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Protocol Book

Thermo Scientific PathoProof Mycoplasma-8 kit

Instructions for Use

PF8100/PF8500
Revision Date: December 29, 2015

Contents

1. Introduction 1
2. Kit components and storage conditions 2
  2.1 KingFisher Large kit 3
  2.2 Manual Large kit 4
  2.3 Manual Small kit 5
  2.4 Real-time PCR reagent description 6
    2.4.1 PathoProof Mycoplasma-8 kit for Applied Biosystems 7500 and 7500 Fast instrument 6
    2.4.2 PathoProof Mycoplasma-8 kit for Agilent Mx3005P instrument 6
3. Materials required but not supplied 7
4. General guidelines 8
  4.1 Avoiding carryover contamination 8
5. PathoProof Assay Workflow 9
  5.1 DNA extraction 10
    5.1.1 DNA extraction for KingFisher Large kits 11
    5.1.2 DNA extraction for Manual Large kits 13
    5.1.3 DNA extraction for Manual Small kits 14
  5.2 Real-time PCR setup 15
  5.3 Real-Time PCR instrument settings, PCR run and data export 17
    5.3.1 Applied Biosystems 7500 Real-Time PCR System 17
    5.3.2 Agilent Mx3005P qPCR System 19
6. Troubleshooting 21
  6.1 Real-time PCR 21
  6.2 DNA extraction 22
  6.3 Warranty 23
  6.4 Exclusive terms of sale 23
  6.5 Recommended guidelines for safe use of the products 23
  6.6 Permissible use 23
  6.7 Notice to user 23

1. Introduction

Thermo Scientific™ PathoProof™ Mycoplasma-8 kit is designed for accurate same-day identification of mastitis-causing microorganisms from bovine milk using real-time polymerase chain reaction (PCR). PCR technology is used for amplifying DNA in a test tube enabling further analysis of DNA. In the PathoProof kits, real-time PCR detects and amplifies the DNA of mastitis-causing microorganisms in a milk sample. The microorganism in question is identified based on its unique DNA. The kit includes all the necessary reagents for bacterial DNA extraction and PCR. The test has been optimized for use with even the most challenging fresh, frozen and preserved milk samples.

Real-time PCR has become the “gold standard” method for food pathogen testing and quality assurance. Based on this advanced technology, PathoProof kits offer several advantages over the conventional culture-based methods:

- Results are obtained substantially faster.
- Risk of carry-over contamination in the laboratory is minimized because the tests are performed in closed reaction vessels that are not opened after the run.
- Fewer “no growth” results because the test identifies and quantifies DNA, so it accurately detects viable, dead and growth-inhibited microorganisms.
- Applicable for use with preserved milk samples, thus eliminating the need for cooling during sample transportation.
- Can be integrated into milk recording programs using preserved milk.

The PathoProof Mastitis Mycoplasma-8 kit identifies 8 mastitis-causing microorganisms. The microorganisms are detected in two separate PCR reactions. The PathoProof Mastitis Mycoplasma-8 kit identifies the following targets:

- *Staphylococcus aureus*
- *Streptococcus agalactiae*
- *Mycoplasma bovis*
- *Mycoplasma alkalescens*
- *Mycoplasma bovigenitalium*
- *Mycoplasma californicum*
- *Mycoplasma canadense*
- *Mycoplasma* spp.

2. Kit components and storage conditions

PF8100 - PathoProof Mycoplasma-8 kit is for use with the Applied Biosystems™ 7500 and Applied Biosystems™ 7500 Fast Real-Time PCR Systems.

PF8500 - PathoProof Mycoplasma-8 kit is for use with the Agilent™ Mx3005P™ Real Time PCR System.

The PathoProof Mycoplasma-8 kit contains all the necessary reagents (except ethanol) for DNA extraction and real-time PCR. The kits are stable for six months from the packaging date when stored and handled properly.

The tables in Section 2.1 - 2.3 list the components included in the kits.

2.1 KingFisher Large kit

The Thermo Scientific™ KingFisher™ Large kit uses the Thermo Scientific™ KingFisher Flex™ instrument for sample preparation. The KingFisher Large kit is designed for high throughput laboratories, and/or for laboratories requiring a less hands-on time protocol. The kit is sufficient for 4 x 96 reactions when at least 40 samples are analyzed per run.

The PF8100LKF and PF8500LKF kits contain the following components:

Box	Component	Size	Storage conditions
PF8100LKF/ PF8500LKF	Buffer AW1[1]	2x121 ml	Room Temperature
	Buffer AW2[2]	2x68 ml	
	Tween® 20 Solution	5x50 ml	
	Buffer AE	110 ml	
	Buffer RLT[1]	3x35 ml	
	Suspension G2	21 ml	
	F-871L Lysis Solution 1[2]	200 ml	
	Collection Microtubes (racked)	4x96	
	Caps for Collection Microtubes	1x55	
	F-872L Lysis Solution 2	4x10 ml	-20°C
	F-873LKF Proteinase K	2x12 ml	
PF8100LB/ PF8500LB	F-882 PathoProof Master mix	16x1.1 ml	
	F-929 Universal Amplification Standard	350 µl	
PF8100LB only[3]	F-973L Primer Mix 1	2x1.1 ml	-20 °C Protect from light
	F-974L Primer Mix 2	2x1.1 ml	
PF8500LB only[4]	F-971L Primer Mix 1	2x1.1 ml	
	F-972L Primer Mix 2	2x1.1 ml	

1. Contains chaotropic salt. Not compatible with disinfecting agents containing bleach.
2. Contains sodium azide as a preservative.
3. Included in PF8100LKF kit for use with Applied Biosystems 7500 and 7500 Fast instruments only. Not compatible with Agilent Mx3005P instrument
4. Included in PF8500LKF for use with Agilent Mx3005P instruments only with the following filter sets: FAM, Cy5, ROX, ATTO and HEX/JOE. Not compatible with Applied Biosystems 7500 or 7500 Fast instruments.

