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DIAGNOSIS OF DIARRHEAGENIC *ESCHERICHIA COLI* AND *SHIGELLA* USING LIGHTCYCLER™

1. LightCycler equipment and supplies

- LightCycler Instrument (Roche Diagnostics, Mannheim, Germany; Cat. No. 2 011 468)
- LightCycler™ - Centrifuge Adapters (Cat. No.1 909 312)
- LightCycler™ - Sample Carousel (Cat. No. 1 909 282)
- LightCycler™ - Color Compensation Set (Cat. No. 2 158 850)
- LightCycler™ - DNA Master Hybridization Probes (Cat. No. 2 158 825)
- LightCycler™ - Capillaries (Cat. No. 1 909 339)
- Optional: LightCycler™ Carousel Centrifuge (Cat. No. 2 189 682)

2. Additional equipment and supplies:

- "Clean" hood / PCR chamber
- Conventional block cycler or boiling water bath
- Table top centrifuge
- Two sets of pipettors: "clean" set for setting up the mastermixes and another set for preparation of the DNA template
- Filtered pipette tips
- 0.5 ml and 1.5 ml sterile eppendorf tubes
- Forceps

3. Primers and probes:

- Recommended supplier: TIB Molbiol LLC, Adelphia, NJ (Tel. 877-696-5446; Fax 877-696-5456; www.tibmolbiol.com)
- Working concentration for primers: 25 μM.
  - **Exception 1**: *ipah-Stx*$_1$-multiplex for *Shigella dysenteriae*: working concentration for EIEC-1 and EIEC-2 3.125 μM
  - **Exception 2***:* *ST*$_h$-*ST*$_p$ –multiplex: working concentration for STIB-1 and STIB-2 6.25 μM

- Working concentration for probes: 5 μM
- Use the water provided by the LightCycler™ kits to prepare the working concentrations
- Store the working concentrations at +4^{o}C.

Note: probes are light sensitive. Minimize the time they are exposed for light. Working concentrations usually preserve for months, primers in particular for up to a year.

- Allocate the primer and probe stock concentrations and store them at -20^{o}C. Freeze and thaw only once.

- Quality control (QC) for primers and probes:
  - A set of strains that represent different variants (different T_m:s) of *E coli* virulence factors should form a QC test panel
  - Extract a large volume of DNA from the QC strains and use the same template to test each new lot of primers and probes
  - The crossing point for each strain should not vary more than one cycle between different lots
  - The T_m should not vary more than 1°C between different lots

- Sequences for primers (from'5 to '3):

***stx₁* and *stx₂*:**	STEC-1	gAR[a] CRA AAT AAT TTA TAT gTg
	STEC-2	TgA TgA TgR CAA TTC AgT AT
***eae*:**	eaeAF	gAC CCg gCA CAA gCA TAA gC
	eaeAR	CCA CCT gCA gCA ACA AgA gg
E-*hly*:	hlyAF	gCA TCA TCA AgC gTA CgT TCC
	hlyAR	AAT gAg CCA AgC Tgg TTA AgC T
***uidA*:**	O157-PT-2	gCg AAA ACT gTg gAA TTg gg
	O157-PT-3	TgA TgC TCC ATC ACT TCC Tg
***LT*:**	LT-1	gCg TTA CTA TCC TCT CTA TgT g
	LT-2	AgT TTT CCA TAC TgA TTg CCg C
***ST$_h$*:**	STIB-1	CTg TAT TgT CTT TTT CAC CT
	STIB-2	ATT AAT AgC ACC Cgg TAC AA
***ST$_p$*:**	STIA-1	CTg TAT TAT CTT TCC CCT CT
	STIA-2	TTT AAT AAC ATg gAg CAC Ag
***ipa*H-plasmid:**	EIEC-1	gTT CCT TgA CCg CCT TTC CgA
	EIEC-2	gCC ggT CAg CCA CCC TCT gA

[a] IUB code for A or G (wobble base)

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

***stx₁*:**

STEC I-HP-1	TTT ACg TTT TCg gCA AAT ACA gAg ggg AT-[FAM[a]]
STEC I-HP-2	[Red 640[b]]-TCg TAC AAC ACT ggA TgA TCT CAg Tgg g-Ph[c]

For *ipah*-*Stx₁*-multiplex:

STECI-HP-2	[Red 705[b]]-TCg TAC AAC ACT ggA TgA TCT CAg Tgg g-Ph[c]

