DNA DIAGNOSTIC

User Manual

Enterit 4 calves B

DNA extraction - qPCR - Interpretation

USER MANUAL
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1. PURPOSE OF THE TEST

Calf diarrhea also known as scours, is a major problem in dairy and beef productions because it can result in serious economic implications. The calves can die in severe cases, however calves that survive will have poorer performance compared to healthy calves. Several pathogens are associated with calf diarrhea including bacteria, viruses and parasites.

Enterit 4 calves B is a fast, reliable and easy-to-use method for the detection of 4 major bacterial pathogens (*Clostridium perfringens*, *Clostridium perfringens* type B and C, *Salmonella* Dublin and *Escherichia coli* F5) linked to calf diarrhea. Using a simple 96 Deep Well-based extraction protocol followed by an accurate, sensitive and highly specific qPCR reaction, the entire protocol from DNA extraction to result can be carried out in 3 hours and 30 minutes.

2. PRINCIPLE OF THE TEST

Enterit 4 calves B allows for the detection of *Clostridium perfringens*, *Clostridium perfringens* type B and C, *Salmonella* Dublin and *Escherichia coli* F5 in feces samples from calf. It includes the following three main steps:

The *DNA extraction step* uses the extraction kit for DNA/RNA extraction from 0.1 g of feces sample. The sample is dissolved in resuspension buffer and transferred to a plate containing bead solution and then mixed, spun and washed. The DNA is extracted by the lysis mix buffers at 37 °C for 20 minutes following by 95°C for 15 minutes and debris are pelleted by centrifugation. Now the DNA is in the supernatant.

The PCR step uses 5 µL of DNA template which is added to a 96 well qPCR plate containing E4CB qPCR master mix. The qPCR reaction runs for approximately 52 minutes. Each qPCR reaction contains four sets of primers and fluorescence probes for specific detection of three bacterial pathogens. A fifth set of primers and probe detects an internal amplification control (IAC). Each of the four probes emit a specific light (color) enabling the identification of bacteria present in the sample. The tests use the standard 96 well plate format and ready-to-use reaction mixes. The test requires only pipettes, a plate mixer, a vacuum aspirator, a centrifuge, incubation oven, a thermal heating block and a qPCR instrument.

The *interpretation step* involves inspection of the amplification plots. The functionality of the qPCR reaction is controlled by the presence of IAC amplicons resulting in an ATTO signal with a Ct value at 27-32. The CY5, ROX, HEX and FAM signals ≤37 show the sample is positive for *Clostridium perfringens*, *Clostridium perfringens* type B and C, *Salmonella* Dublin and *Escherichia coli* F5 amplicons, respectively. No Ct value or Ct > 37 indicate that the sample is negative.

3. KIT COMPONENTS AND STORAGE

The Enterit 4 calves B contains material for testing 384 samples.

The Enterit 4 calves B contains **one box** for storage at room temperature (RT) and **one box** for storage at -20ºC:

BOX	COMPONENTS	AMOUNT	STORAGE
I	Buffer F	5 bottle of 110 mL	RT
	96 deepwell plates (2mL), empty	4 x 96 Deep Well Plate	RT
	Sealed 96 Deep Well Plates with bead solution	4 x 96 Deep Well Plate	RT
	Wash Buffer	4 bottle of 110 mL	RT
	Buffer P	2 bottles of 30 mL	RT
	96 Well Plates (0.2 mL clear tubes) with cap	4 x 96 well plate (0.2 mL clear tubes) with cap	RT
	Adhesive seals for Deep Well plates	8 pieces	RT
	Silicone seals	8 pieces	RT
	Cap mats for the qPCR 96 Well Plates	2x 96 cap for qPCR tube	RT
II	Lysis Buffer-I	2 bottle of 12 mL	-20ºC
	Mix I Additive for Lysis Buffer-I	2 tube of 1334 µL	-20ºC
	Sealed 96 Well Plates (0.2 mL white tubes) with E4CB qPCR Master Mix	4 x 96 well plates	-20ºC

4. REQUIRED EQUIPMENT AND MATERIALS NOT INCLUDED IN THE KITS

- Centrifuge for 96 well plates. Centrifuge **must be capable of running at 5000xg.**
  Example: Heraeus Multifuge X3R Centrifuge with HIGHPlate 6000 rotor.

- Plate washer / vacuum system capable of aspirating supernatant from Deep Well plates.
  Recommended: Bio-Rad DW40 plate washer.
  Alternative: Vacuum system with pipette tips (35mm from tip to rim stop).

- qPCR instrument, Stratagene Mx3005 with filters for ATTO (440nm-492nm), FAM (492nm-516nm), HEX (535nm-555nm), ROX (585nm-610nm), CY5 (635nm-665nm).