2.2 Manual Large kit

The Manual Large kits are designed for laboratories performing DNA extraction on large numbers of samples in each session. The extraction is performed in 8 well strips and in 96 column well plates. The kit is sufficient for 4 x 96 reactions when at least 20 samples are analyzed per run.

The PF8100L and PF8500L kits contain the following components:

Box	Component	Size	Storage conditions
PF8100L/ PF8500L	96 column well plates	4	Room Temperature
	S-Blocks[1]	2	
	Collection Microtubes (racked)	4x96	
	Elution Microtubes CL (racked)	4x96	
	Caps for Collection Microtubes	4x55	
	Caps for Elution Microtubes	1x50	
	Microporous tape sheet	25 sheets	
	Buffer AL[2]	2x54 ml	
	Buffer AW1[2] (concentrate)	95 ml	
	Buffer AW2[3] (concentrate)	66 ml	
	Buffer AE	110 ml	
	F-871L Lysis Solution 1[3]	200 ml	
	F-872L Lysis Solution 2	4x10 ml	-20 °C
	F-873L Proteinase K	2x6 ml	
PF8100LB/ PF8500LB	F-882 Master Mix	16x1.1 ml	
	F-929 Universal Amplification Standard	350 µl	
PF8100LB only[4]	F-973L Primer Mix 1	2x1.1 ml	-20 °C Protect from light
	F-974L Primer Mix 2	2x1.1 ml	
PF8500LB only[5]	F-971L Primer Mix 1	2x1.1 ml	
	F-972L Primer Mix 2	2x1.1 ml	

1. Reusable, wash or autoclave after use.
2. Contains chaotropic salt. Not compatible with disinfecting agents containing bleach.
3. Contains sodium azide as a preservative.
4. Included in PF8100L kit for use with Applied Biosystems 7500 and 7500 Fast instruments only. Not compatible with Agilent Mx3005P instrument
5. Included in PF8500L for use with Agilent Mx3005P instruments only with the following filter sets: FAM, Cy5, ROX, ATTO and HEX/JOE. Not compatible with Applied Biosystems 7500 or 7500 Fast instruments.

2.3 Manual Small kit

The Manual Small kit is suitable for laboratories handling small numbers of samples in each DNA extraction session. The DNA extraction is performed in individual tubes. The kit is sufficient for 50 reactions when at least 12 samples are analyzed per run.

The PF8100S and PF8500S kits contain the following components:

<table>
<tr><th>Box</th><th>Component</th><th>Size</th><th>Storage conditions</th></tr>
<tr><td rowspan="7">PF8100S/
PF8500S</td><td>Mini Spin Columns</td><td>50</td><td rowspan="7">Room Temperature</td></tr>
<tr><td>Collection Tubes (2 ml)</td><td>150</td></tr>
<tr><td>Buffer AL[1]</td><td>12 ml</td></tr>
<tr><td>Buffer AW1[1] (concentrate)</td><td>19 ml</td></tr>
<tr><td>Buffer AW2[2] (concentrate)</td><td>13 ml</td></tr>
<tr><td>Buffer AE</td><td>12 ml</td></tr>
<tr><td>F-871S Lysis Solution 1[2]</td><td>30 ml</td></tr>
<tr><td rowspan="4">PF8100SB/
PF8500SB</td><td>F-872S Lysis Solution 2</td><td>4x1.3 ml</td><td rowspan="4">-20 °C</td></tr>
<tr><td>F-873S Proteinase K</td><td>2x1.8 ml</td></tr>
<tr><td>F-882 Master Mix</td><td>2x1.1 ml</td></tr>
<tr><td>F-929 Universal Amplification Standard</td><td>350 µl</td></tr>
<tr><td rowspan="2">PF8100SB only[3]</td><td>F-973S Primer Mix 1</td><td>300 µl</td><td rowspan="4">-20 °C
Protect from light</td></tr>
<tr><td>F-974S Primer Mix 2</td><td>300 µl</td></tr>
<tr><td rowspan="2">PF8500SB only[4]</td><td>F-971S Primer Mix 1</td><td>300 µl</td></tr>
<tr><td>F-972S Primer Mix 2</td><td>300 µl</td></tr>
</table>

1. Contains chaotropic salt. Not compatible with disinfecting agents containing bleach.
2. Contains sodium azide as a preservative.
3. Included in PF8100S kits for use with Applied Biosystems 7500 or 7500 Fast instruments only. Not compatible with Agilent Mx3005P instrument.
4. Included in PF8500S kit for use with Agilent Mx3005P instruments only with the following filter sets: FAM™, Cy5™, ROX™, ATTO and HEX™/JOE™. Not compatible with Applied Biosystems 7500 or 7500 Fast instruments.

