Implementation of E. coli STEC analysis according to ISO standard 13136:2012

DVFA Laboratory

Section for Microbiology

Danish Veterinary and Food Administration

Background for implementation of ISO method

- DTU National Food Institute appointed as NRL in Denmark (for feed and food) for *E.coli* STEC
- Laboratory function delegated to DVFA (Danish Veterinary and Food Administration) as the reference laboratory holding accreditation
- At the moment, the ISO standard 13136:2012 has not been implemented for the analysis of *E.coli* STEC
- <u>Aim of this presentation:</u>
  - Share our considerations when setting up the ISO method
  - Present the results from initial tests

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Current setup for detection of E.coli STEC

- DuPont commercial Bax system for detection of STEC
- No accreditation
- DVFA have participated in EURL proficiency tests since implementation of Bax system

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Implementation of ISO 13136:2012

- Why implement ISO 13136?
  - NRL expected to have an ISO reference method
  - Possibility to add new serotypes (e.g. O104) to the setup
  - Allign with the procedures used by other laboratories
    - Profiency tests
  - Obtain accreditation from DANAK (The Danish Accreditation Fund)

EU Reference Laboratory for *E. coli*
Department of Food Safety, Nutrition and Veterinary Public Health
Microbiological Food safety and Food-borne Diseases Unit
Istituto Superiore di Sanità

Detection and identification of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 in food by Real Time PCR

1. Aims and field of application:

The large outbreak of STEC infections in Germany was caused by a STEC strain belonging to serotype O104:H4, a serotype (and serogroup), not comprised among those usually associated with severe infections in Europe and worldwide.

The characteristics of the outbreak strain are the following:

1. It produces Stx2, and harbors the *stx2a* gene subtype.
2. It lacks the gene coding for the adherence factor intimin (*eae* gene), which is considered as a hallmark of the pathogenic STEC.
3. It possesses the genetic markers of typical of Enteroaggregative *Escherichia coli* (EAggEC): the *aggR*, *aatA*, *aaiC* and *aap* genes.

Therefore, the specific genetic markers to be considered for the molecular screening of *stx*-positive enrichment cultures are the O104 antigen-associated gene wzx_{O104} and the gene encoding the H4 flagellar antigen, $fliC_{H4}$.

The markers associated with the enteroaggregative adhesion should be considered for confirmation and characterization of the isolated strains. A laboratory method for such tests is available at the EURL-VTEC website.

The proposed method aims at the identification of the presence of O104 antigen-associated gene (wzx_{O104}) in *stx*-positive enrichment cultures. The molecular design of this Real Time PCR has been described in the literature

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EURL-VTEC_Method_04_Rev 2 04/03/2021

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Initial studies before implementation

- Detection of stx1, stx2 and eae genes
  - O157 and O111
  - ATCC (stx1-, stx2- and eae-negative)
- Template DNA concentrations
  - Boiling lysate for extracting DNA
  - Concentrations tested: 10 ng, 1 ng, 0.1 ng and 0.01 ng
  - Calculate PCR efficienies and linearity
- Testing of an Internal Amplification Control (IAC)
  - Commercial IAC from ThermoFisher tested