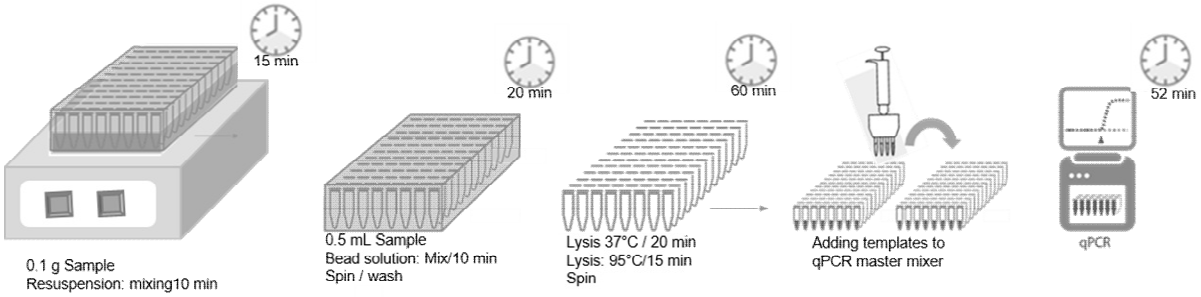
- Vortexer for 96 well plates.

- Ice bucket or 0-4°C cooling block.

- Standard lab pipettes and sterile filter tips.

- Mixer for a Deep Well plate.

5. PROTOCOL



DNA extraction:

1. Add 1.2 mL Buffer F to a well in the empty 2 mL deep well plate (or single 2 mL tubes).
2. Homogenize the feces sample and transfer 0.1g ± 0.03g feces to a corresponding well of the 96 well plate (or 2 mL tube) with buffer F.
   Note: If feces sample is liquid or watery, transfer 100 µL of sample.
3. Seal wells with silicone seal and mix vigorously for 10 minutes on a Deep Well mixer with even pressure on top of the seal to avoid cross contamination. Check that samples are completely homogenized, otherwise prolong mixing time or intensity.
   Note: The pressure on top of the seal can be achieved with a solid plastic square (top) and a rubber mat (bottom), both the size of the deep well plate, secured with rubber bands to the deep well plate.
4. Spin Deep Well plate at 1000x*g* for 1 minutes.
5. Carefully open Deep Well and discard the seal. Transfer 500 µL of supernatant to a 96 Deep Well plate (or 2 mL tubes) containing bead solution. Seal the plate with adhesive seal.
6. Spin Deep Well plate containing bead solution and samples at 5000x*g* for 5 minutes.
7. Remove supernatant with the plate washer. It is important to remove the supernatant completely. Be careful not to touch or remove the pellets.
   Note: It is important to remove all supernatant from the pellet. Therefore, correct adjustment of the plate washer syringes is required.
8. Add 1 mL Wash Buffer to Deep Well plate.
9. Cover wells with adhesive seal and vortex briefly.
10. Centrifuge the plate at 5000xg for 5 minutes.
11. Remove supernatant with the plate washer. It is important to remove the supernatant completely. Be careful not to touch or remove the pellets.
    Note: It is important to remove all supernatant from the pellet. Therefore, correct adjustment of the plate washer syringes is required.
12. Prepare fresh Lysis-I Mix by adding 6 µL Mix I additive to 54 µL Lysis Buffer-I and 60 µL *Buffer P*.

Lysis-I Mixer	1 reaction	9 reaction	50 reaction	100 reactions
Lysis Buffer-I	54 µL	486 µL	2700 µL	5400 µL
Mix I additive	6 µL	54 µL	300 µL	600 µL
Buffer P	60 µL	540 µL	3000 µL	6000 µL
Total	120 µL	1080 µL	6000 µL	12000 µL

13. Add 120 µL Lysis-I Mix, to each pellet and cover with new Silicone seal. Mix vigorously for 15 minutes on a Deep Well mixer with even pressure on top of the seal to avoid cross contamination.
    Note: The pressure on top of the seal can be achieved with a solid plastic square (top) and a rubber mat (bottom), both the size of the deep well plate, secured with rubber bands to the deep well plate

14. Spin down shortly to bring the liquid to the bottom (e.g. 10 seconds at 1500 x g). Transfer of 60 µL (up to 120 µL) from each sample into the corresponding tubes in the 96 well plate (clear 0.2 mL) and close the tubes with the caps. Avoid cross contamination.

15. Incubate the 96 well plate at 37°C for 20 minutes and at 95°C for 15 minutes.

16. Cool the 96 well plate on ice for 5 minutes
    Note: The incubation at 37°C and 95°C can be done using a PCR instrument programmed: 37°C for 20 min. → 95°C for 15 min. → 4°C for 5 minutes.

17. Centrifuge the plate at 5000xg for 5 minutes.

    If lysate sample will subsequently be used for virus/parasites detection, follow the step 18 on Enterit 4 calves V manual protocol.

18. Carefully transfer 5 µL of each aqueous phase directly to the corresponding well of the 96 well plate with E4CB qPCR Master Mix in step 21.
    Note: The remaining DNA can be stored at -20°C for long time storage.

qPCR analysis:

19. Take a E4CB qPCR master mix plate from freezer and thaw it on ice. Briefly spin the qPCR plate to collect the qPCR master mix in the bottoms.

20. Remove and discard the seal from qPCR plate.

21. Carefully transfer 5 µL supernatant from the 96 well 0.2 mL plate (step 18) to the corresponding well in the E4CB qPCR master (white 0.2 mL) mix plate.
    Note: It is important to keep Master Mix on ice while loading template and to run the qPCR within 15 minutes.

