bactotype® MAP real-time PCR — combining optimized sample extraction with sensitive detection

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of paratuberculosis (Johne's disease), a chronic inflammatory intestinal disease of ruminants, which occurs worldwide. The purpose of this study was to increase the sensitivity of MAP detection by combining a sensitive and specific amplification and detection method with an optimized protocol for the extraction of MAP DNA from fecal samples. Culture from fecal samples is generally regarded as the gold standard for MAP detection in ruminants. However, this is labor intensive and can take up to several weeks. Therefore, direct fecal PCR is becoming more widely used which enables test results within hours. The challenges for extracting MAP DNA from fecal samples include MAP clusters in the sample, thick mycobacterial cell walls, and PCR inhibitors.

Optimized fecal sample preparation

To meet these challenges, we have developed a special fecal sample pretreatment by combining three strategies:

- Lysis buffer
- Mechanical disruption using bead-beating instruments and lysis tubes
- Heat treatment of the sample

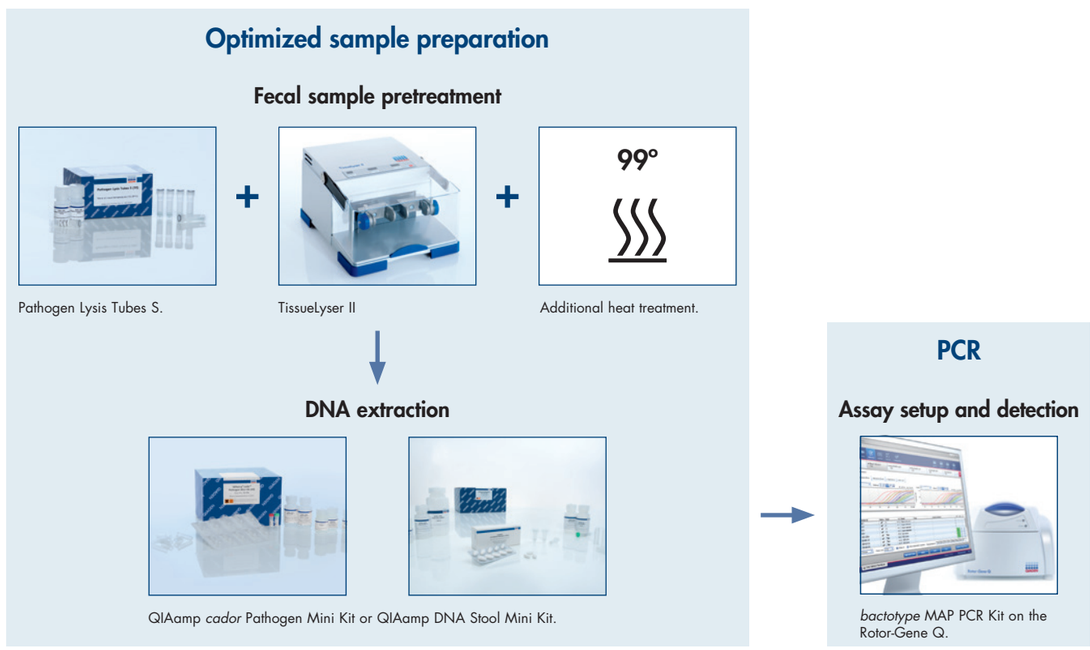
After fecal sample pretreatment MAP DNA extraction is performed using either QIAamp® *cador*® Pathogen Mini Kit or the QIAamp DNA Stool Mini Kit.

Sensitive real-time PCR

For amplification and detection of MAP DNA we have developed a highly sensitive and easy-to-use PCR kit — the *bactotype* MAP PCR Kit. The kit is a duplex real-time PCR and features:

- A ready-to-use master mix
- A heterologous extraction and amplification control.
- TaqMan® based chemistry that can be used on real-time PCR cyclers commonly used in veterinary laboratories, with a total amplification time of about 1.40 hours (on the Rotor-Gene® Q).

Materials and methods



QIAGEN supplementary protocols for purification of MAP DNA from fecal samples are available under Resources at www.qiagen.com/DNAstool and www.qiagen.com/cadorpathogen.

100% accuracy in MAP detection

The workflow was validated using the 2012 Johne's Disease Fecal Proficiency Panel. Samples were extracted using our fecal sample pretreatment and one of the kits shown, with PCR on the Rotor-Gene Q. The workflow was compared with a real-time PCR kit from Supplier L after extraction using the MagMAX™ Total Nucleic Acid Isolation Kit and PCR on the Applied Biosystems® 7500 Real-Time PCR System.