***stx₂*:**

STEC II-HP-1	TCA ggC ACT gTC TgA AAC TgC TCC TgT gTA-[FAM[a]]
STEC II-HP-2	[Red 705[d]]-ACC ATg ACg CCg ggA gAC gTg gAC CT-Ph[c]

eae:

eaeA-HP-1	ACA gTT CTg AAA gCg AAA TgA TgA Agg C-[FAM[a]]
eaeA-HP-2	[Red 640[b]]- CCT ggT CAg CAg ATC ATT TTg CCA CT-Ph[c]

E-*hly:*

hlyA-HP-1	gCA Tgg CTC TTg ATg AAT TgC TgA gA-[FAM[a]]
hlyA-HP-2	[Red 705[d]]-CAA Cgg gAA ggA gAg gAT ATA AgT CAg-Ph[c]

uidA:

O157-HP-1	CgT AAT TAT gTg ggC AAC gTC Tgg T-[FAM[a]]
O157-HP-2	[Red 640[b]]-TCA gCg CgA AgT CTT TAT ACC gAA Agg-Ph[c]

LT:

LT-HP-1	Agg ATg AAg gAC ACA TTA AgA ATC ACA T-[FAM[a]]
LT-HP-2	[Red 640[b]]- TC TgA CCg AgA CCA AAA TTg ATA AAT T-Ph[c]

***ST_h* and *ST_p*:**

STIB-HP-1	gTC CTg AAA gCA TgA ATA gTA gCA AT-[FAM[a]]
STIA-HP-1	AAA TCA gAA AAT ATg AAC AAC ACA T-[FAM[a]]
STI-HP-2	[Red 705[d]]-AC TgC TgT gAA TTg TgT TgT AAT CCT-Ph[c]

***ipa*H-plasmid:**

EIEC-HP-1	TgC gTT TCT ATg gCg TgT Cgg-[FAM[a]]
EIEC-HP-2	[Red 640[b]]-Tg ACA gCA AAT gAC CTC CgC ACT-Ph[c]

[a] [FAM], flourescein
[b] [Red 640], LightCycler™-Red 640-N-hydroxy-succinimide ester;
[c] Ph, 3'-phosphate
[d] [Red 705], LightCycler™-Red 705-phosphoramidite

♦ An example how to calculate working concentrations for primers:

STEC-1:

- 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 the average weight of a DNA base (330):
20 x 330 = 6600

The DNA concentration of the working stock has to be changed
from µM to µg/µl using the molecular weight of the primer:
1 M = 6600 g/l; 1µM = 0.0066 µg/µl; 25 µM = 25 x 0.0066 µg/µ = 0.165 µg/µl

The amount of primer stock needed (Y) is calculated by using equation
vol_1 x $conc_1$ = vol_2 x $conc_2$:

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:
  STECI-HP-1:
  - 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

- **Note**: large quantities of primers and probes are usually shipped in dry form. The concentrations in dry form are often expressed in nmols. For long term storage, a convenient stock concentration is 100 µM. In order to prepare a 100 µM stock, dissolve 1:10 the amount of primer / probe nmols into distilled water.
  For example: the amount of primer / probe in dry form: 53 nmol. Add 530 µl of dH_2O → 100 µM stock.

4. Getting started:

- Turn on the computer and the LightCycler.

- Click on the LightCycler software icon → the front screen will appear

- Click "Run" → Programming screen will appear

- In about 15 s, self test window will appear → run the self test by clicking on the "Start Self Test" button → After the self test is completed and passed click "ok" in the "Self Test Passed window that appears → the programming screen will become active

  - It is recommended to run the self test once a day before making any runs
  - The self test window will appear automatically only when you open the software. If you haven't turned off the instrument for the night, go to the "Tools" and select "Start self test" from the drop down menu.
  - If the instrument does not pass the self test, an error notification window will appear → contact the Roche Technical Service

5. Create a color compensation file: follow the instructions in the insert of LightCycler Color Compensation Set

- The color compensation file corrects the overlap in the emission spectrum between channels 2 and 3 and is needed for multiplex PCR
- You only have to create the color compensation file once. The same file can be used at least for six months.
- Always create a new color compensation file after the instrument has been calibrated or serviced
- If a universal color compensation file is not working appropriately, you can create an assay specific customized color compensation file. For instructions, see Appendix 1.

