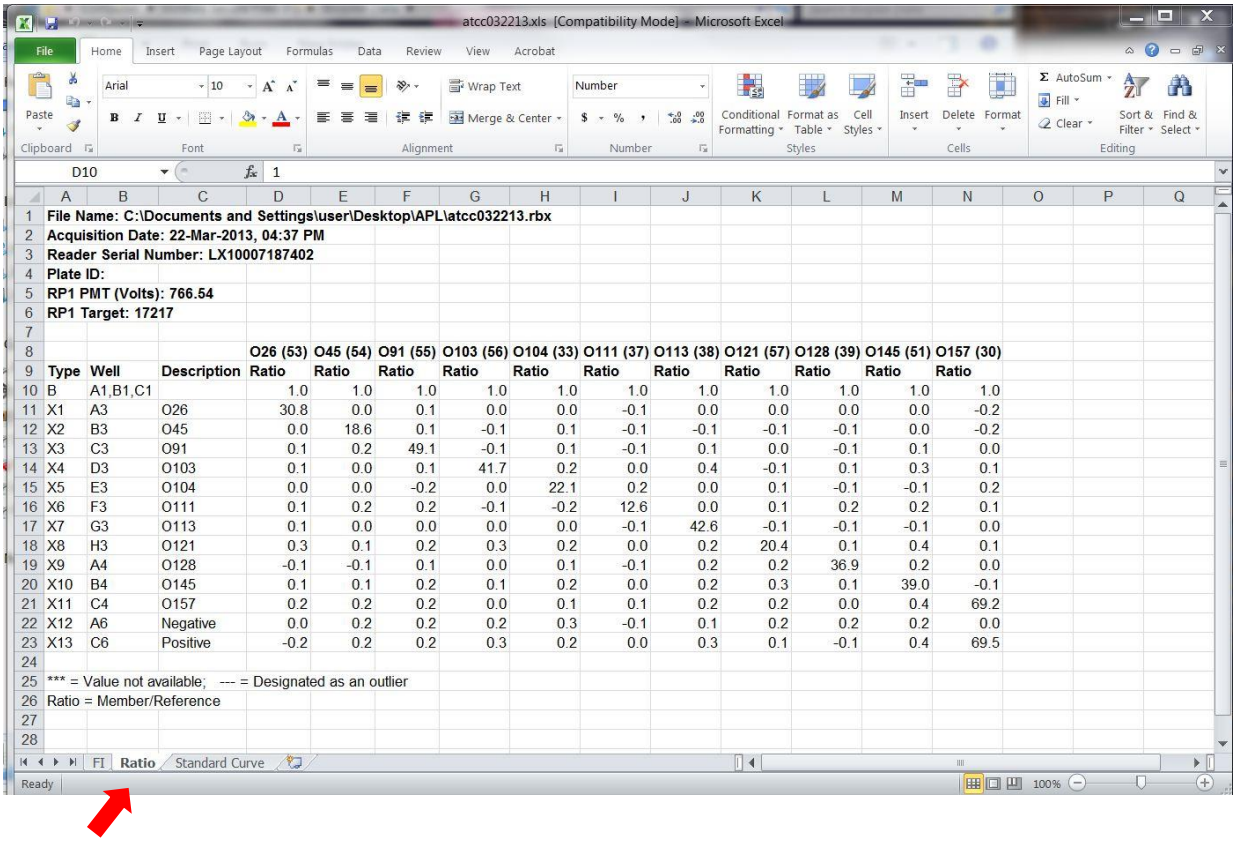
D10 244.7

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	**File Name: C:\Documents and Settings\user\Desktop\APL\atcc032213.rbx**																
2	**Acquisition Date: 22-Mar-2013, 04:37 PM**																
3	**Reader Serial Number: LX10007187402**																
4	**Plate ID:**																
5	**RP1 PMT (Volts): 766.54**																
6	**RP1 Target: 17217**																
7																	
8				**O26 (53)**	**O45 (54)**	**O91 (55)**	**O103 (56)**	**O104 (33)**	**O111 (37)**	**O113 (38)**	**O121 (57)**	**O128 (39)**	**O145 (51)**	**O157 (30)**			
9	**Type**	**Well**	**Description**	**FI**	**FI**	**FI**	**FI**	**FI**	**FI**	**FI**	**FI**	**FI**	**FI**	**FI**			
10	B	A1,B1,C1		244.7	236.8	291.8	310.3	834.0	298.0	279.2	272.0	282.2	232.7	282.0			
11	X1	A3	O26	7772.5	245.0	330.5	298.5	838.0	262.0	268.0	279.5	275.5	243.0	241.0			
12	X2	B3	O45	236.5	4643.0	333.0	268.5	928.0	281.0	246.0	249.0	265.0	243.0	235.5			
13	X3	C3	O91	268.5	292.5	14632.0	293.0	876.5	275.5	313.5	270.0	258.5	246.0	270.5			
14	X4	D3	O103	273.0	236.5	318.0	13237.0	974.5	309.0	399.0	254.5	298.5	302.0	314.0			
15	X5	E3	O104	246.0	231.5	247.0	300.5	19235.0	368.5	284.0	307.0	261.0	222.0	323.0			
16	X6	F3	O111	276.5	271.5	342.5	282.0	708.0	4051.0	274.0	296.0	327.5	289.5	299.0			
17	X7	G3	O113	269.5	247.0	295.0	297.0	839.0	262.0	12170.0	236.0	245.0	222.0	293.0			
18	X8	H3	O121	319.0	259.0	357.0	414.5	968.0	307.0	320.0	5816.0	310.0	327.0	308.5			
19	X9	A4	O128	230.0	223.0	323.0	316.0	910.0	284.0	320.0	320.0	10686.0	274.5	292.0			
20	X10	B4	O145	279.5	265.0	342.5	348.0	974.0	297.5	327.5	355.0	316.0	9311.0	252.0			
21	X11	C4	O157	293.0	277.0	346.5	305.5	920.5	330.5	343.0	324.5	291.0	320.0	19788.0			
22	X12	A6	Negative	249.5	287.5	362.0	371.0	1045.0	255.0	296.5	336.0	351.0	282.5	281.0			
23	X13	C6	Positive	195.5	289.0	353.0	389.0	1011.0	294.5	365.0	294.0	266.5	324.0	19872.5			
24																	
25	*** = Value not available; --- = Designated as an outlier																
