Title: Testing cheese samples for presence of STEC using classical and molecular techniques			
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Testing cheese samples for presence of STEC using classical and molecular techniques

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SECTION 1 INTRODUCTION

1.1 Purpose

To test cheese samples for presence of STEC using classical and molecular techniques.

This test follows the instructions set by FDA BAM (Diarrheagenic *Escherichia coli*) with the exception that different PCR primers are used for the final multiplex PCR. The PCR primers used in this SOP are part of the STEC multiplex PCR described in FDA BAM (see E. coli 6-gene multiplex PCR). With these PCR primers we were able to get 10 times lower detection limit than with PCR primers proposed by FDA. See the Troubleshoot section for PCR primers and PCR conditions to perform the multiplex PCR proposed by FDA BAM.

1.2 Scope

This SOP applies to the MQIP and the FSL.

1.3 Definitions

STEC: Shiga Toxin producing *E. coli*

Stx: Shiga Toxin (codded by *Stx1* and *Stx2*)

Slt: Shiga-like Toxin (codded by *SltI* and *SltII*). Equivalent to Stx (*Stx1* and *Stx2*) but often times referred to as Shiga-like toxin because the genes (*SltI* and *SltII*) are not in Shigella and the toxin is not produced by Shigella.

mBPWp: modified Buffered Peptone Water with added pyruvate

ACV: Three-part supplement: Acriflavin-Cefsulodin-Vancomycin

SMAC: MacConkey agar with added Sorbitol

L-EMB: Levine's Eosin-Methylene Blue agar

TBE: Tris/Borate/EDTA. Buffer used in electrophoresis.

EDTA: Ethylenediaminetetraacetic acid

mBPWp positive control: A positive control which is confirming the mBPWp enrichment is actually enriching the STEC present in the sample and giving a positive PCR result. This is performed by inoculating mBPWp with a STEC control strain.

mBPWp negative control: A negative control which is confirming that the process of anylsis is not introducing any outside contamination.

PCR matrix control: A positive control which is confirming that there is no inhibition of PCR reaction by components which are coming in to reaction with the mBPWp enrichment lysate.

PCR positive control: A positive control which is confirming the PCR reaction is working properly.

PCR negative control: A negative control which is confirming that process of seting up a PCR reaction is not introducing any outside contamination.

BLAST: Basic Local Alignment Search Tool. On-line tool for comparing and searching the nucleotide and amino acid sequences kept by NCBI.

NCBI: National Center for Biotechnology Information

SECTION 2 MATERIALS

- **An overnight culture (on BHI plate) of: FSL F6-0699 (*E. coli* O157:H7); this is our STEC positive control strain.**
- **Sterile stomacher bags**
- **Balance**
- **Sterile spoons**
- **Sterile 250 mL bottle or measuring cylinder**
- **Incubators 37°C/42°C**
- **Pipettes**
- **Sterile 1.5 mL Eppendorf tubes with plastic safety attachments**
- **0.2 mL PCR tubes**
- **Sterile Q-Tips**
- **Safety Glasses**
- **Mini bench-top centrifuge**
- **PCR cycler**
- **Electrophorese**
- **mBPWp:**

Peptone	10.0 g
NaCl	5.0 g
Na_2HPO_4	3.6 g
KH_2PO_4	1.5 g
Casamino acids	5.0 g
Yeast extract	6.0 g
Lactose	10.0 g
Sodium Pyruvate	1.0g
Distilled water	1000 ml (Use 500 ml for 2× strength)

pH to 7.2 ± 0.2

Note: commercial BPW (20 g per L) can be substituted for the first four ingredients.

Sterilize by autoclaving.

- **ACV:**

	conc. in mBPWp	**Conc. In stock**	**Amt. of stock/ 225mL**
Acriflavin	10mg/L	1.125 g/ 500 mL	1 mL
Cefsulodin	10mg/L	1.125 g/ 500 mL	1 mL
Vancomycin	8mg/L	0.90 g/ 500 mL	1 mL

Filter, sterilize.