6. Create a protocol: follow the programming instructions in the LightCycler™ Operator's Manual Version 3.5 (see pages 45-48). Set up a protocol "**ecolivir**" which is used for all assays except *uidA.*

- Start the programming by clicking "New Experiment" → "save window" will appear
- Name your experiment "ecolivir" and save it → "please name new program run" window will appear
- Type in the name of the first segment of the protocol: "Denaturation" and click "ok" → Define conditions in the temperature target segment: 95°C 30 s with temperature transition rate 20°C / s
- Click "Add" → "please name new program run" window will appear again
- Type in the name of the second segment of the protocol: "PCR" and click "ok" → Define cycling conditions in the temperature target segment (use the "Ins" button to enter a new temperature segement): 40 cycles of denaturation 95°C 0 s, annealing 50°C 20 s, extension 72°C 30 s; temperature transition rate 20°C / s in all steps; Acquisition Mode " Single" at the annealing step; Analysis Mode "Quantification"
- Click "Add" → "please name new program run" window will appear again
- Type in the name of the third segment of the protocol: "Melting curves" and click "ok" → Define conditions: Rapid denaturation 95°C 0 s with temperature transition rate 20°C / s, 10 s hold at 40°C with temperature transition rate 20°C / s, slow denaturation 95°C with temperature transition rate of 0.2°C / s; Acquisition Mode "Continuous" during the slow denaturation step; Analysis Mode "Melting Curves"
- Click "Add" → "please name new program run" window will appear again
- Type in the name of the fourth segment of the protocol: "Cooling" and click "ok" → Define conditions: 40°C 2 min with temperature transition rate 20°C / s
- Select the color compensation file you created by clicking "Choose CCC File" button. Click "Use Color Compensation" and save your conditions by clicking "Save Experiment File" button. Your default is now that the color compensation file is always on.

- Cycling conditions in "uidA":
  - Initial denaturation 95°C 30 s with temperature transition rate 20°C / s
  - PCR: 40 cycles of denaturation 95°C 0 s, annealing 65°C 10 s, extension 72°C 20 s ; temperature transition rate 20°C / s in all steps
  - Melting curve analysis to determine the T_m values for the sequence targeted by the hybridization probes:
    a. Rapid denaturation 95°C 0 s with temperature transition rate 20°C / s
    b. 10 s hold at 55°C with temperature transition rate 20°C / s

c. Slow denaturation (95°C) with temperature transition rate of 0.2°C / s
- Cooling 40°C 2 min

7. Preparation of 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 2 µl / reaction
  - Enrichment broth: boil 500 µl of broth culture for 10 min → centrifuge 4,500 rpm 2 min → use 2 µl / reaction

- Purified DNA:

  - PureGene™ kit (Gentra Systems, Minneapolis, MN; Cat No. D-5000A): suspend half of loop (10 µl loop) of overnight growth from agar plate in 600 µl PureGene Lysis Buffer and proceed by following manufacturer's instructions → use 2 µl / reaction
  - DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA; Cat. No. 69506): suspend half of loop (10 µl loop) of overnight growth from agar plate in 180 µl DNeasy ATL Buffer and proceed by following manufacturer's instructions → use 2 µl / reaction
  - MagnaPure automatic DNA extractor (Roche Diagnostics; Cat. No. 2 236 931): make 1" sweep (1 µl loop) from the confluent first streaking area of overnight bacterial growth → suspend in 230 µl 0.01 M PBS (pH 7.2) → boil for 10 min → use 200 µl as a sample input volume in MagnaPure and proceed by following manufacturer's instructions → use 2 µl / reaction
    Note. MagnaPure LC DNA Isolation Kits I and III and Total Nucleic Acid Isolation Kit all perform equally well with pure cultures. Total Nucleic Acid Isolation Kit performs best with primary samples, such as stool swabs

8. Mastermixes for one sample (**Note**: keep reagents in ice or in a cooling block):

* HP mastermix = LightCycler™ - DNA Master Hybridization Probes, 10x
* Use the water and $MgCl_2$ provided by the kit to adjust the final reaction volume and $MgCl_2$ concentration, respectively

- ***stx_1 & stx_2*** (Shiga toxin genes 1 and 2 in STEC):

dH_2O	11.4 µl
HP mastermix	2.0 µl
$MgCl_2$ (25mM)	1.6 µl
STEC-1 primer (25 µM)	0.4 µl
STEC-2 primer (25 µM)	0.4 µl
STEC I-HP-1 (5 µM)	0.8 µl
STEC I-HP-2^{-LC640} (5 µM)	0.8 µl
STEC II-HP-1 (5 µM)	0.8 µl
STEC II-HP-2^{-LC705} (5 µM)	0.8 µl
	= 19.0 µl