26	Ratio = Member/Reference																
27																	
28																	

FI Ratio Standard Curve

Ready Average: 1237.3 Count: 154 Sum: 190539.7 100%

Click on Ratio Tab to display ratio of sample: no template control



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	File Name: C:\Documents and Settings\user\Desktop\APL\atcc032213.rbx																
2	Acquisition Date: 22-Mar-2013, 04:37 PM																
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4	Plate ID:																
5	RP1 PMT (Volts): 766.54																
6	RP1 Target: 17217																
7																	
8				O26 (53)	O45 (54)	O91 (55)	O103 (56)	O104 (33)	O111 (37)	O113 (38)	O121 (57)	O128 (39)	O145 (51)	O157 (30)			
9	Type	Well	Description	Ratio	Ratio	Ratio	Ratio	Ratio	Ratio	Ratio	Ratio	Ratio	Ratio	Ratio			
10	B	A1,B1,C1		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			
11	X1	A3	O26	30.8	0.0	0.1	0.0	0.0	-0.1	0.0	0.0	0.0	0.0	-0.2			
12	X2	B3	O45	0.0	18.6	0.1	-0.1	0.1	-0.1	-0.1	-0.1	-0.1	0.0	-0.2			
13	X3	C3	O91	0.1	0.2	49.1	-0.1	0.1	-0.1	0.1	0.0	-0.1	0.1	0.0			
14	X4	D3	O103	0.1	0.0	0.1	41.7	0.2	0.0	0.4	-0.1	0.1	0.3	0.1			
15	X5	E3	O104	0.0	0.0	-0.2	0.0	22.1	0.2	0.0	0.1	-0.1	-0.1	0.2			
16	X6	F3	O111	0.1	0.2	0.2	-0.1	-0.2	12.6	0.0	0.1	0.2	0.2	0.1			
17	X7	G3	O113	0.1	0.0	0.0	0.0	0.0	-0.1	42.6	-0.1	-0.1	-0.1	0.0			
18	X8	H3	O121	0.3	0.1	0.2	0.3	0.2	0.0	0.2	20.4	0.1	0.4	0.1			
19	X9	A4	O128	-0.1	-0.1	0.1	0.0	0.1	-0.1	0.2	0.2	36.9	0.2	0.0			
20	X10	B4	O145	0.1	0.1	0.2	0.1	0.2	0.0	0.2	0.3	0.1	39.0	-0.1			
21	X11	C4	O157	0.2	0.2	0.2	0.0	0.1	0.1	0.2	0.2	0.0	0.4	69.2			
22	X12	A6	Negative	0.0	0.2	0.2	0.2	0.3	-0.1	0.1	0.2	0.2	0.2	0.0			
23	X13	C6	Positive	-0.2	0.2	0.2	0.3	0.2	0.0	0.3	0.1	-0.1	0.4	69.5			
24																	
25	*** = Value not available; --- = Designated as an outlier																
26	Ratio = Member/Reference																
27																	
28																	

A Ratio of > 5.0 is considered positive for that analyte. Report O serogroups of each sample or "-" for unidentified samples.

