User Manual Pneumo 4B

DNA extraction – qPCR - Interpretation

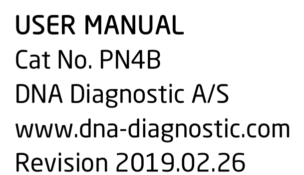


TABLE OF CONTENTS

1. PURPOSE OF THE TEST 3
2. PRINCIPLE OF THE TEST 4
3. KIT COMPONENTS AND STORAGE 5
4. REQUIRED EQUIPMENT AND MATERIALS NOT INCLUDED IN THE KITS 6
5. PROTOCOL 7

Trademarks:
Black Hole Quencher® (BHQ®) and CAL Fluor® dye technologies incorporated in this product are used under licensing agreement with Biosearch Technologies, Inc. and protected by U.S. and world-wide patents issued, or in application.

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 deaths are common and are generally connected with bacterial infections.

Pneumo4 is a fast, reliable and easy-to-use method for the detection of 4 major pathogens (*Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*) link to calf pneumonia. Using a simple 96 deepwell-based extraction protocol followed by an accuracy, sensitive and highly specific qPCR reaction, the entire protocol from DNA extraction to result can be carried out in as little as 3.30 hours.

Pneumo4 is validated to detect down to 15 CFU per 0.5 mL of tracheal aspirate fluid sample.

2. PRINCIPLE OF THE TEST

Pneumo4 allows the detection of *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* in tracheal aspirate fluid sample from calf. It includes the following three main steps:

The *DNA extraction step* uses the Pneumo4 DNA Extraction Kit for DNA 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 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 well in a 96 well qPCR plate containing Pneumo4 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 four bacteria. 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. Probes with CY5, ROX, HEX and FAM fluorophores are present for detection of *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* amplicons, respectively. 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 for *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* amplicons, respectively. No Ct value shows that the sample is negative.

3. KIT COMPONENTS AND STORAGE

The Pneumo4 kit contains material for testing 384 samples (cat. No. PN4B).

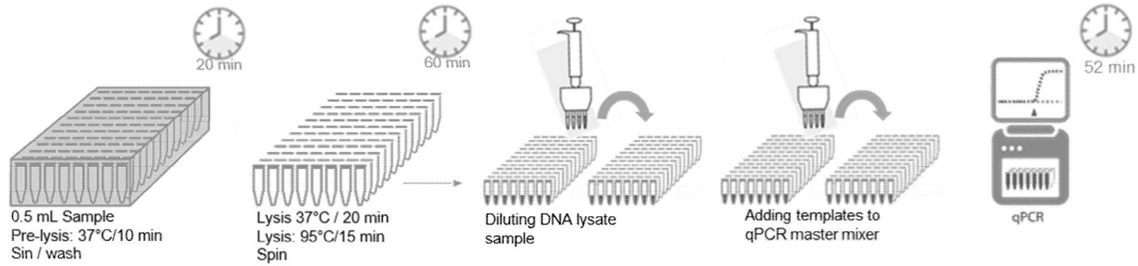
The Pneumo4 kit contains **one box** for storage at room temperature (RT) and **one box** for storage at -20°C:

BOX	COMPONENTS	AMOUNT	STORAGE
		Cat. No. PN4B	
I	Sealed 96 Deep Well Plates containing pre-lysis solution	4 x 96 Deep Well Plate	RT
	Wash Buffer	4 bottle of 110 mL	RT
	96 Well Plates (0.2 mL clear tubes) with cap	8 x 96 well plate (0.2 mL clear tubes) with cap	RT
	Adhesive seals for Deep Well plates	16 pieces	RT
	Cap mats for the qPCR 96 Well Plates	4x 96 cap for qPCR tube	RT
	H_2O	4 bottle of 15 mL	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 Pneumo 4 qPCR Master Mix	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 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



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 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.

    If lysate sample will subsequently be used for virus detection, follow the step 17 on Pneumo 4V manual protocol.

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 lysate samples. 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. Avoid pellet/precipitate.
    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 mixer to the bottoms.
20. Carefully transfer 5 µL of each aqueous phase directly to the corresponding well of the 96 well plate with qPCR Master Mix in step 23.
    Note: The remaining diluted DNA can be stored at -20°C for long time storage.

qPCR analysis:

21. Take a Pneumo 4 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.
22. Remove and discard the seal from qPCR plate.
23. Carefully transfer 5 µL supernatant from the 96 well 0.2 mL plate (step 20) to the corresponding well in the Pneumo 4 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.
24. Carefully cover the qPCR reaction plate with new lids and spin at 1000-1500 xg for 30 seconds to 1 minute.
25. Insert qPCR plate into the qPCR instrument.
26. 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".

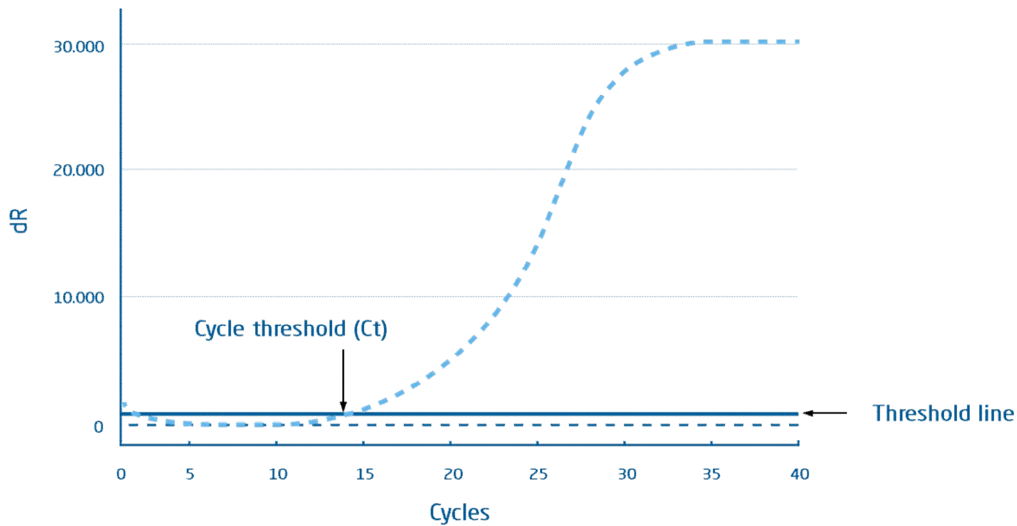
Activate the following filters: ATTO, CY5, ROX, HEX, FAM.

qPCR running program

Condition	Temperature	Duration	Number of cycles	Stage
Heat activation	95°C	1 min	1	Pre-incubation
Denaturing	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 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.

27. 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 theoretical 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****.*

28. 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**
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	
IAC	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.

For more information, contact

DNA Diagnostic A/S
Voldbjergvej 14
8240 Risskov
Denmark
Tel.: +45 8732 3050
E-mail: info@dna-diagnostic.com
www.dna-diagnostic.com

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.

Cat. No.: PN4B **USER MANUAL**