2.4 Real-time PCR reagent description

2.4.1 PathoProof Mycoplasma-8 kit for Applied Biosystems 7500 and 7500 Fast instrument

- **F-882**: PathoProof Master Mix. PCR master mix in an optimized buffer, containing $MgCl_2$, deoxynucleoside triphosphates and hot start DNA polymerase.
- **F-929**: PathoProof Universal Amplification Standard. Control DNA for all targets for use as a positive control and for calibration of PathoProof Norden Lab Studio General Edition with the real-time PCR instrument and the reagents.
- **F-973**: PathoProof Mycoplasma-8 kit Primer Mix 1 for Applied Biosystems 7500 and 7500 Fast instruments. PCR primer mix for PCR reaction 1, including oligonucleotides for identification of *S. aureus*, *Str. agalactiae, M. bovis, M.* species and an Internal Amplification Control (IAC). IAC template DNA is also included.
- **F-974**: PathoProof Mycoplasma-8 kit Primer Mix 2 for Applied Biosystems 7500 and 7500 Fast instruments. PCR primer mix for PCR reaction 2, including oligonucleotides for identification of *M. alkalescens*, *M. bovigenitalium*, *M. californicum*, *M. canadense* and an IAC. IAC template DNA is also included.

2.4.2 PathoProof Mycoplasma-8 kit for Agilent Mx3005P instrument

- **F-882**: PathoProof Master Mix. PCR master mix in an optimized buffer, containing $MgCl_2$, deoxynucleoside triphosphates and hot start DNA polymerase.
- **F-929**: PathoProof Universal Amplification Standard. Control DNA for all targets for use as a positive control and for calibration of PathoProof Norden Lab Studio General Edition with the real-time PCR instrument and the reagents.
- **F-971**: PathoProof Mycoplasma-8 Primer Mix 1 for Agilent Mx3005P instruments. PCR primer mix for PCR reaction 1, including oligonucleotides for identification of *S. aureus*, *Str. agalactiae, M. bovis, M.* species and an IAC. IAC template DNA is also included..
- **F-972**: PathoProof Mycoplasma-8 Primer Mix 2 for Agilent Mx3005P instruments. PCR primer mix for PCR reaction 2, including oligonucleotides for identification of *M. alkalescens*, *M. bovigenitalium*, *M. californicum*, *M. canadense* and an IAC. IAC template DNA is also included.

3. Materials required but not supplied

The materials required but not supplied with the PathoProof Mycoplasma-8 kit are listed below. Please contact your local Technical Support for ordering details.

- Disposable powder-free gloves
- Ethanol (96–100%); do not use denatured ethanol
- Pipettes
- Sterile pipette tips with filter
- PCR plates compatible with the real-time PCR instrument
    - For ABI7500 Fast system: order catalogue number AB-1900 - 96-well Fast PCR Plate
    - For ABI7500 system: order catalogue number AB-1400 - 96-well PCR Plate
    - For Agilent Mx3005P/Mx3000P systems: order catalogue number PF0016A 96-well PCR Plate
- Optically clear PCR vessel caps: order catalogue number PF0866A - Optically clear flat 8 Cap Strips
- Vortex mixer
- Microcentrifuge compatible with the spin tubes and strip tubes
- Either 1.5 ml or 2 ml microcentrifuge tubes for sample preparation
- Strip tubes for sample preservation after DNA extraction (optional)
- Real-time PCR instrument compatible with PathoProof kits:
    - Applied Biosystems 7500 and 7500 Fast Real-Time PCR System with the following dyes calibrated: FAM, CY5, Texas Red, VIC and TAMRA
    - Agilent Mx3005P qPCR System with filter sets for: FAM, CY5, ROX, HEX/JOE and ATTO.

Depending on the extraction platform used, materials or instruments not supplied with the PathoProof Mycoplasma-8 kit are listed below. Please contact your local Technical Support for ordering details.

KingFisher Large kits:

- KingFisher Flex instrument
- Plate centrifuge capable of 1500 xg
- Microtiter 96 DeepWell Plate
- Kingfisher Flex 96 Tip Comb
- Cap Mat for 96 DeepWell Plate or Adhesive Plate Seal

Manual Large kits:

- Decapping tool
- Thermo Scientific™ Heraeus™ Multifuge X3 plate centrifuge
- Two dry bath incubators for +37°C and +55°C
- Two dry bath 96 blocks for Collection Microtubes

Manual Small kits:

- Microcentrifuge for 1.5 or 2 ml tubes that is capable of 20 000 xg
- Two dry bath incubators for +37°C and +55°C
- Two or more dry bath blocks for 1.5 ml or 2 ml tubes

4. General guidelines

The following general guidelines should be followed throughout the PathoProof Mycoplasma-8 kit protocol:

- Use protective gloves
- Thaw all frozen reagents thoroughly prior to use
- Mix all solutions well before use
- Spin down reagents after mixing

4.1 Avoiding carryover contamination

Due to their high sensitivity, real-time PCR kits are susceptible to carryover contamination of DNA. The contaminating DNA is typically an amplification product from a real-time PCR run, but can also originate from samples containing high quantities of target bacterial DNA.

The PathoProof Mycoplasma-8 kit does not require opening of the real-time PCR vessels once the real-time PCR program has been started. While this kit design significantly reduces the risk of cross-contamination, the following general guidelines should be followed, in addition to other precautions mentioned in this instruction manual, in order to minimize such risks:

- Designate physically separated working areas for:
  - DNA extraction (handling of milk samples or other samples containing bacteria)
  - PCR preparation
- Use different laboratory equipment (disposable gloves, pipettes, pipette tip boxes, vortexes, centrifuges, laboratory coats etc.) in each working area.
- Change gloves frequently and always before leaving an area.
- Use aerosol-resistant pipette tips.
- Use new and/or sterilized plastic ware.
- After starting a real-time PCR run, do not open the real-time PCR vessels under any circumstances.
- Always dispose the real-time PCR vessels into a dedicated, closed trash container and make sure that the vessels do not open accidentally.