- ***eae &* E-*hly*** (intimin and enterohemolysin genes in STEC and EPEC, and STEC, respectively):

dH_2O	11.0 µl
HP mastermix	2.0 µl
$MgCl_2$ (25 mM)	1.6 µl
eaeAF primer (25 µM)	0.4 µl
eaeAR primer (25 µM)	0.4 µl
eaeA-HP-1 (5 µM)	0.8 µl
eaeA-HP-2^{-LC640} (5 µM)	0.8 µl
hlyAF primer (25 µM)	0.2 µl
hlyAR primer (25 µM)	0.2 µl
hlyA-HP-1 (5 µM)	0.8 µl
hlyA-HP-2^{-LC705} (5 µM)	0.8 µl
	= 19.0 µl

- ***uidA*** (1 bp-mismatch in *uidA*-gene of O157:H7):

dH_2O	13.0 µl
HP mastermix	2.0 µl
$MgCl_2$ (25 mM)	1.6 µl
O157-PT-2 primer (25 µM)	0.4 µl
O157-PT-3 primer (25 µM)	0.4 µl
O157-HP-1 (5 µM)	0.8 µl
O157-HP-2^{-LC640} (5 µM)	0.8 µl
	= 19.0 µl

- ***LT*** (thermolabile enterotoxin gene in ETEC):

dH_2O	13.0 µl
HP mastermix	2.0 µl
$MgCl_2$ (25 mM)	1.6 µl
LT-1 primer (25 µM)	0.4 µl
LT-2 primer (25 µM)	0.4 µl
LT-HP-1 (5 µM)	0.8 µl
LT-HP-2^{-LC640} (5 µM)	0.8 µl
	= 19.0 µl

- ***ST_h* and *ST_p*** [thermostable enterotoxin gene variants **h**uman (*STIb*) and **p**orcine (*STIa*) in ETEC]:

dH_2O	11.4 µl
HP mastermix	2.0 µl
$MgCl_2$ (25 mM)	1.6 µl
STIB-1 primer (6.25 µM)	0.4 µl
STIB-2 primer (6.25 µM)	0.4 µl
STIA-1 primer (25 µM)	0.4 µl
STIA-2 primer (25 µM)	0.4 µl
STIB-HP-1 (5 µM)	0.8 µl
STIA-HP-1 (5 µM)	0.8 µl
ST-HP-2^{-LC705} (5 µM)	0.8 µl
	= 19.0 µl

- ***ipa*H** (*ipa*H-plasmid EIEC and *Shigella*):

dH_2O	13.0 µl
HP mastermix	2.0 µl
$MgCl_2$ (25 mM)	1.6 µl
EIEC-1 primer (25 µM)	0.4 µl
EIEC-2 primer (25 µM)	0.4 µl
EIEC-HP-1 (5 µM)	0.8 µl
EIEC-HP-2^{-LC640} (5 µM)	0.8 µl
	= 19.0 µl

- ***ipaH* & *stx_1*** (*ipaH*-plasmind and *Stx_1*-gene in *Shigella dysenteriae* type 1):

dH_2O	11.4 µl
HP mastermix	2.0 µl
$MgCl_2$ (25 mM)	1.6 µl
EIEC-1 primer (3.125 µM)	0.4 µl
EIEC-2 primer (3.125µM)	0.4 µl
EIEC-HP-1 (5 µM)	0.4 µl
EIEC-HP-2^{-LC640} (5 µM)	0.4 µl
STEC-1 primer (25 µM)	0.4 µl
STEC-2 primer (25 µM)	0.4 µl
STECI-HP-1 (5 µM)	0.8 µl
STECI-HP-2^{-LC705} (5 µM)	0.8 µl
	= 19.0 µl

9. Controls:

- Positive controls:
  - EDL 933 (STEC O157:H7; ATCC 43895): positive for s*tx*$_1$, *stx*$_2$, *eae*, E-*hly* and *uidA*
  - S1191(STEC O139:H1; CDC); positive for *stx*$_{2e}$
  - 3157-02 (STEC O91:NM; CDC) positive for *stx*$_1$, *stx*$_2$-variant and E-*hly*
  - H10407 (ETEC O78:H11; ATCC 35401): positive for *LT*, *ST*$_p$ and *ST*$_h$
  - EDL 1284 (EIEC O124:NM); ATCC 43893): positive for *ipa*H
  - F1641 (*S. dysenteriae* type 1; CDC): positive for *ipa*H and *stx*$_1$

- Negative controls: negative DNA control (non-pathogenic *E. coli* strain) and no DNA (water) control

10. Place a required number of LightCycler capillaries in precooled LightCycler centrifuge adapters. Don't touch the capillaries with your fingers. We suggest you handle the capillaries with forceps. Use **18.0 µl of mastermix** per reaction. Add **2 µl of DNA template**. Place the cap on the capillary with the forceps and press the cap gently with the end of the forceps until the capillary is properly sealed. Now transfer the sealed capillaries in the LightCycler sample carousel. Work swiftly to avoid non-specific amplification due to warming of the PCR mastermixes.