Appendix A: Calculation Worksheet

Example of calculations for preparation of reagents. # of reactions to prepare for should be at least number of reactions needed (# of samples + 3 no template controls + positive and negative control) *1.1 (for extra)

PCR Master Mix Set Up				
1x	Reagents	20x	50x	100x
1.62µL	STEC Primer Mix	32.4µL	81µL	162µL
12.5µL	HotstarTaq Master Mix	250µL	625µL	1250µL
8.88µL	Nuclease Free Water	177.6µL	444µL	888µL
23µL	Total	460µL	1150µL	2300µL
2µL	Template DNA			
STEC Bead Hybridization Reaction				
1x	Reagents	20x	50x	100x
0.33µL	STEC Bead Pool 100x	6.6µL	16.5µL	33µL
32.67µL	1.5X TMAC	653.4µL	1633.5µL	3267µL
12µL	TE	240µL	600µL	1200µL
45µL	Total	900µL	2250µL	4500µL
5µL	PCR Reaction			
Reporter				
1x	Reagents	20x	50x	100x
0.3µL	SAPE 1mg/mL	6µL	15µL	30µL
74.7µL	1.0X TMAC	1494µL	3735µL	7470µL
75µL	Total	1500µL	3750µL	7500µL

Appendix B: Supplies Needed

- Sterile microcentrifuge tubes
- Sterile loops or needles
- TSA or TSA-YE (as described in BAM M152 and M153 respectively)
- Hotstar Taq Master Mix (Qiagen #203443 or 203445)
- Nuclease free water (Ambion AM9937)
- Quickstrip tubes with caps (Phenix; Fisher NC9909941)
- Conventional thermocycler (such as ABI GenAmp 9700)
- Low profile 96-well microplates (BioRad MLL-9601).
- Microseal "A" adhesive film (MSA-5001) or equivalent plate sealer.
- Strepavidin-R-Phycoerythrin (SAPE) 1 mg/ml, 1 ml (Invitrogen S-866), stored in the dark at 2-8^{o}C.
- 1.5 X TMAC (Appendix 3)
- 1.0 X TMAC (Appendix 3)
- 1 X TE, pH 8.0 (Appendix 3)
- Sonicating water bath (Fisher 15-337-22)
- LifeSep 96F Magnetic Separation Unit (2501008-1)
- VWR Modular Heat Block for Titer Plates (13259-295)
- STEC Primer Mix, stored at -20^{o}C
- STEC Bead pool 100x (stored in the dark at 2-8^{o}C, DO NOT FREEZE)
- Control DNA extracted from ATCC strains stored at -20^{o}C

Suggested ATCC control cultures

O serogroups	ATCC number
O26	BAA2196*
O45	BAA 2193*
O91	51435*
O103	BAA 2215*
O104	BAA 2326*
O111	BAA 2440*
O121	BAA 2219*
O128	BAA 1704*
O145	BAA 2192*
O157	43894 or 43895*†
-	25922*†