5. PathoProof Assay Workflow

This section contains instructions for performing DNA extraction, real-time PCR setup and PCR run. The instructions for performing results analysis with Thermo Scientific™ PathoProof™ Norden Lab Studio software are given in the PathoProof Norden Lab Studio Software manual. An overview of the laboratory workflow is presented in **Figure 1** below.

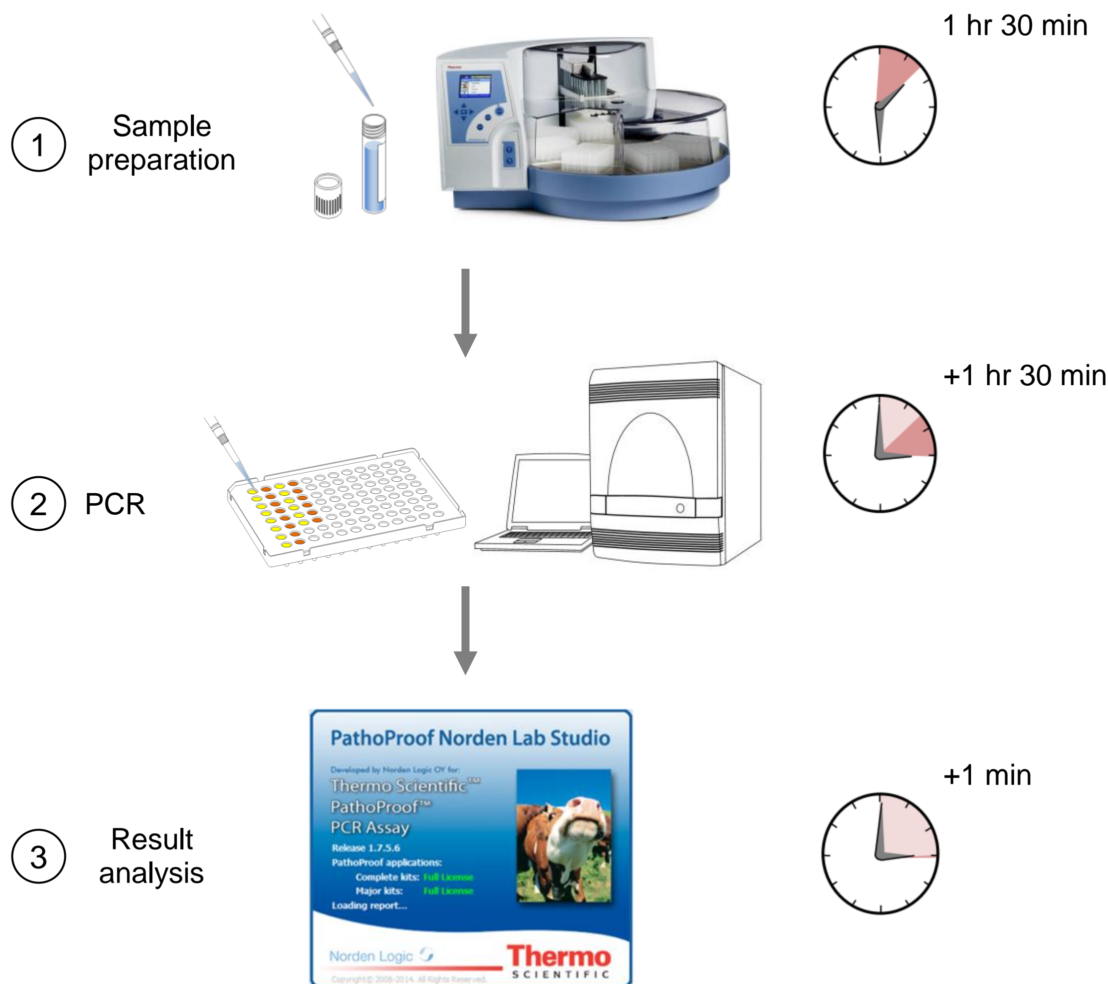


Figure 1. Schematic illustration of the PathoProof assay workflow in three easy steps.

1. DNA is extracted from fresh, frozen or preserved bovine milk samples.
2. Extracted DNA is used to set up the PCR reactions in a 96-well PCR plate. Two separate PCR reactions are run for each sample.
3. The obtained data is interpreted, reported and data-based with Norden Lab Mastitis Studio.

PathoProof Norden Lab Studio is a software application designed for interpreting, reporting and storing the results obtained using PathoProof kits. This software facilitates data analysis and is highly recommended as an integral part of the procedure for the PathoProof Mycoplasma-8 kit.

When using PathoProof kit for the first time, it is necessary to calibrate PathoProof Norden Lab Studio software. Calibration may also be required when changing real-time PCR plastics or when the real-time PCR instrument has undergone maintenance. The calibration runs and experiment runs must be performed using the same real-time PCR instrument, the same type of vessels/plastics, and same optically clear caps for sealing the PCR plate.

Dye calibration is also required for Applied Biosystems™ 7500 instruments that have not previously been calibrated for dyes FAM, CY5, TAMRA, VIC and TEXAS RED.

5.1 DNA extraction

Things to do before starting:

- Buffers AW1 and AW2 are supplied as concentrates. Before using them for the first time, add the amount of ethanol (96–100%) indicated on the bottles.
- Equilibrate Buffer AE to room temperature.
- Plan sample layout before starting the DNA extraction (KingFisher Large and Manual Large kit extraction only).