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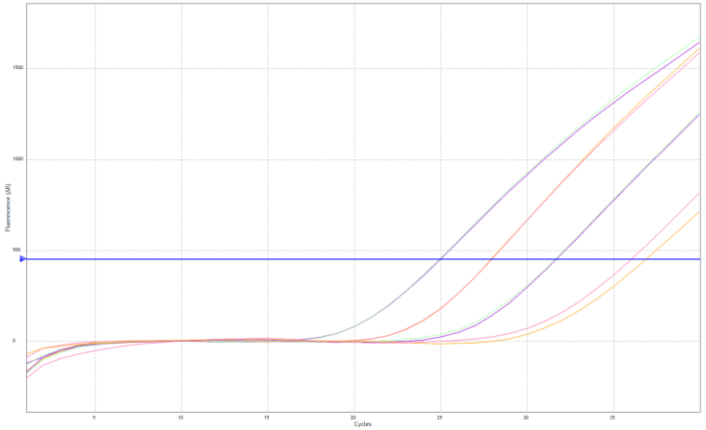
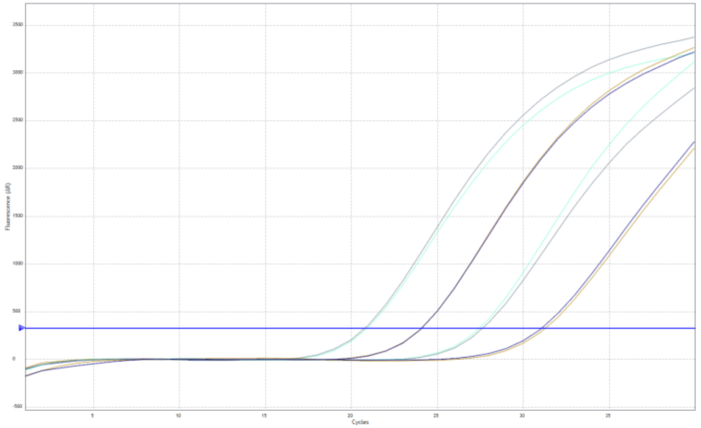
Detection of stx1, stx2 and eae genes

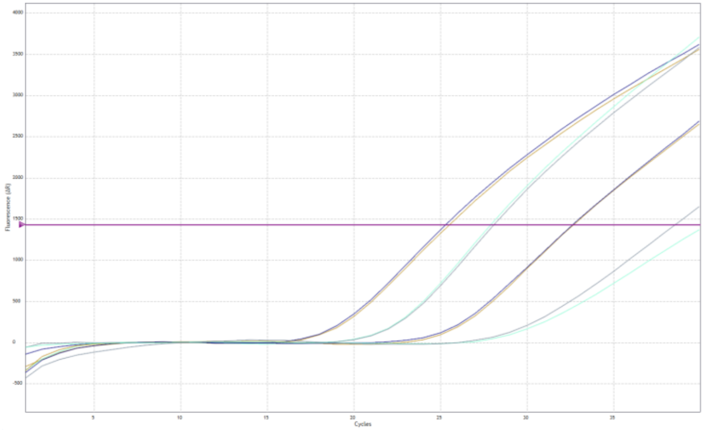
- PCR setup:
  - Duplex for stx1 and stx2
  - Singleplex for eae
- O111: stx1, stx2 and eae detected
- O157: no signal for stx1 and stx2 (eae detected)
- Optimisations:
  - Annealing temperatures
  - Primer/probe concentrations
  - Choice of mastermix and concentrations
  - DNA purification



