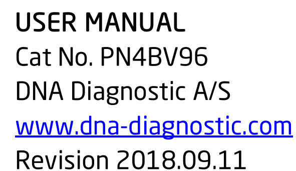
User Manual Pneumo 4BV

RNA/DNA extraction – cDNA synthesis - qPCR - Interpretation



USER MANUAL
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1. PURPOSE OF THE TEST

Calf pneumonia is a major problem in dairy and beef herds worldwide because it can result in serious economic loss due to the direct costs of calf losses and treatment. Calf pneumonia can be viral or bacterial in origin, but most often an initial viral infection is followed by a bacterial infection.

Pneumo 4BV is a fast, reliable and easy-to-use method for the detection of the primary bacterial and viral pathogens [*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis*, Bovine parainfluenza virus, Bovine coronavirus, Bovine Respiratory Syncytial virus, Bovine viral diarrhea virus (BVD) and Bovine Herpesvirus 1(IBR)] linked to calf pneumonia. Using a simple 96 deepwell-based extraction protocol followed by a sensitive and highly specific qPCR reaction, the entire protocol from RNA/DNA extraction to result can be carried out in 5 hours.

2. PRINCIPLE OF THE TEST

Pneumo 4BV allows the detection of *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis,* Bovine parainfluenza virus, Bovine coronavirus, Bovine Respiratory Syncytial virus, Bovine viral diarrhea virus (BVD) and Bovine Herpesvirus 1(IBR) in tracheal aspirate fluid sample from calves. It includes the following four main steps:

The DNA/RNA extraction step uses the Pneumo 4 Extraction Kit for DNA/RNA extraction from 0.5 mL of sample. The sample is incubated with pre-lysis buffer at 37 °C for 10 minutes and then spun and washed. The DNA/RNA is extracted by incubating sample in the lysis mix buffers at 37 °C for 20 minutes followed by 95°C for 15 minutes and debris is pelleted by centrifugation. Now the RNA/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 Pneumo 4V cDNA master mix. The cDNA synthesis reaction runs for 1 hour and 15 minutes.

The PCR step uses 2 µL of cDNA template which is added to a well in a 96 well plate containing Pneumo4V qPCR master mix for detection of vial pathogens. For detection of bacterial pathogens, 5 µL of diluted DNA template is added to a well in a 96 well plate containing Pneumo4B 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 four bacteria. A fifth set of primers and probe detects an internal amplification control (IAC). Each of the four bacteria probes emit a specific light (color) enabling the identification of bacteria or virus 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 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 shows that the sample is negative.

3. KIT COMPONENTS AND STORAGE

The Pneumo 4BV kit contains material for testing 96 samples.

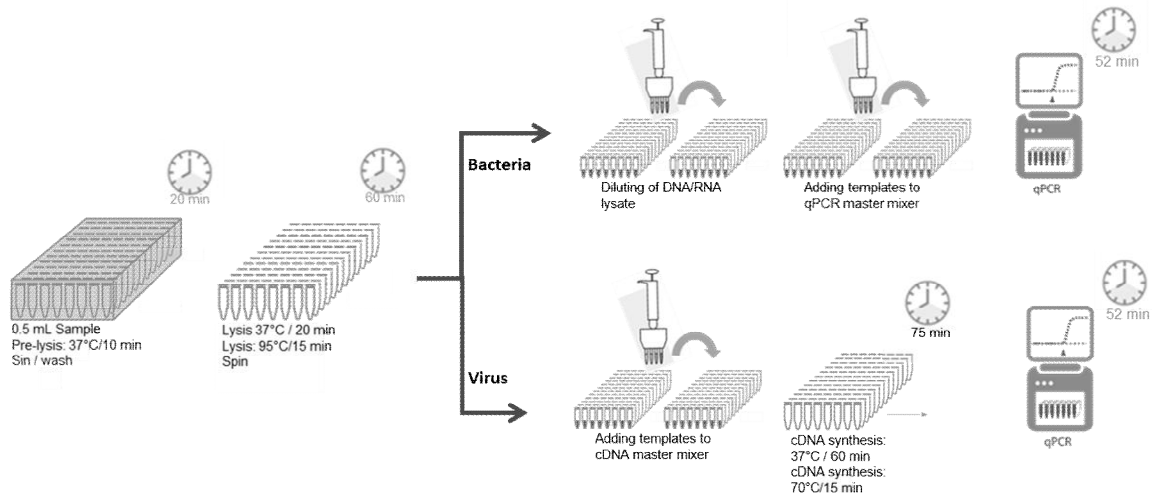
The Pneumo 4BV kit contains **one box** for storage at room temperature (RT) and **one box** for storage at -20°C:

BOX	COMPONENTS	AMOUNT	STORAGE
I	Sealed 96 Deep Well Plates containing pre-lysis solution	1 x 96 Deep Well Plate	RT
	Wash Buffer	1 bottle of 110 mL	RT
	96 Well Plates (0.2 mL clear tubes) with cap	2 x 96 well plate (0.2 mL clear tubes) with cap	RT
	Adhesive seals for Deep Well plates	4 pieces	RT
	Cap mats for the qPCR 96 Well Plates	2x 96 cap for qPCR tube	RT
	H_2O	1 bottle of 15 mL	RT
II	Lysis Buffer-I	1 bottle of 12 mL	-20°C
	Mix I Additive for Lysis Buffer-I	1 tube of 1334 µl	-20°C
	Sealed 96 Well Plates (0.2 mL clear tubes) with Pneumo 4V cDNA Master Mix	1 x 96 well plates	-20°C
	Sealed 96 Well Plates (0.2 mL white tubes) with Pneumo 4B and Pneumo 4V qPCR Master Mix	2 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 5000 x g at 37°C.**
  Example: Heraeus Multifuge X3R Centrifuge with HIGHPlate 6000 rotor.
- Incubator at 37°C.
- 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°C cooling block.
- Standard lab pipettes and sterile filter tips.

5. PROTOCOL



RNA/DNA extraction:

1. Centrifuge a 96 Deep Well Plate containing 150 µl of pre-lysis buffer at 1000 xg for 1 minute and remove seal.
2. Transfer 0.5 mL of samples to the corresponding well of the 96 well plate with pre-lysis buffer.
3. Pipette briefly up and down to mix the sample and cover wells with seal.
4. Incubate at 37°C for 10 minutes.
5. Spin Deep Well plate at 5000 x*g* for 5 minutes at 37°C.
6. Remove supernatant with the plate washer. 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.*
7. Add 1 mL Wash Buffer to Deep Well plate.
8. Cover wells with seal and vortex briefly.
9. Centrifuge the plate at 5000 xg for 5 minutes.
10. Remove supernatant with the plate washer. It is important to remove the supernatant completely. Be careful not to touch or remove the bacterial pellets.
11. Prepare fresh Lysis-I Mix by adding 6 µL Mix I additive to 54 µL Lysis buffer-I.

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
Total	60 µL	540 µL	3000 µL	6000 µL

12. Add 60 µL Lysis-I Mix, to each pellet. Vortex and spin 20 seconds at 1000 xg to bring Lysis mixer to the bottoms.

13. Transfer of 60 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.
14. Incubate the 96 well plate at 37°C for 20 minutes and at 95°C for 15 minutes.
15. 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.
16. Centrifuge the plate at 5000 xg for 5 minutes.
    Note: The RNA/DNA lysate can be stored at -20°C until use. Due to the instability of RNA, it is recommended to perform the cDNA synthesis immediately before storing samples. **For cDNA synthesis follow step 21-27.**

17. Add 120 µL of diluting Pneumo 4 H_2O into the wells of a new 96 well plate (clear 0.2 mL).
18. Carefully remove the caps from the 96 well plate containing DNA/RNA lysates. Use an 8-channel pipette with filter tips to transfer 30 µL of each aqueous phase directly to the corresponding well of the 96 well plate containing 120 µL of diluting Pneumo 4 H_2O from step 17. Avoid pellet/precipitate. Add lids.
    Note: The remaining purified DNA can be stored at -20°C for long time storage.
19. Vortex and spin 20 seconds at 1000 xg to bring mix to the bottom.
20. Carefully transfer 5 µL of each diluted sample directly to the corresponding well of the 96 well plate with Pneumo 4B qPCR Master Mix (step 30).
    Note: The remaining diluted DNA can be stored at -20°C for long time storage.

cDNA Synthesis (step 21-27 is performed for detection of viral pathogens):