- **PBS (Phosphate-Buffered Slaine)**

NaCl	7.650 g
Na_2HPO_4, anhydrous	0.724 g
KH_2PO_4	0.210 g
Distilled water	1 liter

Dissolve ingredients in distilled water. Adjust pH to 7.2 (with 1 N NaOH). Autoclave 15 min at 121°C.

- **TBE buffer**

5x Stock Solution

Water	1 L
Tris Base	54 g
Boric acid	27.5 g
EDTA (0.5M)	20 mL

Dissolve ingredients in distilled water. Adjust pH to 8.3 (with 1 N HCl). Make a 10-times dilution to use in the electrophoresis.

- **GoTaq PCR Kit**
- **Qiagen DNA extraction kit**
- **Slt1 and Slt2 PCR primers**

sltI-F (10 µM)	TgTAACTggAAAggTggAgTATAC
sltI-R (10 µM)	gCTATTCTgAgTCAACgAAAAATAAC
sltII-F (10 µM)	gTTTTTCTTCggTATCCTATTCCg
sltII-R (10 µM)	gATgCATCTCTggTCATTgTATTAC

- **Agarose gel**
- **Sterile glass or plastic hockey spreader**
- **SMAC plates**
- **L-EMB plates**
- **BHI plates**
- **Sterile toothpicks**