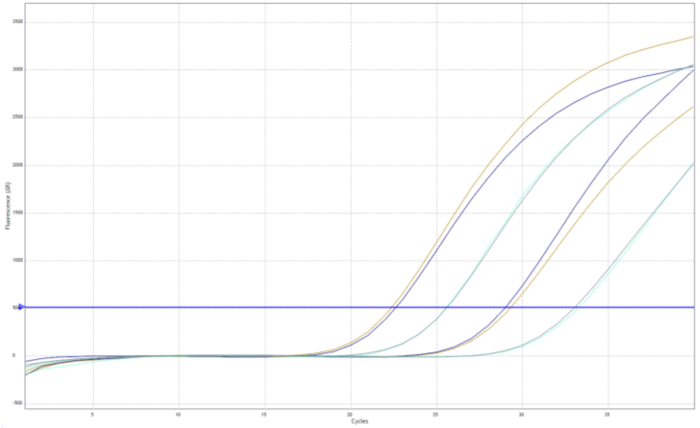
O111: stx1



O111: eae



O111: stx2



O157: eae

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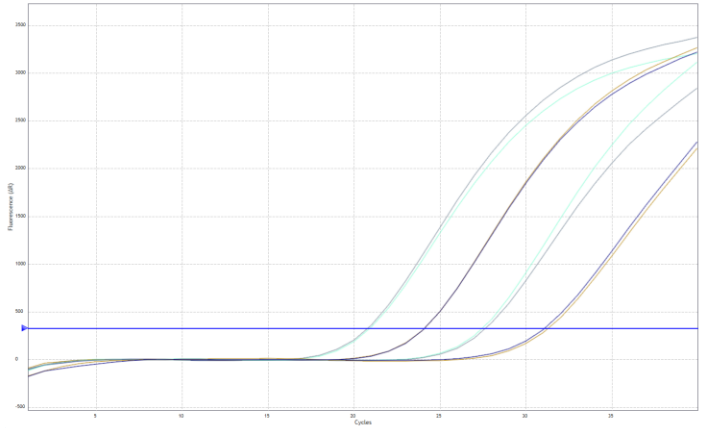
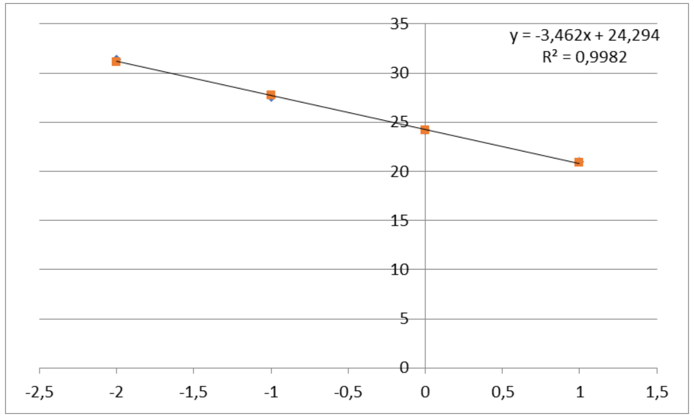
Test of DNA concentrations – O111 eae

- Boiling lysate for extracting DNA
  - Concentrations tested: 10 ng, 1 ng, 0.1 ng and 0.01 ng
- PCR efficiency: 94.5 %
- R^2: 0.9982

O111: eae





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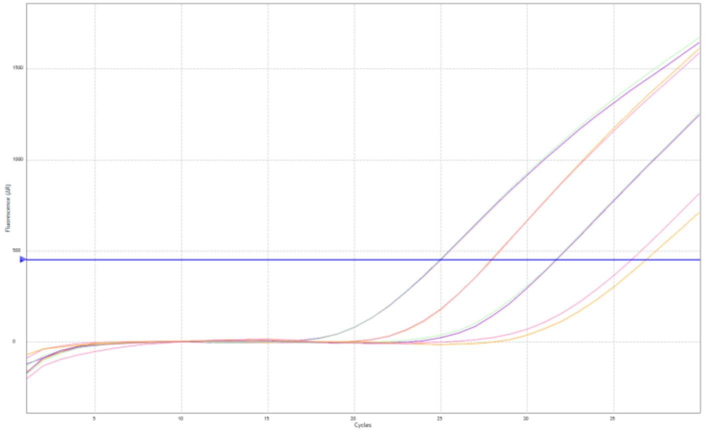
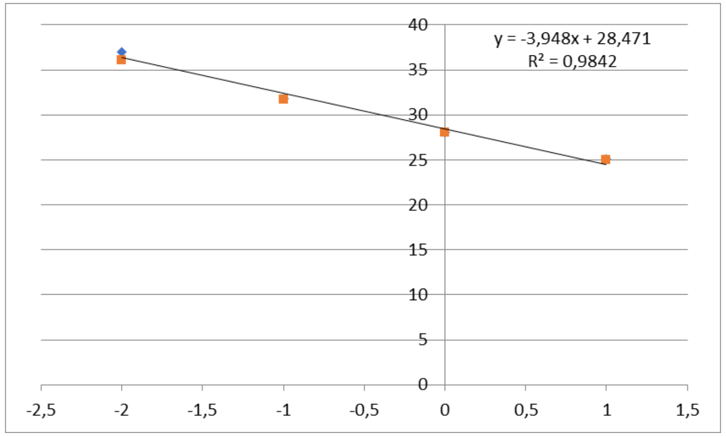
Test of DNA concentrations – O111 stx1