11. Spin down the samples in the LightCycler carousel centrifuge 3 000 rpm 15 s. Inspect the capillaries for air bubbles or pipetting errors.

Note: if you don't have a LightCycler carousel centrifuge, spin down the samples in a regular tabletop centrifuge while the capillaries are still in precooled LightCycler centrifuge adapters. After centrifuging, transfer the capillaries in the LightCycler sample carousel

12. Place the carousel in the LightCycler

13. In the programming screen, click on "Open Experiment File" and select the program "ecolivir" / "*uidA*"

14. Click "RUN" → sample loading screen with "save" window will appear

15. Name the run and save it

- A practical way to name the run is to indicate the run date and the assay, for example "100803stx" or "100903eaehly". The run name will appear in the result printout.
- A practical way to organize the data is to create separate folders for each assay

16. Define the number of samples at maximum position

17. Click "Enter Samples Later" → the running screen will appear and the run will start

18. Click "Edit Samples" and enter the sample data (follow the instructions in the LightCycler™ Operator's Manual Version 3.5 in pages 50-53). After the sample ID:s have been typed in, click "Done"→ The running screen will reappear and you can view the results real-time

19. Analysis of the results (follow the instructions in the LightCycler™ Operator's Manual Version 3.5 in pages 59-85):

- After the run is done, the data analysis front screen will automatically appear
- Select an appropriate fluorescence display mode from data analysis front screen: F2/F1 and F3/F1 for for channels 2 and 3, respectively
- If you are running a multiplex, select the appropriate color compensation file by clicking "Select CC Data"
  Note: you can skip this step, if you saved the color compensation file as being ON as a default, when you created the experiment file
- Click "Select a Program" and from the pull down menu select either quantification or melting curve analysis
- By clicking either "Quantification" or "Melting curve" buttons you can proceed to quantification and melting curve analysis screens, respectively.

- **Quantification screen:**
  - Select "Fit Points" analysis method. Accept the defaults for "Baseline Adjustment " (Arithmetic) and "Number of Points" (2)
  - Click "Step 2: Noise Band" and adjust the noise band manually
  - Print the quantification screen for your record
  - The earlier the crossing point, the higher the number of copies of the target gene in the sample

- **Melting curve analysis screen:**
  - Accept the default for "Calculation Method" (Polynomial)
  - Click "Extra: Manual Tm"
  - Drag the cursors manually to determine T_m:s
  - Print the melting curve analysis screen for your record

- ***stx$_1$ & stx$_2$:***
  - Fluorescence for the targets measured at channels 2 and 3, respectively
    - ✓ Fluorescence variations within a run for *stx$_1$* (F2/F1): 0.01-0.09
    - ✓ Fluorescence variations within a run for *stx$_2$* (F3/F1): 0.01-0.02
  - Most *stx$_1$*-positive strains have a T_m of 68°C (EDL 933). Two variants with T_m: s of 62°C and 65°C have been reported
  - *stx$_2$*, *stx$_{2c}$* and *stx$_{2d}$* have a T_m of 71°C (EDL 933)
  - *stx$_{2e}$* has a T_m of 66°C (S1191)
  - Three variants for *stx$_2$* with T_m:s of 51°C, 55 °C (3157-02) and 63°C have been described

- ***eae* & E-*hly*:**
  - Fluorescence for the targets measured at channels 2 and 3, respectively
    - ✓ Fluorescence variations within a run for *eae* (F2/F1): 0.01-0.14
    - ✓ Fluorescence variations within a run for E-*hly* (F3/F1): 0.01-0.03
  - Six different T_m-groups for *eae* have been described: 56°C, 61°C, 62°C, 63/64°C, 65°C and 67°C. The T_m of EDL 933 is 67°C. The T_m:s of unknown isolates in any particular run should be determined in proportion to the T_m of the EDL 933 control.