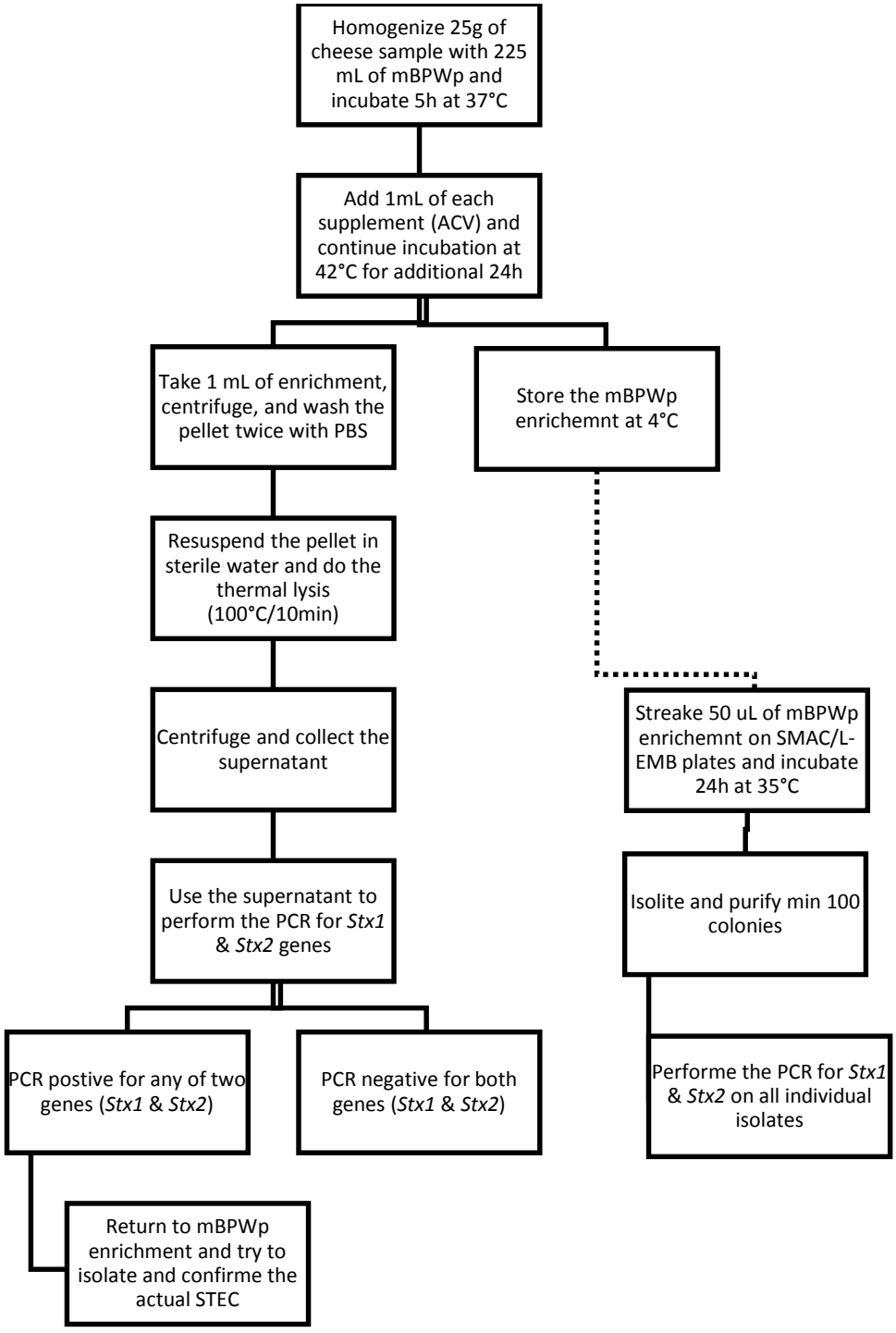

Figure 1: Flow Chart of steps needed to detect, isolate and confirm the presence of STEC in cheese.

SECTION 3 PROCEDURES

3.1 Setting up the enrichment of cheese sample in mBPWp

(i) Obtain and prepare a representative cheese sample and weigh 25g of it in to sterile stomacher bag. Add 225 mL of mBPWp and gently brake the cheese sample with hands to obtain a uniform suspension, if necessary you can use short stomaching (30s, 230RPM). It is very important **not** to perform vigorous stomaching or shaking incubation. E. coli O157:H7 is known to grow better without shaking. Do the same with all cheese samples you have.

(ii) Prepare mBPWp negative control by pouring 250 mL of mBPWp in to sterile stomacher bag.

(iii) Prepare mBPWp positive control by pouring 250 mL of mBPWp in to sterile stomacher bag and inoculating it with 1 mL of diluted (typically diluted in PBS to get $2.5x10^4$ cfu/mL inoculum) over-night culture of FSL F6-0699 (*E. coli* O157:H7). The starting concentration in the mBPWp enrichment should be approximately 100 cfu/mL.

(iv) Incubate sample enrichments together with mBPWp positive and negative controls at 37°C for 5h. After initial incubation (37°C/5h) add in to each sample bag and both control bags 1 mL of each ACV supplement (1 mL of Acriflavin, 1 mL of Cefsulodin, 1 mL of Vancomycin). Gently mix to distribute the supplements and continue the incubation at 42°C for additional 24h.

3.2 Preparing the lysates of mBPWp enriched samples

After incubation (42°C/24h) each cheese enrichment and both mBPWp controls are gently mixed and 1 mL of each is transferred in to 1.5 mL Eppendorf tubes. The suspensions in the tubes are centrifuged at 12 000 x g for 3 min and the supernatant discarded. There can be a fat lair lefover at the top of the tubes. You can use a sterile Q-Tip to remove the fat leftover. Each pellet is re-suspended in 1 mL sterile PBS. This washing step is repeated two more times. The first time the pellet is again re-suspended in PBS and the second time it is re-suspended in sterile water. If needed the process can be postponed at this point. All water suspensions can be stored at -20°C until ready to continue with the lysis step.

The lysis is performed by heating the suspension in a thermoblock (10 min/100°C).
Make sure:

- You are wearing safety glasses during this step.
- All tubes with suspension have safety attachment securely in place (this will prevent tubes from opening during heating).
- The thermoblock is at correct temperature before and during the lysis step (do not rely on the set temperature, use a control thermometer).

After heating cool down the lysate suspension on ice and perform the last centrifugation (12 000 x g 3 min). Transfer the DNA containing supernatant in to fresh Eppendorf tube. The supernatants can be stored at -20°C if the PCR for Stx 1 and Stx 2 is not performed immediately.