- Boiling lysate for extracting DNA
  - Concentrations tested: 10 ng, 1 ng, 0.1 ng and 0.01 ng
- PCR efficiency: 79.2 %
- R^2: 0.9842

O111: stx1





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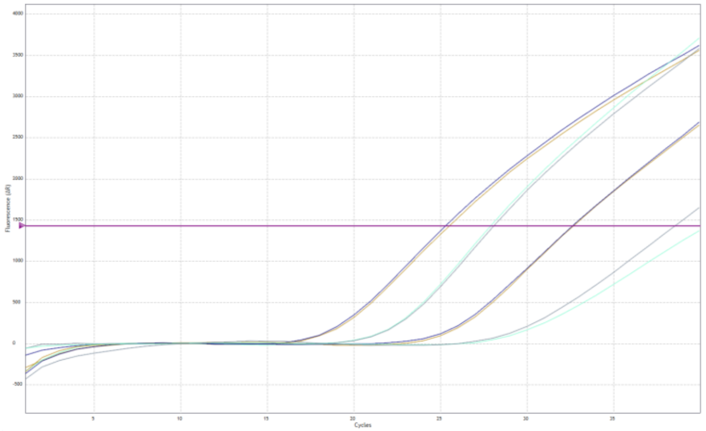
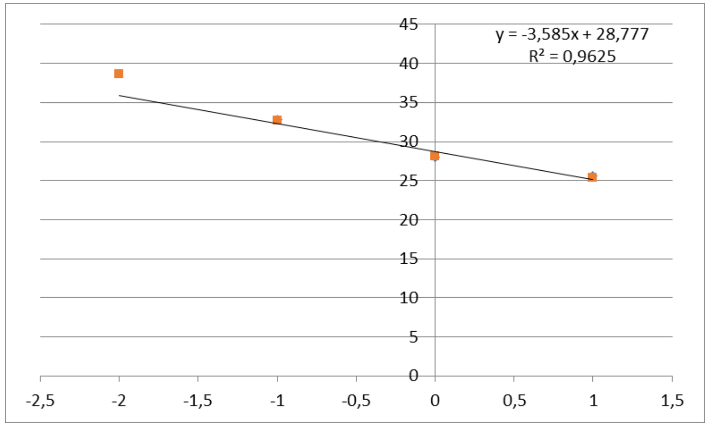
Test of DNA concentrations – O111 stx2

- Boiling lysate for extracting DNA
  - Concentrations tested: 10 ng, 1 ng, 0.1 ng and 0.01 ng
- PCR efficiency: 90.1 %
- R^2: 0.9625

O111: stx2





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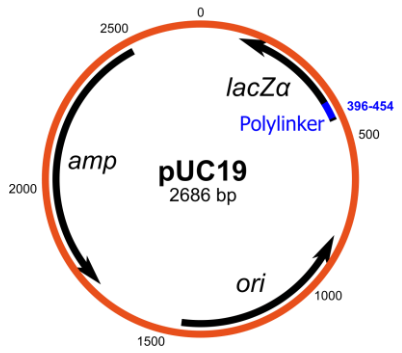
Internal Amplification Control

- 3 differerent options according to ISO standard 13136:2012:
  1. TaqMan® exogenous internal positive control. Reagent kit containing probe, IAC target DNA and blocking solution.
     - At DVFA we have investigated this solution initially
  2. Open formula pUC19 plasmid based internal amplification control IAC.
  3. A recombinant plasmid (pIAC-STEC) containing the stx1/stx2 gene
- Option 2. and 3. may also be used as an extraction control
  - DNA purification step