22. Carefully cover the qPCR reaction plate with new lids and spin at 1000-1500xg for 30 seconds to 1 minute.

23. Insert qPCR plate into the qPCR instrument.

24. Run the qPCR reaction using the instrument setting and the running program below:

Instrument settings for the MX3005P qPCR machine

Filter Gain Settings:

ATTO	CY5	ROX	HEX	FAM
1x	1x	1x	1x	2x

Note: If the raw data signal (R) is lower than 5000 for a color at the cycles 7-11 then increase the filter gain setting during the next runs resulting in a raw data signal (R) of 5000.

The guidelines for setting baseline and threshold should be checked manually to ensure that threshold lines are in the exponentially area of the amplification plot and above the background.

Threshold Fluorescence: Select “Background Based Threshold” to cycles 7-11 and set the “Sigma multiplier” to 10.

To avoid bias of threshold wells including amplification curve with Ct<15 is flag out. The threshold is then looked based on the rest of the sample and the sample with Ct<15 are put into the analysis again.

Baseline Correction: Select “Adaptive baseline”.

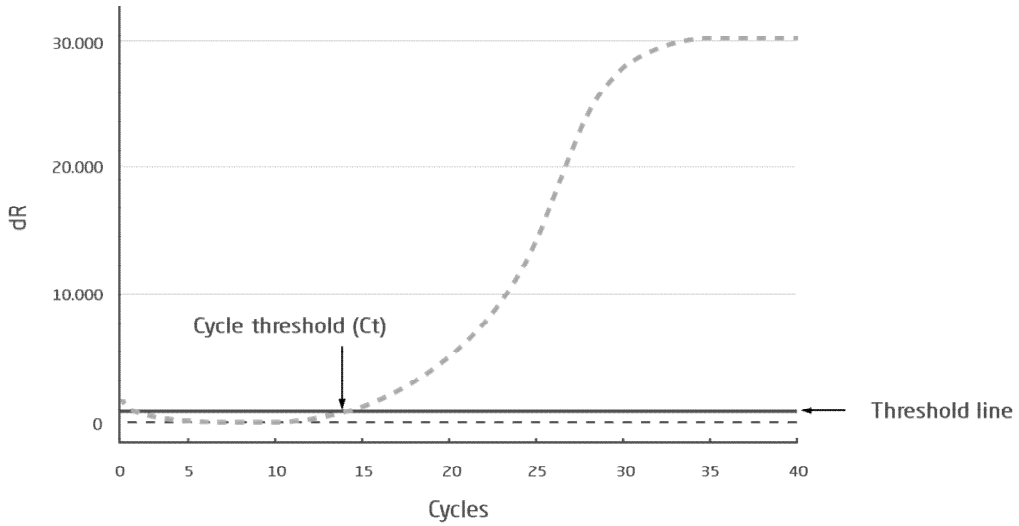
Activate the following filters: ATTO, CY5, ROX, HEX, FAM.

qPCR running program

Condition	Temperature	Duration	Number of cycles	Stage
Heat activation	95ºC	1 min	1	Pre-incubation
Denaturing	95ºC	5 sec	40	Amplification
Annealing/extension	60ºC	25 sec*		

** Read fluorescence for ATTO, FAM, HEX, ROX, CY5 at the end of each annealing/extension step.*

Interpretation of analysis:



Amplification plot with the number of PCR cycles is shown on the x-axis, and the background subtracted fluorescence from the amplification reaction is shown on the y-axis. The threshold line is used to find the Ct value, where it intersects the amplification curve when the fluorescence increases due to PCR amplification. The threshold line is calculated automatically by the instrument giving in this example a Ct value of 14.

25. Check amplification plot for ATTO signals and the signals should be present in all reactions. The Ct must be in the interval 27-32 to confirm the qPCR reaction is functional.
    Note: ATTO Ct can be above 32 or missing if the Ct for one or more of FAM, HEX, ROX, CY5 is low. Then the DNA sample should be diluted 5-10 times and a new qPCR reaction should be analyzed using this diluted DNA. A dilution of 10 times will theoretically move Ct values 3.3 higher. Reactions with no FAM, HEX, ROX, CY5 signal and ATTO signal absent or outside the interval 27-32 ***must be retested****.*

26. Check amplification plot for CY5, ROX, HEX, FAM signals and interpret the results as described in the table below.

Target	Filter	Positive	Negative	Retest
		Ct	**Ct**	**Ct**
Clostridium perfringens	CY5	≤37	>37 or absent	
Clostridium perfringens type B,C	ROX	≤37	>37 or absent	
Salmonella Dublin	HEX	≤37	>37 or absent	
Escherichia coli F5	FAM	≤37	>37 or absent	
IACB	ATTO	≤32		>32 or absent

Note: Sample with Ct values below 37 are considered as true positive.
Sample with Ct values above 37 should be considered negative.

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DNA Diagnostic A/S was established in 1992. DNA Diagnostic A/S is an ISO 13485 certified developer, manufacturer, and worldwide supplier of PCR based in vitro diagnostic kits.

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