21. Take a Pneumo 4V cDNA master mix plate from freezer and thaw it on ice. Briefly spin the plate to collect the cDNA master mix in the bottom.
22. Remove and discard the seal from cDNA master mix plate.
23. Carefully transfer 5 µL supernatant from the 96 well 0.2 mL plate from step 16 to the corresponding well in the Pneumo 4V cDNA master mix plate (clear 0.2 mL).
24. Incubate the cDNA reaction at 37°C for 60 minutes.
25. Inactivate Reverse Transcriptase enzyme by heating to 70°C for 15 minutes.
26. 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.
27. Carefully transfer 2 µL supernatant from the 96 well 0.2 mL clear plate to the corresponding well in the Pneumo 4V qPCR master mix plate (step 30).
    Note: It is important to keep Master Mix on ice while loading template and to run the qPCR within 15 minutes.

qPCR analysis:

28. Take a qPCR master mix plate (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.
29. Remove and discard the seal from qPCR plate.
30. For detection of bacterial pathogens, carefully transfer 5 µL diluted DNA/RNA from the 96 well 0.2 mL plate (step 20) in the Pneumo 4B qPCR master mix. For detection of viral pathogens, carefully transfer 2 µL cDNA from the 96 well 0.2 mL plate (step 27) in the Pneumo 4V qPCR master mix.
Note: It is important to keep Master Mix on ice while loading template and to run the qPCR within 15 minutes.
31. Carefully cover the qPCR reaction plate with new lids and spin at 1000-1500 xg for 30 seconds to 1 minute.
32. Insert qPCR plate into the qPCR instrument.
33. 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”.

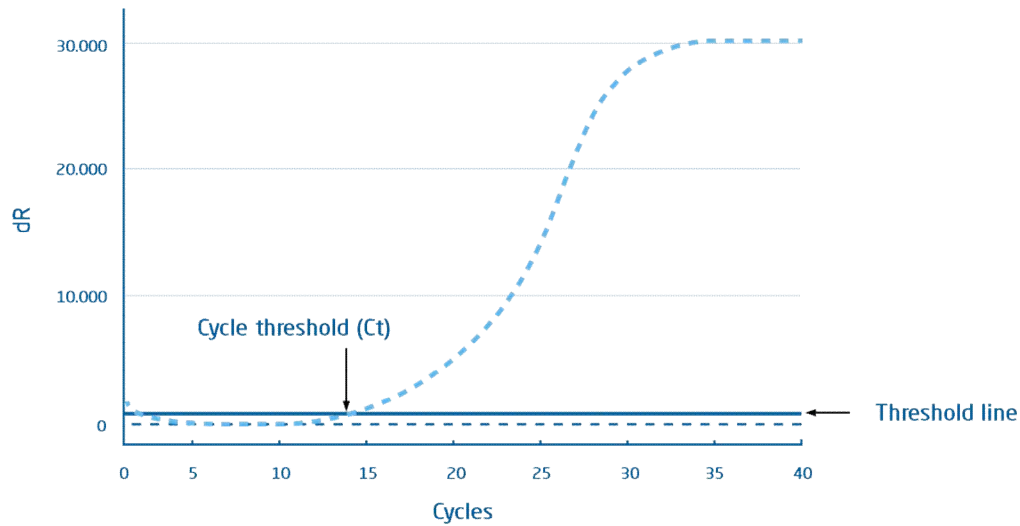
Activate the following filters: ATTO, CY5, ROX, HEX, FAM.

qPCR running program

Condition	Temperature	Duration	Number of cycles	Stage
Heat activation	$95^{o}C$	1 min	1	Pre-incubation
Denaturation	$95^{o}C$	5 sec	40	Amplification
Annealing/extension	$60^{o}C$	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.

34. 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****.*

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

Bacteria target	Filter	Positive	Negative	Retest
		Ct	Ct	Ct
Mannheimia haemolytica	CY5	≤37	>37 or absent	
Pasteurella multocida	ROX	≤37	>37 or absent	
Histophilus somni	HEX	≤37	>37 or absent	
Mycoplasma bovis	FAM	≤37	>37 or absent	
IACPB	ATTO	≤32		>32 or absent

Virus target	Filter	Positive	Negative	Retest
		Ct	Ct	Ct
Bovine parainfluenza virus	CY5	≤37	>37 or absent	
Bovine coronavirus	ROX	≤37	>37 or absent	
Bovine Respiratory Syncytial virus	HEX	≤37	>37 or absent	
Bovine viral diarrhea virus and Bovine Herpesvirus 1	FAM	≤37	>37 or absent	
IACPV	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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