KingFisher Large kits only:

- Buffer RLT is provided in 35 ml bottles. To prepare the mixture be used in the extraction, add 35 ml ethanol (96-100%) and 7 ml Suspension G to the Buffer RLT bottle. Vortex the Suspension G for 3 min before using it for the first time and 1 min before subsequent use. The mixture is stable for one year.
- The 96 Tip Combs are supplied as packages of 2. If opening a new package, store the other 96 Tip Comb within an unused 96 DeepWell Plate.
- Wash and Elution plates can be filled, sealed with Cap Mats or Adhesive Plate Seals and preserved at room temperature for one month. If using Adhesive Plate Seals ensure that the rims of the plates are dry and clean before sealing. Ensure that no liquid has evaporated from the wells (especially from the corner wells) before using the prefilled plates for extraction. Small amount of evaporation (10-25%) does not affect the efficiency of the extraction. Prefilled plates should not be shaken or heated.

Manual Large and Small kits only:

- Set two incubators with the proper block inserts at +37°C and +55°C.

5.1.1 DNA extraction for KingFisher Large kits

1. Prepare eight 96 DeepWell plates according to the table below.

Plate name	Reagent	Volume per well
Tip Comp Plate	Place Tip Comp plate into 96 DW plate	-
Sample	Proteinase K F-873LKF	40 µl
Wash 1	Buffer AW1	800 µl
Wash 2	Buffer AW1	500 µl
Wash 3	Buffer AW2	500 µl
Wash 4	Buffer AW2	500 µl
Wash 5	Tween® 20 solution	600 µl
Elution	Buffer AE	150 µl

- Add reagents only to wells allocated for each sample and the extraction negative control. Example sample layout for a total of 60 samples and negative control is presented in **Figure 2.**
- Mark the seven 96 DeepWell plates accordingly from Sample, Wash 1 to Wash 5 and Elution for later identification.

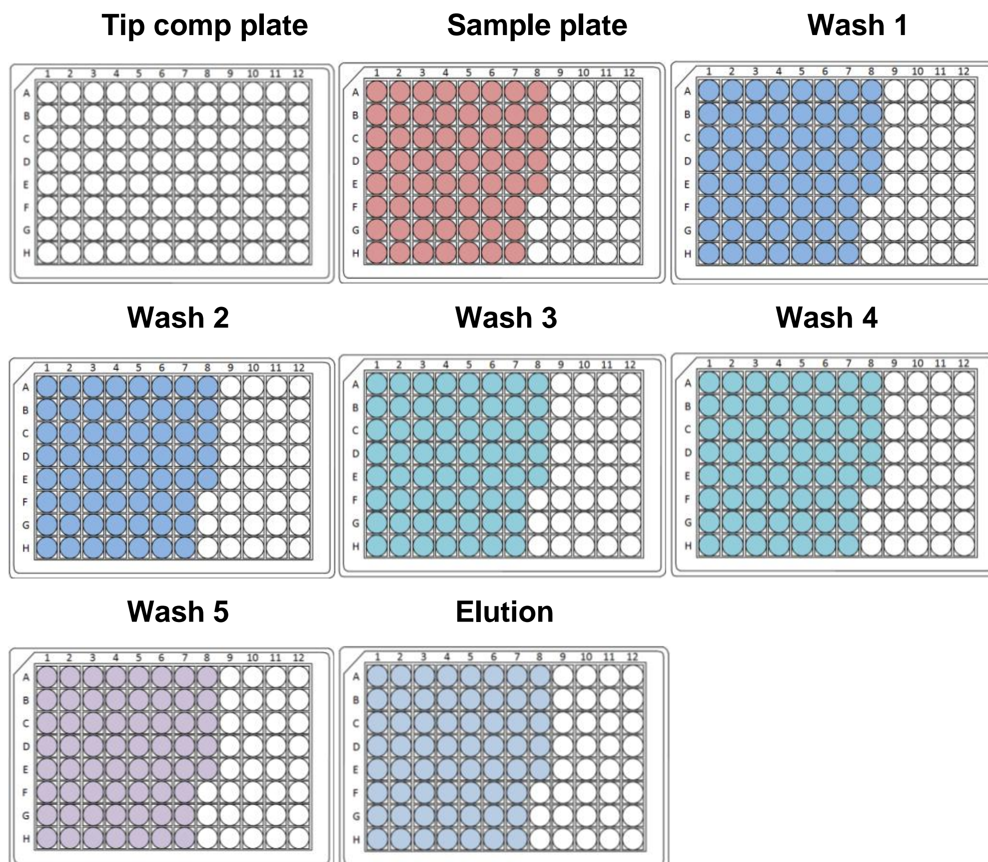


Figure 2. Filling the Thermo Scientific™ KingFisher™ 96 DeepWell plates for KingFisher Large kit. This setup is an example for 60 samples and 1 extraction negative control.

- After filling the plates, put these plates aside for the sample preparation process (steps 2 – 5). The prefilled plates can be stacked (96 Tip Comb plate at the top) to avoid contamination of the extraction reagents.

2. Depending on the sample type, prepare samples for centrifugation using the instructions below:

    - For individual quarter milk samples: Add 400 µl of Lysis Solution 1 and 400 µl milk sample into racked Collection Microtubes. Avoid pipetting milk clots into the reaction vessels. However, this may not always be possible as the quality of samples can vary remarkably.
    - For DHI samples: Prepare a fresh mix containing the following volumes per sample: 5 µl Proteinase K and 400 µl Lysis Solution 1. If using a multichannel pipette, add in an extra 1-2 reactions to ensure sufficient volume. Add 400 µl of the Lysis Solution 1/Proteinase K mix and 600 µl milk sample into racked Collection Microtubes.

    In addition to the milk samples, include at least one negative control (reagents only) in each DNA extraction run. Seal the tubes using the caps provided for Collection Microtubes.