3.3 Running the Multiplex Touch-down PCR for Stx1 and Stx 2

Each PCR run is performed in parallel duplicates. One duplicate set represents the samples where we are looking PCR products which will indicate presence of Stx1 and Stx 2 genes. The second duplicate set represents a PCR matrix control for each individual sample which will indicate if there is any inhibition of the PCR reaction by the lysate matrix.

Parallel 1:	Parallel 2:
Sample (1)	Sample (1) matrix control
.	.
.	.
.	.
Sample (n)	Sample (n) matrix control
mBPWp negative control	mBPWp matrix control
mBPWp positive control	mBPWp matrix control
PCR negative control	PCR positive control

The PCR master mix is prepared in double amount and divided in to two separate tubes. One tube is used to test samples (Parallel 1) and the other tube is supplemented with the positive control DNA (FSL F6-0699 (*E. coli* O157:H7)) and used for PCR matrix controls (Parallel 2).

How to prepare positive control DNA:
Prepare an over-night culture of *E. coli* O157:H7 (FSL F6-0699) (BHI, 32°C/24h). Extract DNA from 1 mL of over-night culture using Qiagen kit (Protocol for Gram-negative bacteria). The final concertation of extracted DNA should be approximately 500 ng/µL. The extracted DNA is further diluted 1000 times to obtain the final positive control DNA (approximate DNA concertation 0.5 ng/ µL). If the original concertation of extracted DNA is different adjust the dilution accordingly to obtain the same concertation in the final positive control DNA. The positive control DNA is stored at -20°C, divided in to parallels to avoid repeated freezing and thawing cycles at every use. Use individual parallel of positive control in a way that you don't freeze and thaw it more than five times.

Multiplex PCR master-mix for 1 cheese sample and 1 corresponding PCR matrix control:

dH_20	28,75 µL
5x GoTaq Flexi Buffer	10,0 µL
$MgCl_2$ (25mM)	5,0 µL
dNTPs (10mM)	1,0 µL
slt I-F (10uM)	1,0 µL
slt I-R (10uM)	1,0 µL
slt II-F (10uM)	1,0 µL
slt II-R (10uM)	1,0 µL
GoTaq DNA polymerase	0,25 µL
Total volume	**49 µL**

N=number of cheese samples + 4
M=(Nx49)/2

To determine the final volume of each component of PCR master mix, multiply each component by N. Prepare the PCR master-mix in one tube. Transfer M amount of PCR master-mix in to new Eppendorf tube (this is half of the master-mix). Label first tube as 1 and the second tube as 2. Add N amount (in µL) of positive control DNA to the master-mix in tube 2 and mix gently by pipetting up and down.

Distribute 24 µL of master-mix from tube 1 in to N-1 number of PCR tubes (0.2 mL). This represents the parallel set 1 (the sample set).
Distribute 25 µL of master-mix in to N-1 number of PCR tubes. This will represent the parallel set 2 (the matrix control set).

Place all PCR tubes in a rack to have tubes from Parallel 1 above tubes from Parallel 2. In the next step take the first sample lysate and transfer 1 µL of it in to first tube of Parallel 1 and another 1 µL in to first tube of Parallel 2. Continue doing so with the rest of the sample lysates, putting 1 µL of second sample lysate in to second tube of both Parallel 1 and Parallel 2 set. Third sample lysate in to third, fourth in to fourth and so on. After you have put the last sample lysate in to both Parallel sets, do the same with both positive and negative mBPWp control. Put 1 µL of each in to tubes of both Parallel 1 and 2 sets. The last tube within each Parallel set remains as it is, nothing is added in to these PCR mixes/tubes. The last tube within Parallel 1 set represents a negative PCR control and the last tube within Parallel 2 set represents a positive PCR control.

After all PCR mixes are prepared the tubes are securely locked and a short spin-down step is performed on mini bench-top centrifuge in order to collect entire mixture on the bottom without any air bubbles. The tubes are placed in to PCR cycler and the touch-down PCR reaction performed under following conditions.