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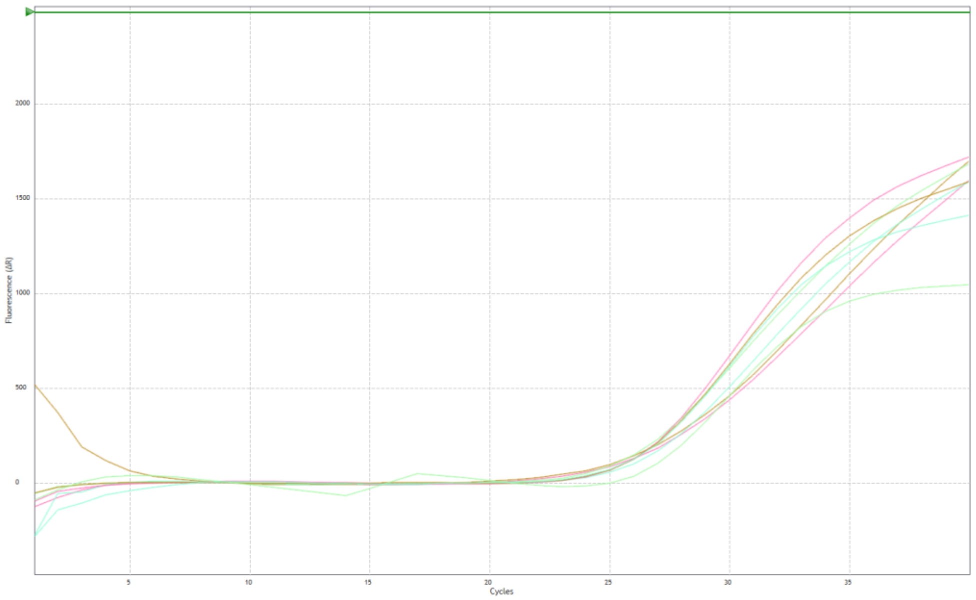
Internal Amplification Control

- TaqMan™ Exogenous Internal Positive Control Reagent from ThermoFisher Scientific
  - Contains a pre-optimized internal positive control (IPC) with pre-designed primers and TaqMan™ probe.
  - Spiked into samples to distinguish true target negatives from PCR inhibition.
  - The concentration of the IPC primers in the PCR reaction is limiting so that the amplification efficiency of the target reaction is not compromised.
- ThermoFisher recommends end-point readings (amplification/no amplification) based on their properitary PCR machines



Points to consider

- **Sample Preparation**:
  - Extract DNA from the food matrix of interest using an appropriate extraction method.
  - Test of different kits for DNA purification
- **Controls (according to ISO 22174)**:
  - Process controls
  - Amplification controls
  - Negative extraction control
  - PCR controls
- **PCR:**
  - **Reaction Setup**:
    - Concentrations of primers, DNA template, buffer, dNTPs, and DNA polymerase.
  - **Thermal Cycling Conditions**:
    - Establish and optimize the PCR cycling parameters (e.g., denaturation, annealing, and extension temperatures, as well as cycle number).
  - **Amplification Efficiency and Sensitivity**:
    - Determine the amplification efficiency by running a dilution series of known DNA concentrations to assess linearity and sensitivity.

Verification plan

- **According to ISO 22118:**
  - **Specificity Testing**:
    - Perform specificity testing to confirm that the primers amplify only the target sequence.
  - **Reproducibility and Precision**:
    - Perform replicates of the assay using different operators, instruments, and on different days to assess reproducibility.

Conclusion

- **Detection of stx1, stx2 and eae genes**
  - Optimisations of PCR reactions needed for stx1 and stx2
  - Include ROX reference dye
- **PCR efficiencies and linearity accessed**
- **Internal Amplification Control (IAC)**
  - One of the options listed in ISO was successfully tested
  - Investigate one of the other options in order to include an extraction control
- **Recommendations and advices from you are more than welcome ☺**

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