- The T_m of E-*hly* is from 66-67 ^oC (EDL 933)

- *uidA*:
  - Fluorescence for the target measured at channel 2
    - ✓ Fluorescence variations within a run for *uidA* (F2) 0.45-0.9 and (F2/F1) 0.03-0.04
  - The T_m of *uidA* is from 66-67 ^oC (EDL 933)

- ***LT:***
  - Fluorescence for the target measured at channel 2
  - The T_m for *LT* is 63^oC (E2539-C1). A rare 61^oC variant has been described

- ***ST_h* and *ST_b*:**
  - Fluorescence for the target measured at channel 3
  - The T_m:s for prototype ***ST_h*** and ***ST_p*** are 64^oC and 57^oC (H10407), respectively. A 49^oC variant for *ST_p* has been described. *ST_h*-variants with T_m:s of 59^oC and 62^oC have been reported. If you are uncertain of the *ST*-subtype, rerun the samples with ***ST_h*** and ***ST_p***- primers separately in order to differentiate the two subtypes.

- ***ipa*H:**
  - Fluorescence for the target measured at channel 2
  - The T_m for *ipa*H is 67-68^oC

- ***ipa*H-*stx_1*- multiplex for *S. dysenteriae* type 1**
  - Positive for ipaH (T_m 67-68^oC) and *stx_1* (T_m 68^oC) at channels 2 and 3, respectively

20. Discard the capillaries in the sharps container

Note:

With the exception of *eae*-E*hly*-multiplex and *ipa*H-*stx_1*-multiplex, the protocols also work with LightCycler™ - FastStart DNA Master Hybridization probes (Roche Diagnostics; Cat. No. 2 239 272). *Eae* and E-*hly* (and *ipaH* and *stx_1*) will amplify if run separately, but not as a multiplex. If you prefer to use the FastStart kit, the following modifications will apply to the previous protocol:

- Keeping the mastermix cool is not required. Working at room temperature is acceptable.
- Amplification conditions for assays other than *uidA* (program "**ecolivirFS**"; the run takes about 1 h 10 min):
  - Initial denaturation 95^oC 10 min with temperature transition rate 20^oC / s
  - PCR: 40 cycles of denaturation 95^oC 10 s, annealing 50^oC 20 s, extension 72^oC 30 s; temperature transition rate 20^oC / s in each step
  - Melting curve analysis to determine the T_m values for the sequences targeted by the hybridization probes:
    a. Rapid denaturation 95^oC 0 s with temperature transition rate 20^oC / s
    b. 10 s hold at 40^oC with temperature transition rate 20^oC / s

c. Slow denaturation (95°C) with temperature transition rate of 0.2°C / s
- Cooling 40°C 2 min

- Amplification conditions for *uidA* (program "**uidAFS**"; the run takes about 50 min):
  - Initial denaturation 95°C 10 min with temperature transition rate 20°C / s
  - PCR: 40 cycles of denaturation 95°C 10 s, annealing 65°C 10 s, extension 72°C 20 s; temperature transition rate 20°C / s in each step
  - Melting curve analysis to determine the T_m values for the sequences targeted by the hybridization probes:
    a. Rapid denaturation 95°C 0 s with temperature transition rate 20°C / s
    b. 10 s hold at 55°C with temperature transition rate 20°C / s
    c. Slow denaturation (95°C) with temperature transition rate of 0.2°C / s
  - Cooling 40°C 2 min

Trouble shooting (see also the back of the LightCycler™ Operator's Manual):

The fluorescence level varies between the samples:

- Mastermix not mixed well enough before pipetting into capillaries
- Minor pipetting errors when dispensing the mastermix into capillaries ("carry-over") → change pipette tip between each capillary when dispensing the mastermix
- Higher than normal initial fluorescence level is characteristic for some template types (for example whole cell templates from broth cultures)

No amplification. Even the positive control is negative:

- Did you add each component of the mastermix?
- Check whether a new lot of any reagent was started in this run.

  Note: keep track of lot numbers, i.d. write down the lot number and the date when it was started. That might be helpful if you have to troubleshoot.

- Old primer or probe working stocks. Prepare fresh working stocks.
- If you suspect a problem with primers or probes, you can identify the problem by running the contents of the capillary in an agarose gel. (Place the capillary upside down in a 1.5 ml eppendorf tube leaving the lid open. Centrifuge briefly in a tabletop centrifuge. Add 4 µl of 5x loading buffer and load the whole volume.) If no amplification occurred, the primers are more likely to be the problem. If amplification occurred, but was not detected by LightCycler, probes are more likely to be the problem.
- Too much DNA used. Excessive amounts of DNA can be inhibitory for PCR.
- Old positive control template. DNA degrades over time, particularly when stored at fridge for an extended period of time or when frozen and thawed repeatedly. Allocate the positive control DNA and store at -20°C. Store the aliquot that you are working with at +4°C.
- The instrument is not working properly

  Note: it is advisable to run the self test once a day before making any runs. It only takes few minutes and tests the different functions of the LightCycler making sure for example that the equipment reaches the target temperature.