Touch-down PCR cycling conditions:

Temperature:	**Time:**	**Number of cycles:**
94°C	2 min	1x
94°C	30s	20x
59°C (TD -0.50°C per cycle)	1 min	
72°C	1 min	
94°C	30s	20x
49°C	1 min	
72°C	1 min	
72°C	7 min	1x

PCR product size:

Gene fragment:	Amplicon size:
Stx1 (Slt I)	210 bp
Stx2 (Slt II)	484 bp

3.4 Separating PCR products on agarose gel

After the PCR reaction is finished the PCR products are separated on 1.5% agarose gel.

How to prepare 1.5% agarose gel:
Weigh 1.5g of agarose in to 250 mL Erlenmeyer flask and add 100 mL of 0.5x TBE Buffer. Place a folded kimwipe in to the neck of the flask to partial close the opening and prevent excessive evaporation during heating. Heat the suspension for approximately 2 min, using microwave oven. Heat for a minute and swirl flask every 20 s. During the second minute of heating swirl every 7 s. The final gel should be clear without any undissolved particles. Adjust the time of hating accordingly. After heating swirl the flask one last time and let it sit for 2-3 min, use this time to set up the gel tray. Make sure the gel tray you are using is clean and also the combs you are using are clean without dried agarose between the teeth. Each gel tray has two walls, make the two missing walls using labeling tape. Before applying the labeling tape, place the string rubber gaskets in to side grooves of the tray. Place the tray in to bigger holding tray and insert the comb in the top tray groove. Each gel tray has two sets of grooves allowing you to place two combs on a single gel (allowing you to have two rows for loading of samples). When preparing gel for separation of PCR products from Multiplex Touch-down PCR for *Stx1* and *Stx2*, each gel is prepared to have only one/top row for loading. To separate these PCR products you need entire length of the gel, half of the gel is not enough. Stx1 PCR product has only 210 bp and it will not separate sufficiently from the prime-dimer leftover at the bottom of the gel. Amount of primer-dimer leftover depends on level of amplification and this depends on the presence of templet DNA and the amount of templet DNA.

3.5 What to do when cheese sample is PCR positive for Stx1 & Stx2

It is very important to know how to interpret a PCR positive result for any of two Stx1 and Stx2 genes. PCR can be positive for either of two genes or both of them because STEC can have genes and produce either only one of them or both. PCR positive result does NOT count as positive result for presence of STEC in the cheese sample. PCR can be positive because the DNA of a dead STEC cell is still present in cheese (it is very likely that you have STEC in raw milk, but less likely that you have STEC in cheese especially if it is made from pasteurized milk). In order to confirm presence of STEC in cheese you have to isolate the actual live STEC.

When you get a PCR positive result in one of the cheese samples, you have to go back to the original mBPWp enrichment for this cheese sample and try to isolate the STEC. This involves the following procedure:

(1) mBPWp enrichment is diluted in PBS accordingly to obtain countable number of colonies after plating on L-EMB. Usually repeating 7 serial 10-fold dilutions of mBPWp is sufficient.
(2) Plate in triplicate, 100 µL of 6^{th} and 7^{th} serial dilution on each agar media. Use a sterile plastic or glass hockey spreader to distribute the entire volume evenly across the agar surface.
(3) Incubate plates aerobically at 35°C for 24h. After incubation you should have L-EMB plates with number of colonies between 10 and 100.
(4) Use a Sharpie and make a grid on the bottom of a BHI plate. Make a grid with 15 to 25 individual spaces in the grid and number each space you're going to use.
(5) Use sterile toothpicks to transfer individual colonies from L-EMB in to individual grid spaces on BHI plate. It is enough to just touch the colony with a toothpick and again just touch the BHI agar in the designated space. Based on colony morphology and color on L-EMB you can speculate which colonies are *E. coli* (dark red colonies with metallic green sheen) but this is not 100% consistent, it can both ways non-coli with the green sheen and *E. coli* without the green sheen. Based on that it is best to pick as many different representatives as possible. You have to select and pick at least 100 individual colonies.
(6) Once you transferred all of the selected colonies on to BHI plates, incubate the plates at 35°C for 24h. Next day you should have individual colonies growing on the BHI plates, each separated from the rest within the gird.
(7) Use a 96-well PCR plate to prepare lysates of individual colonies keeping track which lysate represent which colony on the plate (see Colony PCR protocol). Finish each 96-well PCR plate with one well dedicated to negative control (empty well) and one to positive control (a colony of positive control strain FSL F6-0699). Using sterile toothpicks transfer each colony in to individual well, again, it is enough to just touch the colony and scrape the toothpick on the bottom of the well.