The *bactotype* MAP PCR Kit correctly detected all samples in the Panel.

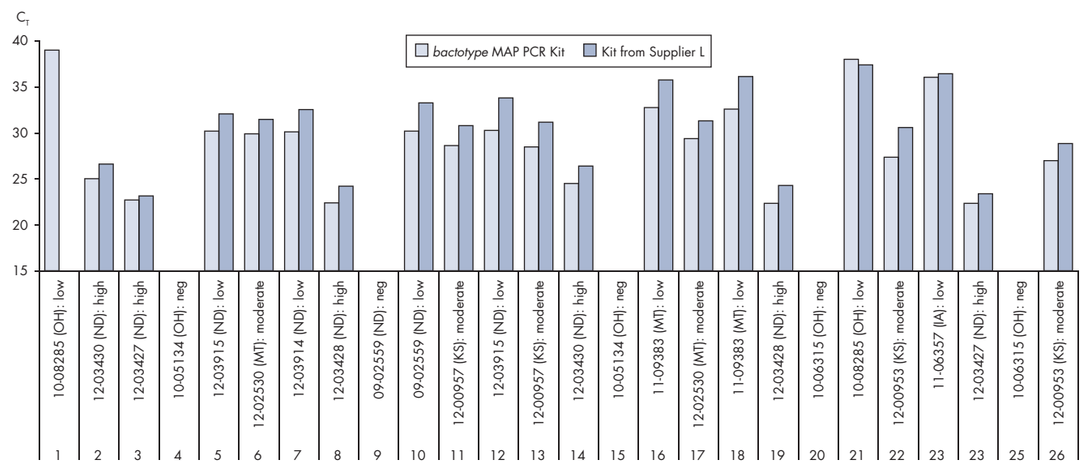
			bactotype MAP PCR Kit				Supplier L	
			QIAamp DNA Stool Mini Kit		QIAamp *cador* Pathogen Mini Kit		MagMAX Total Nucleic Acid Isolation Kit	
No.	Cow ID	Shedding status	MAP	IC	MAP	IC	MAP	IC
1	11-06357 (IA)	Low	33.73	26.43	31.99	26.63	36.84	31.43
2	11-09383 (MT)	Low	32.62	26.76	33.11	27.68	35.30	32.57
3	12-00954 (KS)	Critical – high shedding	23.21	25.81	23.71	26.75	26.67	31.49
4	10-04999 (OH)	Negative	–	26.33	–	27.20	–	32.61
5	11-09383 (MT)	Low	32.71	26.51	32.61	27.46	34.83	32.89
6	12-02530 (MT)	Moderate	29.16	27.15	29.50	27.85	32.22	32.87
7	10-04923 (OH)	Negative	–	26.48	–	26.61	–	32.22
8	11-09381 (MT)	Critical – high shedding	21.25	25.83	21.87	26.51	25.20	32.58
9	11-06359 (IA)	Critical – high shedding	22.76	26.65	22.54	27.01	28.22	32.43
10	10-04922 (OH)	Negative	–	26.91	–	26.99	–	32.25
11	11-09382 (MT)	Low	37.68*	27.03	39.15*	28.62	–	32.58
12	12-00957 (KS)	Moderate	28.07	26.96	28.08	28.01	33.85	34.63
13	10-04999 (OH)	Negative	–	26.43	–	26.68	–	33.00
14	11-09381 (MT)	Critical – high shedding	21.24	25.69	21.16	26.12	25.76	33.15
15	11-09382 (MT)	Low	39.61*	27.16	38.32*	28.32	38.65	33.32
16	11-06361 (IA)	Low	24.95	26.64	25.18	26.69	29.11	31.53
17	12-00957 (KS)	Moderate	27.07	26.63	28.09	27.38	31.58	32.98
18	11-06357 (IA)	Low	34.36	26.71	33.61	27.08	36.22	32.22
19	11-06361 (IA)	Low	24.87	26.37	25.23	26.60	29.36	31.88
20	12-02530 (MT)	Moderate	29.19	27.30	30.43	28.52	34.31	33.82
21	11-06359 (IA)	Critical – high shedding	21.99	26.24	23.82	27.58	28.04	32.87
22	10-04923 (OH)	Negative	–	26.99	–	27.28	–	32.81
23	12-02530 (MT)	Moderate	30.26	27.44	30.68	28.88	33.12	33.20
24	11-06357 (IA)	Low	34.89	26.87	31.53	26.90	28.19	32.01
25	10-04923 (OH)	Negative	–	26.89	–	27.22	–	32.88
26	12-00954 (KS)	Critical – high shedding	22.90	25.99	23.41	26.70	25.98	31.87