Amplification occurred but the fluorescence level is lower than normal

- Check that the probe working concentration was correctly calculated
- Bear in mind that the probe stock concentration could have been measured incorrectly by the manufacturer. Try increasing the probe concentration by two to four-fold.
- Old probe working concentrations. Dyes (LC-640, LC-705, fluorescein) degrade over time.

False positives (negative DNA and water positive with correct T_m) = contamination:

- Prepare fresh working concentrations and open new vials of water, $MgCl_2$ and HP-mastermix
- In order to minimize any contamination, set up and dispense mastermix in a clean hood where no DNA or cultures are handled. Decontaminate the hood every day using UV-light.
- Dedicate separate sets of pipettors for mastermix set up and preparation of DNA template
- Always use filtered tips
- Autoclave the sample carousel regularly and ALWAYS after a capillary breaks in the carousel. Remember also to autoclave the capillary releaser

Note: Occasionally, the automatic second derivative maximum method for quantification in the software 3.5 assigns positive and negative samples incorrectly. Use **fit points** method with baseline adjustment for quantification instead.

True false positives (= no contamination)

- Primer-dimers
- In rare cases, we have seen true false positives for *stx*$_1$ and *stx*$_2$.
- Ways to identify a false positive sample:
  - Typically, the crossing point for a false positive sample is late (after 37 cycles). However, a late crossing point can also indicate a very low target copy number in the sample, for example a mixed culture. Rerun by using purified DNA instead of whole cell template
  - The baseline of the false positive sample (before crossing point) is not even
  - T_m is incorrect
  - The shape of the melting curve is unusual: shallow, wide with no distinct peak.
  - Verify a suspected false positive sample either by running the contents of the capillary in an agarose gel or by using conventional PCR.

Fluorescence curves of some samples dip under the baseline in multiplex PCR:

- Your color compensation file is "overcompensating". This could be a problem with a freshly created color compensation file
  - Use fresh reagents to create a new file. Even if your color compensation kit is not expired the reagents may have degraded after repetitive thawing and freezing
  - Always create a new color compensation file after the LightCycler has been serviced and calibrated
- Problem with a particular template → rerun the assays separately instead of a multiplex

T_m of the positive control varies from run to run:

- Variation of 1^{o}C between the runs is acceptable

T_m of the positive control varies between different probe lots:

- We have noticed significant differences between identical probes prepared by different manufacturers
- Check that the probe sequence is correct. Even one base mismatch can destabilize the probe and change the T_m significantly or cause variation of the T_m for a target that is conserved.

A single isolate has two different peaks in the melting curve analysis:

- One isolate can harbor two different *stx*$_2$ variants
- In rare cases, ETEC can be positive for both *ST*$_h$ and *ST*$_p$

References:

Cebula, T. A., W. L. Payne, and P. Feng. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their shiga-like toxin type by mismatch amplification assay-multiplex PCR. J. Clin. Microbiol. 33, 248-250.

Reischl, U., M. T. Youssef, J. Kilwinski, N. Lehn, W. L. Zhang, H. Karch, and N. A. Strockbine. 2002. Real-time fluorescence PCR assays for detection and characterization of Shiga toxin. Intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli.* J. Clin Microbiol. 40, 2555-2565.

Reischl, U., M. T. Youssef, H. Wolf, E. Hyytiä-Trees, and N. A. Strockbine. 2003. Real-time fluorescence PCR assays for the detection and characterization of heat labile I and heat-stable I enterotoxin genes from enterotoxigenic *Escherichia coli*. Manuscript.