3. Mix well by vortexing or shaking and centrifuge for 3 min at 1500 xg.
4. The presence of fat on top of the supernatant following centrifugation is normal. Remove the supernatant and fat layer on top of supernatant using a pipette without disturbing or removing the pellet. After liquid removal, approximately 50-100 µl of residual fat/liquid on top of the pellet is normal and does not need to be removed.
5. Resuspend the pellet carefully in 100 µl Lysis Solution 2 by pipetting up and down and transfer the sample into a well of the sample plate using a pipette. If the pellet is difficult to resuspend, 100-200 µl sterile water can be added to facilitate the resuspension.
6. Switch on the KingFisher Flex instrument at the power switch. If using the KingFisher Flex instrument through a computer, open the Thermo Scientific™ BindIt™ software from the computer connected to the KingFisher Flex instrument.
7. Select the ''PathoProof'' protocol from the DNA/RNA menu under factory protocols. Press ''Start'' to start the run.
8. Open the cover of the instrument and load the plates into the instrument as indicated on the KingFisher Flex display. After each plate press ''Start'' and after the worktable has rotated load the next plate. After loading the last plate close the protective cover and press ''Start'' to initiate the run.

After completion of the sample incubation steps, the extraction protocol pauses for 2 minutes and offers the option to remove the sample plate. Remove the sample plate immediately when the instrument pauses or wait for the full 2 minutes before removing the plate. At the end of the next step, take caution to only add the plate back when the instrument asks for insertion of the sample plate. This will avoid spillages and potential injury from the moving instrument turntable.

9. Take the sample block from the KingFisher instrument, shake the mixture containing Buffer RLT, ethanol and Suspension G thoroughly for 10 s and add 440 µl to each sample in the sample plate. Load the plate back into the instrument when the instrument asks to do so. Press ''Start'' to continue the run.

If dispensing the mixture into wells with a multichannel dispenser, make sure tips do not touch the well walls or the liquid in the wells.

10. After the samples have been processed remove the plates as instructed by the KingFisher Flex display.
    - If the eluted samples are going to be stored, seal the wells of the elution plate with a Cap Mat or an Adhesive Plate Seal.
    - The presence of a small amount of beads in the eluted DNA is normal and does not affect the PCR step.

    Proceed to Section 5.2 for real-time PCR setup.

5.1.2 DNA extraction for Manual Large kits

Centrifugation of 96 column well plates is performed on the Heraeus Multifuge X3 plate centrifuge.

1. Prepare a fresh mix containing the following volumes per sample: 7 µl Proteinase K and 350 µl Lysis Solution 1. If using a multichannel pipette, add in an extra 1-2 reactions to ensure sufficient volume.
2. Vortex or shake the milk samples thoroughly. Add 350 µl of Lysis Solution 1/Proteinase K mix and 350 µl milk sample into racked Collection Microtubes. Avoid pipetting milk clots to the reaction vessels. In addition to the milk samples, include at least one negative control (reagents only) in each DNA extraction. Seal the tubes using the caps provided for Collection Microtubes.
3. Vortex briefly and incubate at 55°C for 5 min.
4. Centrifuge for 5 min at 5000 rpm.
5. The presence of fat on top of the supernatant following centrifugation is normal. Remove the supernatant and fat using a pipette without disturbing or removing the pellet. Residual fat/liquid on top of the pellet is normal and does not need to be removed.
6. Resuspend the pellet in 100 µl Lysis Solution 2 by pipetting up and down. Seal the tubes using new caps for Collection Microtubes.
7. Incubate at 37°C for 10 min.
8. Prepare a fresh mix containing the following volumes for each sample: 20 µl Proteinase K and 200 µl Buffer AL. If using a multichannel pipette, add in an extra 1-2 reactions to ensure sufficient volume. Add 220 µl of the mix to each sample, taking care not to wet the rims of the Collection Microtubes. Seal the tubes using new caps for Collection Microtubes.
9. Mix thoroughly by shaking vigorously for 15 sec. For efficient lysis, it is essential that the samples and Buffer AL are mixed immediately and thoroughly to yield a homogeneous solution. Hold the racked Collection Microtubes with both hands and shake up and down vigorously.
10. Incubate at 55°C for 10 min. Centrifuge briefly at 3000 rpm to remove drops from inside the caps. Stop the centrifuge as soon as it reaches 3000 rpm.
11. Add 200 µl ethanol (96–100%) to each tube. Seal the tubes using new caps for Collection Microtubes. Shake vigorously for 5 sec. Centrifuge briefly at 3000 rpm to remove drops from inside the caps. Stop the centrifuge as soon as it reaches 3000 rpm.

12. Place the 96 column well plate on top of an S-Block. Mark the plate for later identification. Remove the possible viscous clots from the mixture prepared in step 11 using a pipette. Then, carefully apply the supernatant to the 96 column well plate. Take care not to wet the rims of the wells to avoid aerosol formation during centrifugation.
13. Seal the 96 column well plate with Microporous tape sheet. Load the S-Block and 96 column well plate onto the carrier. Place the carrier in the rotor bucket and centrifuge at 6000 rpm for 4 min.
14. Place the 96 column well plate on top of an empty S-Block. Remove the tape. Carefully add 500 µl Buffer AW1 to each well. Seal the 96 column well plate with a new Microporous tape sheet. Centrifuge at 6000 rpm for 4 min.
15. Place the 96 column well plate on top of an empty S-Block. Remove the tape. Carefully add 500 µl Buffer AW2 to each well. Do not seal the plate, to ensure evaporation of residual ethanol in the following centrifugation step. Centrifuge at 6000 rpm for 15 min. The heat generated during centrifugation ensures evaporation of residual ethanol in the sample (from Buffer AW2) that might otherwise inhibit subsequent reactions.
16. Place the 96 column well plate on top of a rack of Elution Microtubes. To elute the DNA, add 100 µl Buffer AE to each well. Seal the 96 column well plate with a new Microporous tape sheet, and incubate for 1 min at room temperature. Centrifuge at 6000 rpm for 4 min. Seal the wells of the Elution Microtubes using the caps for Elution Microtubes.