* single test result

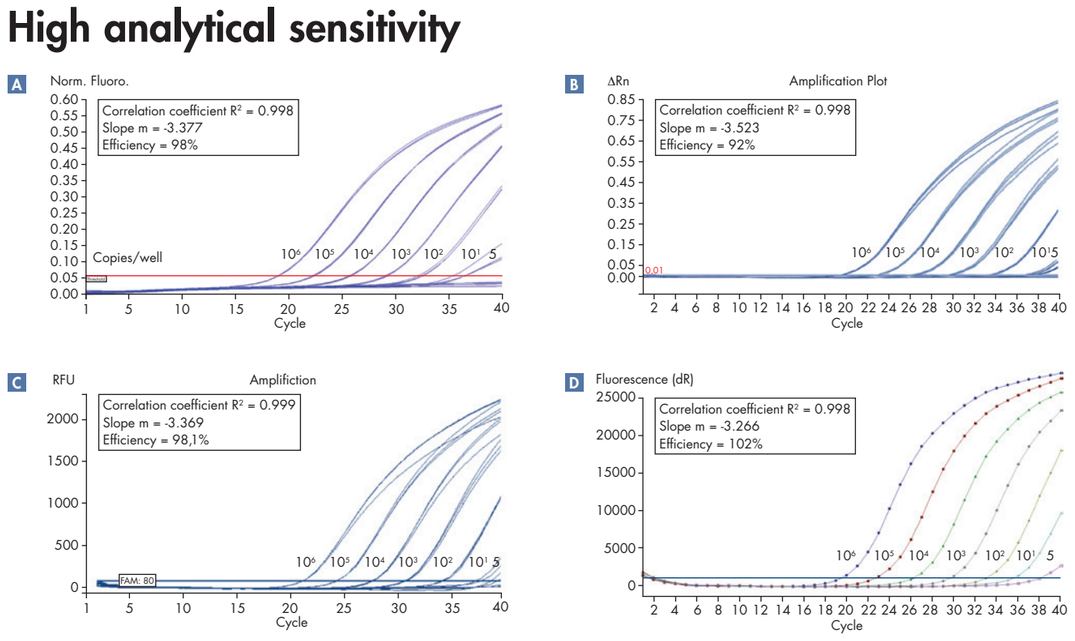
Sensitive detection of MAP in fecal samples

The *bactotype* MAP real-time PCR workflow was further validated using the 2013 Johne's Disease Fecal Proficiency Panel. Samples were extracted using a fecal sample pretreatment in combination with the QIAamp *cador* Pathogen Mini Kit. In addition our workflow was tested in comparison with a real-time PCR kit from Supplier L and magnetic-bead sample extraction using the MagMAX Total Nucleic Acid Isolation Kit on BioSprint 96. PCR was performed using the Applied Biosystems 7500 Real-Time PCR System.

Results: The *bactotype* MAP PCR Kit detected all positive samples in comparison to the alternative method, which failed to detect sample number 1. Furthermore, the C_T values revealed better results using the *bactotype* MAP PCR Kit.



High analytical sensitivity



Proven sensitivity. The high analytical sensitivity of the *bactotype* MAP PCR Kit was proven using a titration series of in vitro DNA [10^6–100 copies/well] performed in triplicate and analyzed using A Rotor-Gene Q B Applied Biosystems 7500 Real-Time PCR System C BioRad CFX96 and D Agilent Mx3005P.

Results: The *bactotype* MAP kit is able to detect 5 MAP DNA copies per sample with a correlation coefficient ≥0.998 and with high efficiency on all instruments tested.

Conclusion

- QIAGEN's workflow for detection of MAP in bovine fecal samples combines a sensitive real-time PCR with optimized sample preparation.
- This method combines a special fecal sample pretreatment, MAP DNA extraction using the QIAamp cador Pathogen Mini Kit or QIAamp DNA Stool Mini Kit, and MAP DNA amplification and detection with the easy-to-use bactotype MAP PCR Kit.
- Our solution can detect 5 copies of MAP DNA per sample with high efficiency and weak positive samples from low shedders.

The Johne's Disease Fecal Proficiency Panels 2012 and 2013 were kindly provided by the U.S. Department of Agriculture (USDA) Veterinary Services. Positive samples were collected from naturally infected cows and negative samples were from animals in non-infected herds.

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