




DNA
DIAGNOSTIC

User Manual PN 4BC55

DNA extraction – 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 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 3.3 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 bacteria probes emit a specific light (color) enabling the identification of bacteria present in the sample. Probes with CY5, Texas Red, 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 a QUASAR705 signal with a Ct value at 27-32. The CY5, Texas Red, 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 96 samples.

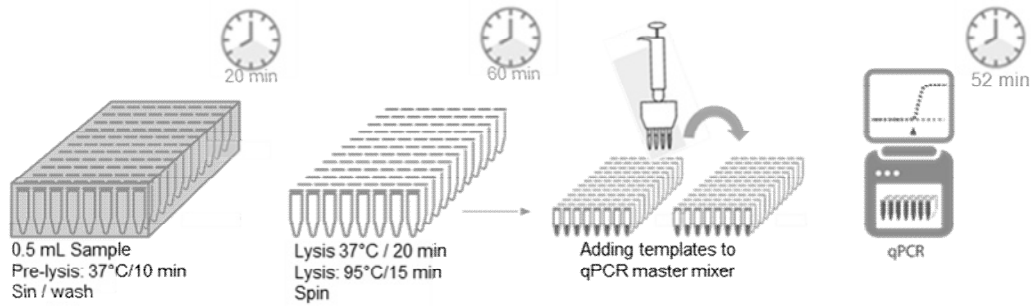
The Pneumo4 kit contains **one box** for storage at room temperature (RT) and **one box** for storage at -20°C:

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	1x 96 cap for qPCR tube	RT
	H ₂ O	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 white tubes) with Pneumo 4 Master Mix PNBC55	1 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 e.g Biorad CFX96 touch with filters for QUASAR705 (440nm-492nm), FAM (492nm-516nm), HEX (535nm-555nm), Texas Red (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 xg 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.
17. Carefully remove the caps from the 96 well plate. 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 buffer. Avoid pellet/precipitate.
Note: The remaining purified DNA can be stored at -20°C for long time storage.
18. Vortex and spin 20 seconds at 1000 xg to bring mix to the bottom.
19. Carefully transfer 5 µL of each aqueous phase directly to the corresponding well of the 96 well plate with qPCR Master Mix in step 22.
Note: The remaining diluted DNA can be stored at -20°C for long time storage.

qPCR analysis:

20. Take a Pneumo 4 Master Mix PNBC55 plate from freezer and thaw it on ice. Briefly spin the qPCR plate to collect the qPCR master mix in the bottoms.
21. Remove and discard the seal from qPCR plate.
22. Carefully transfer 5 µL supernatant from the 96 well 0.2 mL plate (step 19) to the corresponding well in the Pneumo 4 Master Mix PNBC55 plate.
Note: It is important to keep Master Mix on ice while loading template and to run the qPCR within 15 minutes.
23. Carefully cover the qPCR reaction plate with new lids and spin at 1000-1500 xg for 30 seconds to 1 minute.
24. Insert qPCR plate into the qPCR instrument.
25. Run the qPCR reaction using the instrument setting and the running program below:

Instrument settings for the CFX96 touch qPCR machine

Threshold settings:

Fluorophore	QUASAR705	FAM	HEX	Texas Red	CY5
Threshold	375	375	375	375	375
Baseline	“User defined” 10-15	“User defined” 10-15	“User defined” 10-15	“User defined” 10-15	“User defined” 10-15

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.

Baseline Correction. Select “Baseline subtracted curve fit”.

Activate the following filters: FAM, HEX, Texas Red, Cy5 and QUASAR705.

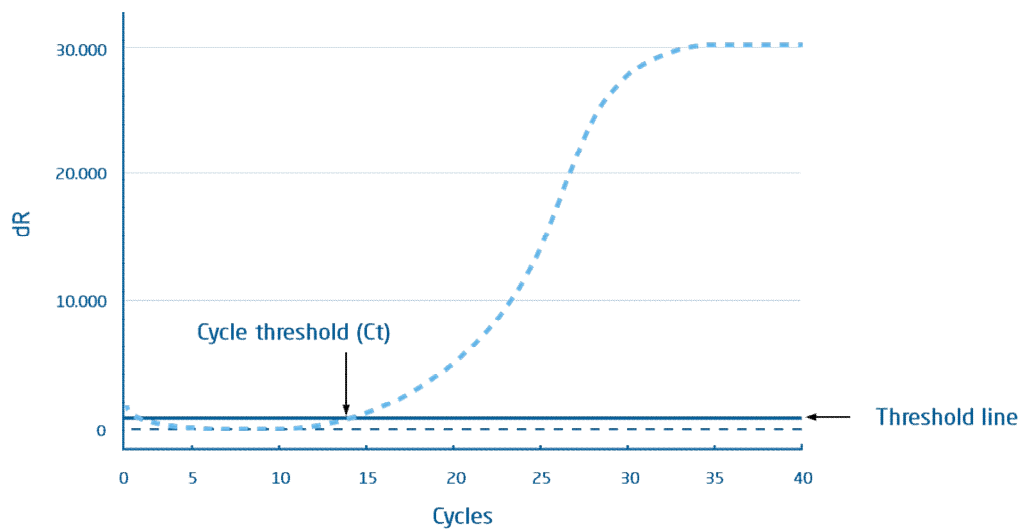
qPCR running program

Condition	Temperature	Duration	Number of cycles	Stage
Denaturation	95°C	1 min	1	Pre-incubation
Denaturation	*95°C	5 sec	40	Amplification
Annealing/extension	60°C	**25 sec		

*Only ramp rate should be set for 2.5 °C / sec without setting increment temp.

** Read fluorescence for FAM, HEX, Texas Red, CY5, QUASAR705 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.

26. Check amplification plot for QUASAR705 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: QUASAR705 Ct can be above 32 or missing if the Ct for one or more of FAM, HEX, Texas Red, 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, Texas Red, CY5 signal and QUASAR705 signal absent or outside the interval 27-32 **must be retested**.

27. 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
<i>Mannheimia haemolytica</i>	CY5	≤37	>37 or absent	
<i>Pasteurella multocida</i>	Texas Red	≤37	>37 or absent	
<i>Histophilus somni</i>	HEX	≤37	>37 or absent	
<i>Mycoplasma bovis</i>	FAM	≤37	>37 or absent	
IAC	QUASAR705	≤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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