(8) The plate is microwaved at high for 1 min.

(9) After cooling add 50 µL of multiplex PCR master mix for Stx1 and Stx2.
(10) Follow the instructions under 3.3 and 3.4 to perform the PCR reaction.
(11) Positive PCR reaction for ether of two genes allows you to go back to the original colony for additional purification and re-confirmation.

3.6 Confirm PCR reaction for Stx1 and Stx2 by sequencing the PCR products

The final confirmation of the positive PCR result for Stx1 and Stx2 is sequencing the PCR fragments. PCR is repeated with PCR primer pair for only one Stx gene, the volume of the other PCR primer pair is replaced by water in the multiplex PCR master mix (technically this is no longer a multiplex PCR). This way for each positive sample reactions for the two Stx genes are performed in two separate reactions. After confirming the amplification on agarose gel, each reaction mix is prepared for sequencing. Follow the protocol 'PCR product purification using ExoSAP' to clean the PCR reaction of primers and free dNTPs. After Exo/SAP purification prepare the sample for sequencing using the protocol 'Sample submission to BRC'. After editing the sequences you can use BLAST to confirm the sequences are really representing Stx1 and/or Stx2 genes.

SECTION 4 TROUBLESHOOTING

Problem:	**Explanation:**	**How to salve it:**
You have amplification of *Stx1* &*Stx2* fragments in PCR positive control but one or both PCR products in one or more of the PCR matrix controls is missing	There is an inhibition of PCR reaction present. Since the only part that is different is the presence of sample lysate.	Return to the stored enrichment (mBPWp) and repeat the process of obtaining the lysates for the samples with the missing amplicons and for both mBPWp positive and mBPWp negative control. With the new lysates repeat the PCR for *Stx1* and *Stx2*.
After repeating the process of obtaining lysates, you still don't get amplification in your matrix control	The inhibition of PCR is still present.	Use 10-times dilution of the lysate to perform the PCR for Stx1 and Stx2.
The PCR primers for Stx1 and Stx2 used in this SOP are for any reason not acceptable. You want to confirm the results with a second PCR reaction.		Use the original primers and multiplex PCR conditions proposed by FDA BAM: <u>Primers for Stx1 (348 bp):</u> LP30 … 5' - CAGTTAATGTGGTGGCGAAGG - 3' LP31 … 5' - CACCAGACAATGTAACCGCTG - 3' <u>Primers for Stx2 (584 bp):</u> LP43 … 5' - ATCCTATTCCCGGGAGTTTACG - 3' LP44 … 5' - GCGTCATCGTATACACAGGAGC - 3' <u>PCR reaction mix:</u> d H_2O 27.75 µL 5x Go Taq Flexi Buffer 10.00 µL $MgCl_2$ 25mM 6.00 µL dNTP's 10mM 1.25 µL LP30 10uM 1.00 µL LP31 10uM 1.00 µL LP43 10uM 1.00 µL LP44 10uM 1.00 µL Go Taq DNA Polymerase 0.25 µL 48 µL master mix + 2 µL of sample

		The PCR conditions: 95ºC/15 min 1 cycle 95ºC/1 min 56ºC/1 min 25 cycles 72ºC/1 min . 72ºC/5 min 1 cycle

SECTION 5 REFERENCES

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