* Test for QC of reagents

† Test with every sample analysis

Appendix C: Molecular Serotyping Reagent Recipes

Table: Primers and probes Sequences

O serogroup	Gene	Sequence	gene position (bp)
O26	*wzx*	ttttatctggcgtgctatcg	557-577
		Biotin-cggggttgctatagactgaa	784-804*
		Uni-link-tggcactcttgcttcgcctg	720-740
O45	*wzy*	tacgatttcacaagcttcca	769-789
		Biotin-tgcaatcgcataaggaaata	1003-1023*
		Uni-link-tcgcgggctcccttattgtg	917-937
O91	*wzx*	catgctgctcattcttctca	266-286
		Biotin-tggagtttgcaacaaacaaa	380-400*
		Uni-link-aaatggtttgctgcgacgct	358-378
O103	*wzx*	gggcttgtattgttgtaccg	896-916
		Biotin-agtggcaaacagccaactac	1045-1065*
		Uni-link-tcggggattttctgcggatt	1025-1045
O104	*wzx*	tgcgggattaatatcctttg	591-611
		Biotin-acgccctagaaacctgactt	854-874*
		Uni-link-cgcaggttttattgtcgcgc	780-800
O111	*wzx*	caatccaatttgcatcttca	75-95
		Biotin-accgcaaatgcgataataac	294-314*
		Uni-link-tggaggatgttccgcatgga	189-209
O113	*wzx*	tgaccttacttcctgcgaat	752-772
		Biotin-agcaccacgataggattgaa	977-997*
		Uni-link-cctgggaggaggctgcaaaa	953-973
O121	*wzy*	tggatggcattcctcagtat	809-829
		Biotin-agcaagccaaaacactcaac	1043-1063*
		Uni-link-ttaacacgggcgtggttgga	920-940
O128	*wzx*	tcgatcgtcttgttcaggtt	1123-1143
		Biotin-gaatgcaatgggcaattaac	1298-1318*
		Uni-link-gggttgcacaattggcctcc	1184-1204
O145	*wzy*	tgttcctgtctgttgcttca	224-244
		Biotin-atcgctgaataagcaccact	495-515*
		Uni-link-tgggctgccactgatgggat	442-462
O157	*wzx*	ataatccagccagcaaagtg	1026-1046
		Biotin-ggtgctgctctgacattttt	1141-1161*
		Uni-link-gcccaccactaatttgccga	1047-1067

***** Reverse compliment

5' biotinylated primers should be ordered HPLC purified. All primers should be rehydrated to 100µM in nuclease free water. A primer mix with all primers is made as follows:

Primer Mix for 100 reactions

Primer	amount of 100µM stock to use in Primer Mix	Primer Mix Stock Concentration	Final Concentration (1.62µL stock/25µL Reaction)
O26F	3 µL	1.85µM	120nM
BiotinO26R	15 µL	9.26µM	600nM
O45F	2 µL	1.23µM	80nM
BiotinO45R	10 µL	6.15µM	400nM
O91F	2 µL	1.23µM	80nM
BiotinO91R	10 µL	6.15µM	400nM
O103F	2 µL	1.23µM	80nM
BiotinO103R	10 µL	6.15µM	400nM
O111F	5 µL	3.09µM	200nM
BiotinO111R	25 µL	15.43µM	1000nM
O113F	3 µL	1.85µM	120nM
BiotinO113R	15 µL	9.26µM	600nM
O121F	2 µL	1.23µM	80nM
BiotinO121R	10 µL	6.15µM	400nM
O128F	2 µL	1.23µM	80nM
BiotinO128R	10 µL	6.15µM	400nM
O145F	2 µL	1.23µM	80nM
BiotinO145R	10 µL	6.15µM	400nM
O157F	2 µL	1.23µM	80nM
BiotinO157R	10 µL	6.15µM	400nM
O104F	2 µL	1.23µM	80nM
BiotinO104R	10 µL	6.15µM	400nM

100x STEC Bead Mix

Purchase probe sequences described above conjugated to MagPlex C microspheres of the following bead regions (Radix Biosolutions, Georgetown, TX):

MagPlex C Bead Region	Target
53	O26
54	O45
55	O91
56	O103
33	O104
37	O111
38	O113
57	O121
39	O128
51	O145
30	O157

To make 100x concentration STEC Bead Pool, sonicate and vortex each $1x10^7$ bead/mL conjugated microsphere stock for 20 seconds Add 100µL of each microsphere stock to single eppendorf tube. Place eppendorf tube on a 1.5mL magnetic particle concentrator and allow beads to collect 1 minute. Remove liquid and resuspend in 100µL of 1x TE. Store Microspheres 2-8ºC protected from light.

1x TE (Tris-EDTA)

Reagent	Catalog Number	Final concentration	Amount/100 mL
Tris-EDTA Buffer, pH 8.0, 100X	Sigma T-9285-100ml	1 X	1.0 mL
Distilled Deionized Water	------	------	99.0 mL

Filter sterilize and store at room temperature, pH should be 8.0±0.2.

Recommend pipetting 1.0 mL into graduated cylinder, bring to 100 mL with DDI water, then filter sterilize.

