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TRIPLEX PCR FOR DETECTION OF TOXIGENIC *V. CHOLERAE* O1 AND O139 USING SMARTCYCLER®

1. DNA template: suspend a small amount (half the growth of a 2 mm diameter colony) of bacterial growth into 300 µl of 0.01 M TE buffer → boil for 10 min → centrifuge 4,500 rpm 2 min → use 2 µl / reaction. **NOTE: store the templates at –20°C**

2. Reagents:
   - Platinum *Taq* DNA polymerase (5 units / µl) (Invitrogen, Carlsbad, CA; Cat. No. 10966-034)
   - 10 x PCR buffer, Minus Mg (Invitrogen, Carlsbad, CA; Cat.No. Y02028)
   - $MgCl_2$ (50 mM) ((Invitrogen, Carlsbad, CA; Cat.No. Y02016)
   - dNTP mix (10mM) (Roche, Cat. No. 1 814 362)
   - **NOTE 1: store the reagents at –20°C**
   - **NOTE 2: thaw the reagents completely and vortex them thoroughly before use**

3. Primers and probes:
   - Working concentration for primers: 12.5 µM (*ctx* and O1rfb) or 25 µM (O139 *rfb*)
   - Working concentration for probes: 5 µM
   - Store the working concentrations at +4°C. **NOTE: probes are light sensitive. Minimize the time they are exposed for light**
   - Allocate the primer and probe stock concentrations and store them at –20°C. Freeze and thaw only once.
   - Sequences for primers (from '5 to'3)

ctx:	CTX4-F	CGG GCA GAT TCT AGA CCT CCT
	CTX5-R	CGA TGA TCT TGG AGC ATT CCC
O1 *rfb:*	O1RFB4-F	TGG TTT CAC TGA ACA GAT GGG G
	O1RFB4-R	AGG TCA TCT GTA AGT ACA ACA TTC CC
O139 *rfb:*	O139RFB3-F	AGC CTC TTT ATT ACG GGT GGC A
	O139RFB3-R	GGT CAA ACC CGA TCG TAA AGG

   - Sequences for probes (from '5 to '3)

ctx: CTX-HP95
AT GAA ATA AAG CAG TCA GGT GGT CTT ATG CCA
5' = FAM (fluorescein), 3' = BHQ1 (quencher)

O1 *rfb:* O1RFB-HP317
TA ATT ACC CAG ATT GTA AAG CAG GAT GGA AAC ATA TTC
5' = TFA-AL for Texas Red, 3' = BHQ2 (quencher)

O139 *rfb*: O139RFB-HP265
CG CGA TAC T"T"T TCT GTT TGC TTC TGA GTT AAA AGC
5' = Yakima Yellow, "T" = C6dT for qsy7, 3' = Phospate

- Examples how to calculate working concentrations for primers:

CTX4-F (306035):
Mer = 21, conc. 2.46 µg/µl
To prepare 100 µl of 25 µM working stock:

21x330=6930
$6930x25/10^6$=0.17325 µg/µl
100 µl x 0.17325 µg/µl = Y x 2.46 µg/µl
Y= 7.0 µl
Water: 100 µl-7.0 µl= 93.0 µl

CTX5-R (306036):
Mer = 21, conc. 2.18 µg/µl
To prepare 100 µl of 25 µM working stock:

21x330=6930
$6930x25/10^6$=0.17325 µg/µl
100 µl x 0.17325 µg/µl = Y x 2.18 µg/µl
Y= 7.9 µl
Water: 100 µl-7.9 µl= 92.1 µl

O1RFB4-F (410058):
Conc. 344.97 µM
To prepare 100 µl of 12.5 µM working stock:

100 µl x 12.5 µM = Y x 344.97 µM
Y= 3.6 µl
Water: 100 µl-3.6 µl= 96.4 µl

O1RFB4-R (410059):
Conc. 443.45 µM
To prepare 100 µl of 12.5 µM working stock:

100 µl x 12.5 µM = Y x 443.45 µM
Y= 2.8 µl
Water: 100 µl-2.8 µl= 97.2 µl

O139RFB3-F (306041):
Mer = 22, conc. 2.93 µg/µl
To prepare 100 µl of 25 µM working stock:

22x330=7260
$7260x25/10^6$=0.1815 µg/µl
100 µl x 0.1815µg/µl = Y x 2.93 µg/µl
Y = 6.2 µl
Water: 100 µl-6.2 µl= 93.8 µl

O139RFB3-R (306042):
Mer = 21, conc. 2.48 µg/µl
To prepare 100 µl of 25 µM working stock:

21x330=6930
$6930x25/10^6$=0.17325 µg/µl
100 µl x 0.17325 µg/µl = Y x 2.48 µg/µl
Y = 7.0 µl
Water: 100 µl-7.0 µl= 93.0 µl

- Examples how to calculate working concentrations for probes:

CTX-HP95 (306037):
Conc. 223.61 µM
To prepare 200 µl of 5 µM working stock:

200 µl x 5 µM = Y x 223.61 µM
Y = 4.5 µl
Water: 200 µl-4.5 µl= 195.5 µl

O1RFB-HP317 (311888):
Conc. 55.16 µM
To prepare 100 µl of 5 µM working stock:

100 µl x 5 µM = Y x 55.16 µM
Y = 9.1 µl
Water: 100 µl-9.1 µl= 90.9 µl

O139RFB-HP265 (317686):
Conc. 71.98 µM
To prepare 100 µl of 5 µM working stock:

100 µl x 5 µM = Y x 71.98 µM
Y = 6.9 µl
Water: 100 µl-6.9 µl= 93.1 µl

4. Mastermix **(NOTE: keep the reagents in ice or in a cooling block):**

	1 sample	18 samples (full run + 2)
dH_2O	13.5 µl	243.0 µl
CTX4-F (12.5 µM)	0.125 µl (0.0625 µM)	2.25 µl
CTX5-R (12.5µM)	0.125 µl (0.0625 µM)	2.25 µl
CTX-HP95 (5 µM)	1.5 µl (0.3µM)	27.0 µl
O1RFB3-F (12.5 µM)	0.125 µl (0.0625µM)	2.25 µl
O1RFB3-R (12.5 µM)	0.125 µl (0.0625 µM)	2.25 µl
O1RFB-HP317 (5 µM)	1.0 µl (0.2 µM)	18.0 µl
O139RFB3-F (25 µM)	0.5 µl (0.5 µM)	9.0 µl
O139RFB3-R (25 µM)	0.5 µl (0.5 µM)	9.0 µl
O139RFB-HP265 (5 µM)	1.0 µl (0.2 µM)	18.0 µl
10 x PCR buffer	2.5 µl	45.0 µl
$MgCl_2$ (50 mM)	1.0 µl (2.0 mM)	18.0 µl
dNTP (10 mM)	0.5 µl (0.2 mM)	9.0 µl
Taq (5 U/µl)	0.5 µl (2.5 U)	9.0 µl
	= 23.0 µl	= 414.0 µl / 18 = 23 µl

5. Controls:
   - Positive controls:
     - ATCC 39315 (*V. cholerae* O1 Inaba Eltor); positive for *ctxA* and O1 *rfb*
     - F642 (V. *cholerae* O139): positive for *ctxA* and O139 *rfb*
   - Negative control: no DNA (water)

6. Place a required number of reaction tubes in the cooling block. **NOTE:** handle the reaction tube by the ribbed upper portion of the tube. Avoid touching the optical detection windows at the bottom edges of the tube and the diamond-shaped area. Dispense the mastermix: 23 µl / reaction. Add the DNA template: 2 µl / reaction

7. Spin down the mastermix. Carefully check for air bubbles. If there are any, keep spinning down the mastermix until there are no air bubbles. **NOTE:** number the reaction tubes before spinning to avoid mixing the samples.

8. Insert the reaction tubes into the I-Core module

9. Click "Create run" → Name the run → Select FCTC25 for dye set→ Click "add/remove sites" → select "BTtriplex3step" protocol and the required number of sites → click "start run"

Running conditions for "BTtriplex3step"
Initial denaturation 95°C 120 s
30 cycles of 95°C 10 s, 60°C 20 s, 72°C 30 s collect data here = optics on

10. Type in the sample IDs and save the run

11. Viewing the results in real time: click "define graphs"→ click "new graph", then enter "FAM, Yakima Yellow and Texas Red primary with threshold" for the new graph name (with this graph you can view the results from all three channels at the same time) → select "optics on", "channel 1", "channel 2" and "channel 3" (channels 1, 3 and 4 in the SmartCycler version I) and "fluorescence vs. cycle" → click"save graph" → go back to "view results" → click "select graphs" and associate the " FAM, Yakima Yellow and Texas red primary with threshold" graph with the run. It should appear in the "views" list.

NOTE 1: you need to create the graph only once when you make your first run. You can later use the same graph by simple clicking it in the "views list"

NOTE 2: you can view channel 1, 2 and 3 results separately by clicking FAM, TET and TAMRA in the "views" list, respectively

12. Analysis of the results

- After the run is done, click "update analysis"→ click "report"→ Print the report table
  - The crossing points for *ctxA, O139 rfb* and *O1 rfb* are read on channels 1, 2 and 3, respectively
- If you want to print the fluorescence curves, click the appropriate graph(s) in the "views" list and then right click on the graph → select "print graph only" from the pull down menu
- If a negative drift is displayed in the fluorescence curve (= the initial background fluorescence of the sample is high, resulting in a false negative result), decrease the manual threshold fluorescence units (default 30) until you get an acceptable curve.

13. References

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