Proceed to Section 5.2 for real-time PCR setup.

5.1.3 DNA extraction for Manual Small kits

Centrifugation of spin columns is performed using a Microcentrifuge capable of 20 000 xg.

1. Prepare a fresh mix containing the following volumes per sample: 7 µl Proteinase K and 350 µl Lysis Solution 1. Add in an extra 1 - 2 reactions to ensure sufficient volume.
2. Vortex or shake the milk samples thoroughly. Add 350 µl of Lysis Solution 1/Proteinase K mix and 350 µl milk to each reaction vessel. Avoid pipetting milk clots into the reaction vessels. In addition to the milk samples, include at least one negative control (reagents only) in each DNA extraction.
3. Vortex briefly and incubate at 55°C for 5 min.
4. Centrifuge for 5 min at 5000 xg.
5. The presence of fat on top of the supernatant following centrifugation is normal. Remove the supernatant and fat using a pipette without disturbing or removing the pellet. Residual fat/liquid on top of the pellet is normal and does not need to be removed.
6. Resuspend the pellet in 100 µl Lysis Solution 2 by pipetting up and down.
7. Incubate at 37°C for 10 min.
8. Prepare a fresh mix containing the following volumes per sample; 20 µl Proteinase K and 200 µl Buffer AL. Add in an extra 1–2 reactions to ensure sufficient volume. Add 220 µl of the mix to each reaction vessel. Mix by vortexing for 5–10 sec.
9. Incubate at 55°C for 10 min. Briefly centrifuge the reaction vessels to remove drops from inside the caps.

10. Add 200 µl ethanol (96–100%) to each sample and mix by pulse-vortexing for 15 sec. It is essential that the sample, the Buffer AL and the ethanol are mixed thoroughly to yield a homogeneous solution. Do not use alcohols other than ethanol, as this may result in reduced DNA yields. After mixing, briefly centrifuge the reaction vessels to remove drops from inside the caps.
11. Remove the possible viscous clots from the mixture using a pipette. Then, carefully apply the supernatant to the spin column (placed inside a 2 ml collection tube) without wetting the rim. Close each spin column to avoid aerosol formation during centrifugation. Centrifuge at 20,000 xg (~14,000 rpm) for 1 min. Place the spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
12. Carefully open the spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 20,000 xg (~14,000 rpm) for 1 min. Place the spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
13. Carefully open the spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at 20,000 x g (~14,000 rpm) for 3 min.
14. Place the spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the spin column and add 100 µl Buffer AE. Incubate at room temperature for 1 min, and then centrifuge at 20,000 x g (~14,000 rpm) for 3 min to elute the DNA. Discard the spin column.

Proceed to Section 5.2 for real-time PCR setup.

5.2 Real-time PCR setup

Throughout the procedure, follow the general guidelines detailed in Section 4.

Important notes before starting:

- Use at least one negative (no template) PCR control for both PCR solutions in each real-time PCR run.
- If all the samples are expected to be negative for the targets, it is advisable to include the PathoProof Universal Amplification Standard and/or a positive DNA extraction control (such as a milk sample previously tested positive with PathoProof Mycoplasma-8 kit) in each real-time PCR run.
- The experiment runs must be performed using the same real-time PCR instrument, the same type of vessels and the same sealing method (optically clear caps) that was used when calibrating the PathoProof software (see PathoProof Norden Lab Studio Software manual).
- The purified DNA can be stored at +5°C for a few days and for longer periods at -20°C.

1. Vortex briefly the thawed PathoProof Master Mix and PathoProof Primer Mixes 1 and 2. Spin down.
2. Prepare two separate PCR solutions by combining PathoProof Master Mix and PathoProof Primer Mixes 1 and 2 in separate microcentrifuge tubes. Use the formula below to calculate the volumes required. The formula provides excess volume to compensate for volume loss due to reagent pipetting steps.

PCR solution	Reagent	Volume
PCR solution 1	PathoProof Master Mix	N x 11 µl
	PathoProof Primer Mix 1	N x 5.5 µl
PCR solution 2	PathoProof Master Mix	N x 11 µl
	PathoProof Primer Mix 2	N x 5.5 µl
N = Number of samples including: • Negative PCR control (necessary in every run) • PathoProof Universal Amplification Standard (positive control, optional) • Negative DNA extraction control (recommended) • Positive DNA extraction control (optional) • DNA from extracted milk samples		

3. Vortex the PCR solutions briefly and spin down.
4. Add 15 µl of the PCR solutions 1 and 2 into the two wells allocated for each sample. **Figure 3** shows an example for allocating PCR solutions for 13 samples and one negative control.