1.5X TMAC Hybridization Solution (Bead Diluent)

Reagent	Catalog Number	Final concentration	Amount/250mL
5 M TMAC	Sigma T-3411-1L	4.5 M	225 mL
20% Sarkosyl	Sigma L7414	0.15%	1.88 mL
1 M Tris-HCl, pH 8.0	Sigma T-3038-1L	75 mM	18.75 mL
0.5 M EDTA, pH 8.0	Sigma 03690-100ml	6 mM	3.0 mL
Distilled Deionized Water	--------	---------	1.37 mL

Store at room temperature

Handle 5 M TMAC under a fume hood. Recommend measuring 225 mL TMAC into a 250 mL graduated cylinder under the fume, then pipette appropriate volumes of Sarkosyl, Tris, and EDTA using disposable pipettes. Bring to 250 mL with DDI water.

1.0X TMAC Hybridization Solution (Detection Buffer)

Reagent	Catalog Number	Final concentration	Amount/250mL
5 M TMAC	Sigma T-3411-1L	3 M	150 mL
20% Sarkosyl	Sigma L7414	0.1%	1.25 mL
1 M Tris-HCl, pH 8.0	Sigma T-3038-1L	50 mM	12.5 mL
0.5 M EDTA, pH 8.0	Sigma 03690-100ml	4 mM	2.0 mL
Bovine Serum Albumin	Sigma A9418	0.2%	0.5g
Distilled Deionized Water	--------	---------	84.25 mL

Store at room temperature

Handle TMAC under a fume hood. Recommend measuring 150 mL TMAC into a 250 mL graduated cylinder under the fume hood, then pipette appropriate volumes of Sarkosyl, Tris, and EDTA using disposable pipettes. Bring to 250 mL with DDI water.

Shelf Life: CDC recommends a shelf life of 6 months for the above working solutions. Follow the expiration dates on labels or the laboratory QMS for individual components of the working solutions.

Appendix G: Bio-plex Maintenance Log

Bio-Plex Maintenance Log		Month(s):																							
Daily Maintenance Tasks (performed every time the instrument is turned on)	**Date**																								
	Frequency	Initials: (for each task, initial in appropriate column after you perform the task)																							
Instrument optics warm-up (30 minutes).	Before each run																								
Check sheath fluid																									
Check waste level																									
Perform Start Up (can be done during warm-up)																									
Calibration (after 30 min optics warm up)																									
Shut Down																									
Turn Systems Off																									
Weekly Maintenance Tasks (performed weekly if instrument is used daily)	**Date**																								
	Frequency	Initials (for each task, initial in appropriate column after you perform the task)																							
Clean and sonicate sample needle	Weekly or as needed																								
Check for leaks																									
Restart computer and instrument																									
Clean surface of instrument																									
Monthly Maintenance Tasks (performed at least once per month)	**Frequency**	Date	Initials	high or low	Date	Initials	high or low	Date	Initials	high or low	Date	Initials	high or low												
Validation *optics*	At least monthly, or as needed																								
fluidics																									
reporter																									
classify																									
Every 6 months	**Frequency**	Date			Initials			Date			Initials														
Replace syringe seal (171-002033)	At least every 6 months, or as needed																								
Clean ventilation filter																									
Yearly	**Frequency**	Date			Initials			Date			Initials														
Replace sheath filter (171-002038)	At least yearly, or as needed.																								
Replace air intake filter (171-002032)																									
As required	**Frequency**	Date			Initials			Date			Initials														
Replace fuse	As needed.																								
Unclog																									
Comments (e.g. calibration and Validation lot and expiration information.):																									

PRLNW ATC MMMoore 07/29/2010

Bio-Plex Maintenance Log		Month(s):																						
Daily Maintenance Tasks (performed every time the instrument is turned on)	**Date**																							
	Frequency	Initials: (for each task, initial in appropriate column after you perform the task)																						
Instrument optics warm-up (30 minutes).	Before each run																							
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Clean and sonicate sample needle	Weekly or as needed																							
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Clean surface of instrument																								

Monthly Maintenance Tasks (performed at least once per month)	**Frequency**	Date	Initials	high or low	Date	Initials	high or low	Date	Initials	high or low	Date	Initials	high or low
Validation *optics*	At least monthly, or as needed												
fluidics													
reporter													
classify													

Every 6 months	**Frequency**	Date	Initials	Date	Initials
Replace syringe seal (171-002033)	At least every 6 months, or as needed				
Clean ventilation filter					

Yearly	**Frequency**	Date	Initials	Date	Initials
Replace sheath filter (171-002038)	At least yearly, or as needed.				
Replace air intake filter (171-002032)					

As required	**Frequency**	Date	Initials	Date	Initials
Replace fuse	As needed.				
Unclog					

Comments (e.g. calibration and Validation lot and expiration information.):

PRLNW ATC MMMoore 07/29/2010