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bactotype® Mastitis HP2+ PCR Kit Handbook

For simultaneous detection of DNA from Mycoplasma, *Mycoplasma bovis* and *Streptococcus agalactiae*

REF 96 reactions (cat. no. BT280025)

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INDICAL BIOSCIENCE

Contents

Kit contents

bactotype Mastitis HP2+ PCR Kit **Cat. no.** **Number of reactions**	**(96)** **BT280025** **96**
Master Mix (tube with orange cap), includes primers, probes and enzymes	2 x 980 µl
Positive Control (tube with red cap)	1 x 150 µl
Negative Control (tube with blue cap)	1 x 150 µl
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Intended use

The bactotype Mastitis HP2+ PCR Kit is intended for the detection of DNA from Mycoplasma, *Mycoplasma bovis* and *Streptococcus agalactiae* in ruminant milk (quarter milk samples, pool or bulk milk).

For veterinary use only.

Symbols

	Legal manufacturer
	Lot number
	Use by date
	Temperature limitations for storage
	Handbook
	Catalog number
	Material number
	Protect from light
	For ruminant samples

Quality control

In accordance with INDICAL's ISO-certified Quality Management System, each lot of bactotype Mastitis HP2+ PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Storage

The components of the bactotype Mastitis HP2+ PCR Kit should be stored at -30°C to -15°C and are stable until the expiration date stated on the label. Avoid repeated thawing and freezing (>2x), as this may reduce assay sensitivity. Freeze the components in aliquots if they will only be used intermittently.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available from your local sales representative or by Email request under **compliance@indical.com**.

All sample residues and objects that have come into contact with samples must be decontaminated or disposed of as potentially infectious material.

Introduction

The bactotype Mastitis HP2+ PCR Kit is a highly sensitive and specific solution for detection of DNA from *Mycoplasma bovis* (*M. bovis*) and other mastitis-causing Mycoplasma as well as *Streptococcus agalactiae* (*S. agalactiae*) in samples from ruminant milk (quarter milk samples, pool or bulk milk).

Bovine mastitis is the single most costly disease of dairy cattle worldwide, mainly caused by intra-mammary infection (IMI). This may be associated with increased somatic cell counts in bovine milk and a reduction of milk yield and quality.

Mastitis manifests itself as clinical or sub-clinical mastitis. Clinical mastitis can be further classified as mild, moderate or severe/fatal and as chronic mastitis, which often requires antimicrobial therapy.

A wide range of bacteria can cause mastitis and can be subdivided in cow-associated (or contagious mastitis) pathogens (e.g., *S. agalactiae*, *M. bovis*, *S. aureus*) and environmental pathogens (e.g., *S. uberis*, *E. coli*, *Klebsiella*).

The bactotype Mastitis HP2+ PCR Kit detects the most important cow-associated and highly contagious bacteria *M. bovis*, *S. agalactiae* and further mastitis-causing Mycoplasma with high sensitivity.

Mycoplasma species that are detected by the bactotype Mastitis HP2+ PCR Kit are:

- *M. alkalescens*
- *M. arginini*
- *M. bovigenitalium*
- *M. bovis*
- *M. californicum*
- *M. canadense*

Principle

Polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR, the amplified product is identified using fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real time) allows detection of the accumulating product without the need to re-open the reaction tubes afterward.

The bactotype Mastitis HP2+ PCR Kit contains all of the necessary reagents for the simultaneous detection of DNA from Mycoplasma, *M. bovis* and *S. agalactiae* including a positive and negative control.

An internal control excludes the possibility of false-negative results.

The kit uses four specific primer/probe combinations:

- FAM™ fluorescence for DNA from Mycoplasma
- Cy®5 fluorescence for DNA from *M. bovis*
- JOE™ fluorescence for DNA von *S. agalactiae*
- Texas Red® fluorescence for the internal control (β-actin DNA, present within the sample)

A Positive Control serves to verify the functionality of the reaction mix for the amplification of one of the DNA targets per channel.

