DNA
DIAGNOSTIC

User Manual

Enterit 4 calves BV

DNA/RNA extraction - cDNA synthesis - qPCR - Interpretation

USER MANUAL
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DNA Diagnostic A/S
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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 sever 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 BV is a fast, reliable and easy-to-use method for the detection of the pathogenic bacteria, viruses and parasites (*Clostridium perfringens*, *Clostridium perfringens* type B and C, *Salmonella* Dublin, *Escherichia coli* F5, Bovine rotavirus, Bovine coronavirus, *Cryptosporidium parvum* and Eimeria species) linked to calf diarrhea. Using a simple 96 deepwell-based extraction protocol followed by a sensitive and highly specific qPCR reaction, the entire protocol from DNA/RNA extraction to result can be carried out in 5 hours.

2. PRINCIPLE OF THE TEST

Enterit 4 calves BV allows for the detection of *Clostridium perfringens*, *Clostridium perfringens* type B and C, *Salmonella* Dublin, *Escherichia coli* F5, Bovine rotavirus, Bovine coronavirus, *Cryptosporidium parvum* and Eimeria species in feces samples from calves. It includes the following four main steps:

The DNA/RNA 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 cDNA synthesis step uses 5 µL of DNA/RNA template which is added to a well in a 96 well plate containing ready to use RT cDNA master mix. The cDNA synthesis reaction runs for 1 hour and 15 minutes.

The PCR step uses 5 µL of cDNA template which is added to a 96 well plate containing E4CV qPCR master mix for detection of vial and parasitic pathogens. For detection of bacterial pathogens, 5 µL of DNA template is added to a 96 well plate containing E4CB qPCR master mix. The qPCR reaction run for 52 minutes. Each qPCR reaction contains four sets of primers and fluorescence probes for specific detection of pathogenic bacteria, viruses and parasites. 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, virus or parasites 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. No Ct value or Ct > 37 indicate that the sample is negative.

3. KIT COMPONENTS AND STORAGE

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

The Enterit 4 calves BV contains **one box** for storage at room temperature (RT) and **three box** for storage at -20°C:

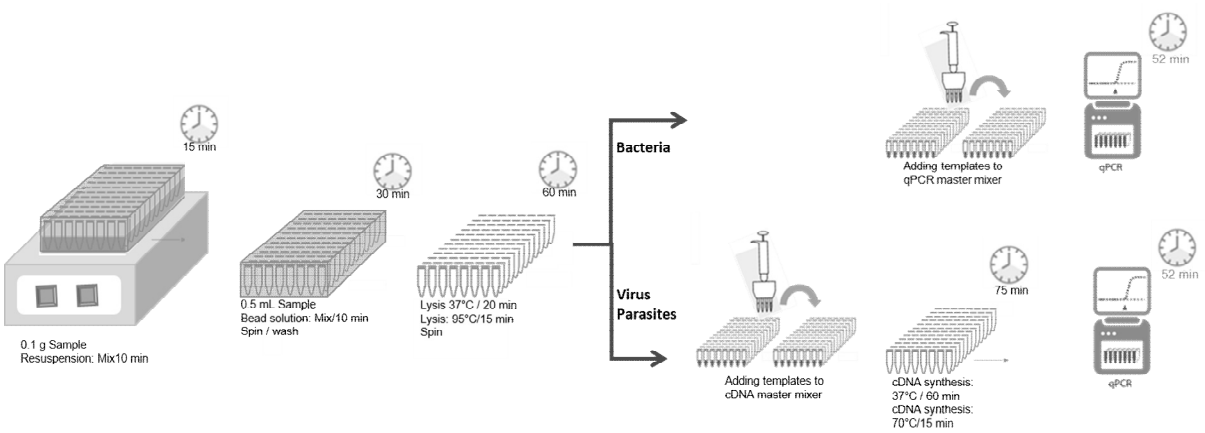
BOX	COMPONENTS	AMOUNT	STORAGE
I	Buffer F	5 bottle of 110 mL	RT
	96 deepwell plates (2mL), empty	4 x 96 Deep Well Plate	RT
	Sealed 96 Deep Well Plates with bead solution	4 x 96 Deep Well Plate	RT
	Wash Buffer	4 bottle of 110 mL	RT
	Buffer P	2 bottles of 30 mL	RT
	96 Well Plates (0.2 mL clear tubes) with 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 PCR 96 Well Plates	12x 96 cap for PCR tube	RT
II	Lysis Buffer-I	2 bottle of 12 mL	-20°C
	Mix I Additive for Lysis Buffer-I	2 tube of 1334 µL	-20°C
	Sealed 96 Well Plates (0.2 mL white tubes) with E4CB qPCR Master Mix	4 x 96 well plates	-20°C
III	Sealed 96 Well Plates (0.2 mL white tubes) with E4CV qPCR Master Mix	4 x 96 well plates	-20°C
IV	Sealed 96 Well Plates (0.2 mL clear tubes) with cDNA Master Mix I	4 x 96 well plates	-20°C

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 Deepwell 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^{o}C cooling block.
- Standard lab pipettes and sterile filter tips.
- Mixer for a Deep Well plate.

5. PROTOCOL



RNA/DNA extraction:

1. Add 1.2 mL Buffer F to a well in the empty 2 mL tubes.
2. Homogenize the feces sample and transfer 0.1g ± 0.03g feces to a corresponding 2 mL tube with buffer F.
   Note: If feces sample is liquid or watery, transfer 100 µL of sample.
3. Close the tube and mix vigorously on a mixer/vortexer until samples are homogenized, otherwise prolong mixing time/intensity.
4. Spin tube at 1000x*g* for 1 minute. Spin can be performed in the tube directly, or by transferring of all sample to a 2 mL Deep Well plate.
5. Carefully open the lid of the 2 mL tube or the seal of 2 mL Deep Well plate containing sample. Transfer 500 µL of supernatant to a 96 Deep Well plate containing bead solution. Mix by pipette up and down. 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 briefly on a Deep Well mixer.