Appendix 1

Preparation of customized color compensation files

If the universal color compensation file created with the Roche Color Compensation Set does not work in a satisfactory manner (i.e. frequent overcompensation = fluorescence dips under the baseline), you might want to try using assay specific color compensation files

1. Mastermixes
<u>*Stx*$_1$-*stx*$_2$ –multiplex</u>

	1 reaction	2 reactions
Capillary 1 = Blank		
dH_2O	14.6 µl	29.2 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
STEC-1 primer (25 µM)	0.4 µl	0.8 µl
STEC-2 primer (25 µM)	0.4 µl	0.8 µl
	= 19.0 µl	= 38.0 µl
Capillary 2 = Fluorescein		
dH_2O	13.0 µl	26.0 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
STEC I-HP1 (5 µM)	0.8 µl	1.6 µl
STEC II-HP1 (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl
Capillary 3 = LC Red 640		
dH_2O	13.8 µl	27.6 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
STEC I-HP2^{-LC640} (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl
Capillary 4 = LC Red 705		
dH_2O	13.8 µl	27.6 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
STEC II-HP2^{-LC705} (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl

eae-EhlyA-multiplex

Capillary 1 = Blank	1 reaction	2 reactions
dH_2O	14.2 µl	28.4 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
eaeA-R (25 µM)	0.4 µl	0.8 µl
hlyA-F (25 µM)	0.2 µl	0.4 µl
hlyA-F (25 µM)	0.2 µl	0.4 µl
	= 19.0 µl	= 38.0 µl
Capillary 2 = Fluorescein		
dH_2O	12.6 µl	25.2 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
hlyA-F (25 µM)	0.2 µl	0.4 µl
hlyA-R (25 µM)	0.2 µl	0.4 µl
eaeA-HP1 (5 µM)	0.8 µl	1.6 µl
hlyA-HP1 (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl
Capillary 3 = LC Red 640		
dH_2O	13.4 µl	26.8 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
hlyA-F (25 µM)	0.2 µl	0.4 µl
hlyA-R (25 µM)	0.2 µl	0.4 µl
eaeA-HP2 (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl
Capillary 4 = LC Red 705		
dH_2O	13.4 µl	26.8 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
eaeA-F (25 µM)	0.4 µl	0.8 µl
hlyA-F (25 µM)	0.2 µl	0.4 µl
hlyA-R (25 µM)	0.2 µl	0.4 µl
hlyA-HP2 (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl

ipaH-stx-multiplex

Capillary 1 = Blank	1 reaction	2 reactions
dH_2O	13.8 µl	28.4 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
EIEC-1 (3.125 µM)	0.4 µl	0.8 µl
EIEC-2 (3.125 µM)	0.4 µl	0.8 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
	= 19.0 µl	= 38.0 µl

Capillary 2 = Fluorescein	1 reaction	2 reactions
dH_2O	12.6 µl	25.2 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
EIEC-1 (3.125 µM)	0.4 µl	0.8 µl
EIEC-2 (3.125 µM)	0.4 µl	0.8 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
EIEC-HP1 (5 µM)	0.4 µl	0.8 µl
STEC I-HP1 (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl

Capillary 3 = LC Red 640	1 reaction	2 reactions
dH_2O	13.4 µl	26.8 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
EIEC-1 (3.125 µM)	0.4 µl	0.8 µl
EIEC-2 (3.125 µM)	0.4 µl	0.8 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
EIEC-HP2 (5 µM)	0.4 µl	0.8 µl
	= 19.0 µl	= 38.0 µl

Capillary 4 = LC Red 705	1 reaction	2 reactions
dH_2O	13.0µl	26.0 µl
HP mastermix	2.0 µl	4.0 µl
$MgCl_2$ (25mM)	1.6 µl	3.2 µl
EIEC-1 (3.125 µM)	0.4 µl	0.8 µl
EIEC-2 (3.125 µM)	0.4 µl	0.8 µl
STEC-1 (25 µM)	0.4 µl	0.8 µl
STEC-2 (25 µM)	0.4 µl	0.8 µl
STEC I-HP2 (5 µM)	0.8 µl	1.6 µl
	= 19.0 µl	= 38.0 µl

2. Dispense the mastermixes in their individual capillaries: 18 µl / capillary
3. Capillary 1 : add 2 µl dH_2O
   Capillaries 2-4: add 2 µl EDL933 DNA template (*stx*$_1$-*stx*$_2$-multiplex and *eae*-*EhlyA*-multiplex) or 2 µl F1641 DNA template (*ipaH*-*stx*-multiplex)
4. Amplification: use the program "ecolivir" or "ecolivirFS" for standard and FastStart reagents, respectively
5. Save the color compensation file
    a. At the data analysis front screen, select the program segment that includes melting program (segment 3)
    b. From the pull down menu, select "Color Compensation"
    c. From the Color Compensation menu, select "Calibration"
    d. Name and save the color compensation file

Note: you have to create new color compensation files after the instrument has been serviced or calibrated and every time you get in new primer or probe lots