DNA extraction

The bactotype Mastitis HP2+ PCR Kit can be used for detection of pathogen DNA from ruminant milk samples. Due to the high sensitivity of the test, individual quarter milk samples, pool milk samples or tank milk samples can be used.

Prior to real-time PCR, bacterial DNA must be extracted from the starting material. INDICAL recommends using the following products for DNA extraction from milk samples.

- DNeasy® Mastitis Mini Kit
- MagAttract® Mastitis Kit

If real-time PCR is not performed immediately after extraction, store the DNA at -30°C to -15°C.

Equipment and reagents to be supplied by user

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Pipets
- Nuclease-free, aerosol-resistant pipet tips with filters
- Sterile 1.5 ml Eppendorf® tubes
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive identification of viral nucleic acids
- Cooling device or ice
- Benchtop centrifuge with rotor for 1.5 ml tubes
- Real-time cycler with appropriate fluorescent channels
- Appropriate software for chosen real-time cycler
- Appropriate strip tubes and caps or 96-well optical microplate with optical sealing film or cover for chosen real-time cycler

Important notes

General precautions

The user should always pay attention to the following:

- Use nuclease-free pipet tips with filters.
- Store and extract positive materials (specimens, positive controls and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Thaw all components on ice before starting as assay.
- When thawed, mix the components by inverting and centrifuge briefly.
- Do not use components of the test kit past the expiration date.
- Keep samples and controls on ice or in a cooling block during the setup of reactions.

Negative control

At least one negative control reaction should be included in each PCR run, containing all the components of the reaction except for the pathogen template. This enables assessment of contamination in the reaction.

Positive control

When performing PCR on unknown samples, it is recommended to perform a positive control reaction in the PCR run, containing a sample that is known to include the targeted bacterial DNA. A positive control serves to prove the functionality of the pathogen assay, e.g., the correct setup of the reaction mix. Use 5 µl of the Positive Control provided with

the bactotype Mastitis HP2+ PCR Kit to test for successful amplification of the target.

Internal control

For increased process safety and convenience, an internal control assay is included in the form of an additional primer/probe set that detects a housekeeping gene (β-actin DNA) present within the sample. This allows both extraction and amplification to be monitored.

Protocol: Real-time PCR for simultaneous detection of Mastitis-causing pathogens

Important points before starting

- Please read „Important notes“ on page 10 before starting.
- Include at least one positive control (Positive Control) and one negative control (Negative Control) per PCR run.
- Before beginning the procedure, read through the protocol and ensure that you are familiar with the operation of the chosen real-time PCR cycler.
- Perform the protocol without interruption.

Things to do before starting

- Thaw all reagents on ice and protect from light.
- Maintain reagents on ice during PCR setup.
- Before use, spin the reagents briefly.

Procedure

1. Pipet 20 µl of the Master Mix into each reaction tube. Then add 5 µl of the sample DNA (Table 1).

   Include positive and negative control reactions.

   Positive Control: Use 5 µl of the positive control (Positive Control) instead of sample DNA.

   Negative Control: Use 5 µl of the negative control (Negative Control) instead of sample DNA.

Table 1. Preparation of reaction mix

Component	**Volume**
Master Mix	20 µl
Sample	5 µl
Total volume	**25 µl**

2. Close the reaction tubes with the corresponding caps.
3. Set the filters for the reporter dyes in the software of your thermal cycler according to Table 2.

   Note: For Agilent Mx3005P cyclers, use the Factory Defaults Filter Gain Setting (Cy5 1x, ROX 1x, HEX/ JOE 1x, FAM 8x).

Table 2. Filter settings for the reporter

Pathogen/ Internal Control	Reporter
Mycoplasma	FAM
M. bovis	Cy5
S. agalactiae	HEX/ JOE[1]
Internal Control	Texas Red/ ROX[2]

1 Use the option appropriate for your thermal cycler.
2 Do not use ROX as passive reference or reference dye on ABI 7500 and Agilent Mx3005P.

