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TRIPLEX PCR FOR DETECTION OF *S. TYPHI* USING SMARTCYCLER®

1. DNA template:
   - Whole cell template: suspend a small amount (half the growth of a 2 mm diameter colony) of bacterial growth into 300 µl of distilled H_2O → boil for 10 min → centrifuge 4,500 rpm 2 min → use 1 µl / reaction
   - Purified DNA: for example PureGene kit (Gentra Systems, Minneapolis, MN; Cat No. D-5000A), MagnaPure automatic DNA extractor (Roche Molecular Diagnostics, Mannheim, Germany); use 1 µl / reaction

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 ***tyv*-primers**: **25 µM**
   - Working concentration for ***viaB*- and *fliCd*-primers 12.5 µM**
   - 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)

***viaB*:**	VIABF2	AGG TTA TTT CAG CAT AAG GAG ACT T
	VIABR2	CTC TTC CAT ACC ACT TTC CGA
***fliCd*:**	FLICCOM-S	AAT CAA CAA CAA CCT GCA GCG
	FLICD-AS2	GCA TAG CCA CCA TCA ATA ACC TTA C
tyv:	TYV3-F	TTG AAG AGG AAG GGA AAT GAA G
	TYV-R	TAG CAA ACT GTC TCC CAC CAT AC

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

viaB: VIAB-HP1032
TT GCT GTT TTC GAT GCG GCA ACA
5'=TFA-AL for **texas red** (TR); 3'=BHQ2

fliCd: FLICD-HP105
CC TGA ACG AAA TCG ACC GTG TAT CCG
5'=**6-FAM**; 3'=BHQ1

tyv: TYV-HP1239
CT TCC TTG GGA GTA ATC TTG CCT CCT TTG C
5'=**Yakima Yellow** (YY); 3'=BHQ1

- An example how to calculate working concentrations for primers:
  - Length of the primer: 20 bases
  - Concentration of the stock: 0.64 μg/μl
  - To prepare 200 μl of 25 μM working stock:

  The molecular weight of the primer is calculated by using a fixed constant (330):
  20 x 330 = 6600

  The DNA concentration of the working stock will be changed
  from μM to μg/μl using the molecular weight of the primer (note: 1 M = 6600 g/l;
  1 μM = 0.0066 μg/μl; 25 μM = 0.165 μg/μl):
  6600 x 25 μg / 10^6 μl = 0.165 μg/μl

  The amount of primer stock needed (Y):
  200 μl x 0.165 μg/μl = Y x 0.64 μg/μl
  Y = 51.56 μl

  The amount of water needed:
  200 μl - 51.56 μl = 148.44 μl

- An example how to calculate working concentrations for probes:
  - Concentration of the stock: 135.17 μM
  - To prepare 200 μl of 5 μM working stock:

  The amount of primer stock needed (Y):
  200 μl x 5 μM = Y x 137.17 μM
  Y = 7.40 μl

  The amount of water needed:
  200 μl - 7.40 μl = 192.60 μl

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

	1 sample	18 samples (full run + 2)
dH_2O	16.0 µl	288.0 µl
VIAB-F2 (**12.5 µM**)	0.125 µl (0.0625 µM)	2.25 µl
VIAB-R2 (**12.5 µM**)	0.125 µl (0.0625 µM)	2.25 µl
FLICCOM-S (**12.5 µM**)	0.125 µl (0.0625 µM)	2.25 µl
FLICD-AS2 (**12.5 µM**)	0.125 µl (0.0625 µM)	2.25 µl
TYV3-F (**25 µM**)	0.25 µl (0.25 µM)	4.5 µl
TYV-R (**25 µM**)	0.25 µl (0.25 µM)	4.5 µl
VIAB-HP1032^{TR} (5 µM)	1.0 µl (0.2 µM)	18.0 µl
FLICD-HP105^{FAM} (5 µM)	1.0 µl (0.2 µM)	18.0 µl
TYV-HP1239^{YY} (5 µM)	0.5 µl (0.1 µM)	9.0 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
	= 24.0 µl	= 432.0 µl / 18 = 24 µl

5. Controls:
   - Positive control: 229-89 (*S.* Typhi phage type A)
   - 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: **24 µl** / reaction. Add the DNA template: **1 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 "Styphitriplex" protocol and the required number of sites → click "start run"

   Styphitriplex
   Initial denaturation 95°C 120 s
   40 cycles of 95°C 15 s, 60°C 60 s collect data here = optics on

10. Type in the sample IDs

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 "automatically added to new runs", graph type "optics", "channel 1", "channel 2" and "channel 3" and "fluorescence vs. cycle" → click "save graph" → go back to "view results"
    If the new graph does not automatically appear in the views list: click "select graphs" and associate the " FAM, TAMRA and Texas red primary with threshold" graph with the run. Now 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 channels 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 *fliC-d, tyv* and *viaB* 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.

14. References

Hirose, K., K.-I. Itoh, H. Nakajima, T. Kurazono, M. Yamaguchi, K. Moriya, T. Ezaki, Y. Kawamura, K. Tamura, and H. Watanabe. 2002. Selective amplification of *tyv* (*rfbE*), *prt* (*rfbS*), *viaB,* and *fliC* genes by multiplex PCR for identification of *Salmonella enterica* Serovars Typhi and Paratyphi A. J. Clin. Microbiol. 40, 633-636.