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.
    Note: Due to the instability of RNA, it is recommended to perform the cDNA synthesis immediately before storing samples. **For cDNA synthesis follow step 19-25.**

18. Carefully transfer 5 µL of each aqueous phase directly to the corresponding well of the 96 well plate with cDNA master mix I (step 21). The rest of lysate is kept for E4CB qPCR in step 28. The remaining lysate can be stored at -20°C until use or for long time storage.

cDNA Synthesis (step 19-25 is performed for detection of viral and parasitic pathogens):

19. Take a cDNA master mix I plate from freezer and thaw it on ice. Briefly spin the plate to collect the cDNA master mix I in the bottom.

20. Remove and discard the seal from cDNA master mix plate.

21. Carefully transfer 5 µL supernatant from the 96 well 0.2 mL plate from step 18 to the corresponding well in the cDNA master mix I plate (clear 0.2 mL).

22. Incubate the cDNA reaction at 37°C for 60 minutes.

23. Inactivate Reverse Transcriptase enzyme by heating to 70°C for 15 minutes.

24. Cool the cDNA reaction tube on ice for 1 minute and spin it again for 1 minute to collect condensate.
    Note: The cDNA samples can be kept at -20°C for long term storage.

25. Carefully transfer 5 µL supernatant from the 96 well 0.2 mL clear plate to the corresponding well in the E4CV qPCR master mix plate (step 28).
    Note: It is important to keep Master Mix on ice while loading template and to run the qPCR within 15 minutes.

qPCR analysis:

26. Take a E4CB and E4CV qPCR master mix plates (white 0.2 mL) from freezer and thaw it on ice. Briefly spin the qPCR plate to collect the qPCR master mix in the bottom.
27. Remove and discard the seal from qPCR plate.
28. For detection of bacterial pathogens, carefully transfer 5 µL lysate sample from the 96 well 0.2 mL plate (step 18) in the E4CB qPCR master mix. For detection of viral and parasite pathogens, carefully transfer 5 µL cDNA from the 96 well 0.2 mL plate (step 25) in the E4CV qPCR master mix.
    Note: It is important to keep Master Mix on ice while loading template and to run the qPCR within 15 minutes.
29. Carefully cover the qPCR reaction plate with new lids and spin at 1000-1500xg for 30 seconds to 1 minute.
30. Insert qPCR plate into the qPCR instrument.
31. Run the qPCR reaction using the instrument settings 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 exponential 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 flagged out. The threshold is then fixed based on the rest of the sample and the sample with Ct<15 are put into the analysis again.

Baseline Correction: Select “Adaptive baseline”.

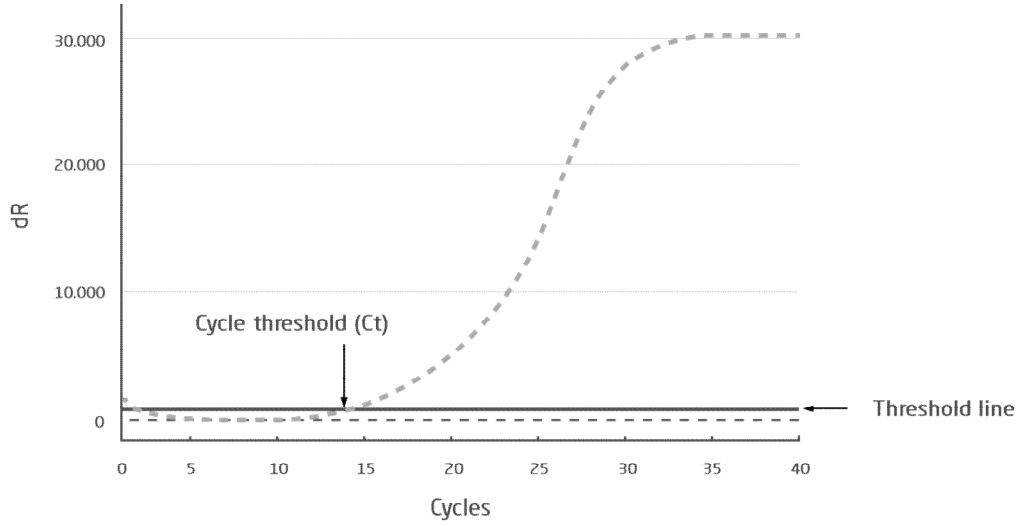
Activate the following filters: ATTO, CY5, ROX, HEX, FAM.

qPCR running program

Condition	Temperature	Duration	Number of cycles	Stage
Heat activation	95°C	1 min	1	Pre-incubation
Denaturation	95°C	5 sec	40	Amplification
Annealing/extension	60°C	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. Ct is optimally determined in the early exponential phase, when the fluorescence starts increasing due to PCR amplification. The threshold line is calculated automatically by the instrument giving in this example a Ct value of 14.

32. 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****.*

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

Bacteria target (E4CB)	**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

Virus/parasites target (E4CV)	**Filter**	**Positive**	**Negative**	**Retest**
		Ct	**Ct**	**Ct**
Bovine rotavirus	CY5	≤37	>37 or absent	
Bovine coronavirus	ROX	≤37	>37 or absent	
Cryptosporidium parvum	HEX	≤37	>37 or absent	
Eimeria species	FAM	≤37	>37 or absent	
IACV	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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