4. Run the real-time PCR protocol according to Table 3.

Table 3. Real-time PCR protocol for bactotype Mastitis PCR kits

Step	Temperature	Time	Number of cycles
Initial Activation	95°C	5 min	1
2-step cycling			
Denaturation	95°C	10 s	40
Annealing/Extension*	57°C	30 s	

* Fluorescence data collection. Approximate run time 65 min (Agilent Mx3005P)

Data analysis and interpretation

Interpretation of results

For the assay to be valid:

- The Positive Control yields a signal in all four channels (FAM, HEX/ JOE, Texas Red/ ROX and Cy5) with a C_T[1] < 35.
- The Negative Control does not yield a signal in any of the four channels.

The following results are possible if working with unknown samples. The possible sample results are also summarized in Table 4 on page 17.

- Check that the sample yields an internal control signal in the Texas Red/ ROX channel. A positive Texas Red/ ROX signal means that extraction and amplification were successful as the housekeeping gene within the sample is amplified.
- The sample is negative if a signal is detected only in the Texas Red/ ROX channel.
- If no signal is detected in all channels (FAM, HEX/ JOE, Texas Red/ ROX and Cy5), the result is inconclusive, and the assay is invalid. The absence of a signal for the housekeeping gene indicates PCR inhibition and/or other malfunctions, such as extraction failure.

To check for inhibition, we recommend 1:5 dilution of the sample DNA in nuclease free water, to repeat the DNA extraction, or repeat the whole test procedure starting with new sample material.

[1] C_T, Threshold cycle (C_T) — cycle at which the amplification plot crosses the threshold, i.e., there is the first clearly detectable increase in fluorescence

Check that there is a fluorescence signal in the pathogen channels (FAM, HEX/ JOE, and Cy5) for the positive control reaction (Positive Control). Absence of a signal for the Positive Control indicates an error, which could be due to incorrect setup of the reaction mix or incorrect cycling conditions.

Internal Control

The assay internal control serves as an extraction and amplification control and is based on a ruminant housekeeping gene. Amplification of the internal control means that nucleic acid extraction and target DNA amplification from milk was successful.

As additional information, the C_T value of the internal control can provide an indication of the amount of somatic cells in the milk sample. A low C_T value of the internal control ($C_T < 29$) may suggest a high somatic cell count in the milk sample.

A low internal control C_T value in absence of a positive signal in the pathogen FAM, HEX/ JOE and Cy5 channels is possible and indicative for high somatic cell count caused by pathogens, different from those detected with this kit.

Table 4. Results interpretation table[1]

FAM	HEX/ JOE	Cy5	Texas Red/ ROX	Sample result
(Pathogen)			(IC)	Positive for
X			X	Mycoplasma (not *M. bovis*)
	X		X	*S. agalactiae*
X		X	X	Mycoplasma and *M. bovis*[2]
X	X		X	Mycoplasma (not *M. bovis*) and *S. agalactiae*
X	X	X	X	Mycoplasma and *M. bovis* and *S. agalactiae*[2]
			X	negative
				inconclusive

1 Interpretation of sample results can be determined provided positive and negative control reactions are performed. The positive control must yield a signal in all channels (FAM, HEX/ JOE, Texas Red/ ROX and Cy5). The negative control must yield no signal in any channel. For further explanation of the results, please refer to “Data analysis and interpretation” on page 15.

2 In cases where the C_T value for the Mycoplasma channel (FAM) is ≥ 2 C_T lower than the C_T value for the *M. bovis* channel (Cy5), the milk sample is positive for *M. bovis* and additional Mycoplasma species (*M. alkalescens*, *M. arginini*, *M. bovigenitalium*, *M. californicum* and/or *M. canadense*).

INDICAL offers a range of ELISA kits and real-time PCR and real-time RT-PCR kits for the detection of animal pathogens.
Visit **www.indical.com** for more information about bactotype, cador, cattletype, flocktype, pigtype and virotype products.

For up-to-date licensing information and product-specific disclaimers, see the respective INDICAL kit handbook or user manual.

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