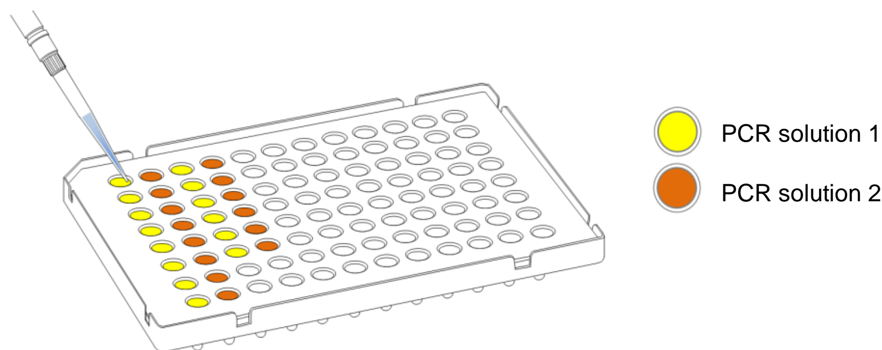


Figure 3. Example setup for pipetting PCR solutions in Mycoplasma-8 kit.

Always add PCR solution 1 to plate columns 1, 3, 5, 7, 9 and 11 PCR Solution 2 to columns 2, 4, 6, 8, 10 and 12. This order must be maintained for correct data analysis when using the PathoProof Norden Lab Studio software.

5. Add 5 µl of the eluate from the DNA extraction protocol (Section 5.1) into the two wells allocated for each sample. **Figure 4** shows an example for pipeting 13 samples and negative control into previously prepared PCR solution plate.

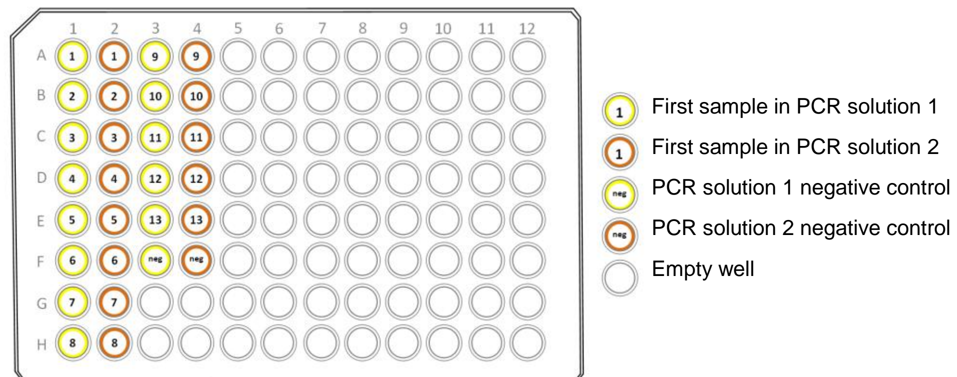


Figure 4. Example setup of pipeting 13 samples and one PCR negative control. Negative PCR control reactions are always allocated to the last available wells.

a. If the PathoProof Universal Amplification Standard is included in the test setup, add 5µl of the standard into two consecutive wells.

b. In the wells allocated for negative (no template) controls, add 5µl of sterile water or Elution Buffer (AE).

6. Close the 96-well PCR plate with **optically clear caps** and centrifuge using a suitable plate centrifuge (1500 g, 5 s).

   Note: It is critically important to use the optically clear caps. Do not use adhesive seal or heat seal to close the PCR plates as performance of the assay will be adversely affected.

   Proceed to instrument-specific instructions in Section 5.3.

5.3 Real-Time PCR instrument settings, PCR run and data export

Instrument specific template files needed for PathoProof calibration and sample runs will be installed into the PathoProof Norden Lab Studio directory, and a link to these files will be created onto the desktop during the software installation process. PathoProof software (and the instrument specific template files) is available to download at www.nordenlogic.com

5.3.1 Applied Biosystems 7500 Real-Time PCR System

PathoProof assays are only compatible with instrument software versions 2.0.6 and above.

Before using Mycoplasma-8 kit, the Applied Biosystems 7500 or 7500 Fast Real-Time PCR System needs to be calibrated for the following dyes:

- FAM
- VIC
- TAMRA
- CY5
- TEXAS RED

If the Applied Biosystems 7500 or 7500 Fast Real-Time PCR System does not have these dyes calibrated, please contact your local technical support to order Spectral Calibration Kits I and II for ABI7500 systems.

1. Open template file

Open the template file: PathoProof_Mycoplasma-8_template.edt.

2. Name samples and load the plate

Name the samples as needed (samples may be named after the run if preferred) in the Define Targets and Samples tab during the Setup phase. The sample names will be automatically added to the plate setup in the same order as in figure 4 (the sample 1 is in wells A1-A2, sample 2 is in wells B1-B2 and so on).

Load the PCR plate into the PCR instrument.

3. Clear empty wells

In Assign Targets and Samples tab, select wells that are not going to be used and select “Clear”.

4. Verify correct settings in the template file

Verify that the settings for plate setup are as follows:

- S.aur, S.aga, My.sp, M.bov and IAC are in columns 1 3, 5, 7, 9 and 11 (highlighted in yellow)
- M.bg, M.can, M.alk, M.cal and IAC are in columns 2, 4, 6, 8, 10 and 12 (highlighted in orange)

Verify that the settings for Run Method are as follows:

Number of cycles: 40

Holding stage:

- Step 1 95˚C 10 min

Cycling stage

- Step 1 95˚C 5 seconds
- Step 2 60˚C 60 seconds
- Endpoint read (Collect data -symbol on step 2)

5. Save the run

Save the file in a separate location and with different file name for easy identification and to ensure, that the default PathoProof Mycoplasma-8 template file is not over written.

6. Start the run

Start the run by clicking the **Start run** -button in the Setup Screen.

7. Export raw data for the PathoProof software

After the real-time PCR run, choose Analysis option from the left side of the screen if this has not yet been selected automatically.

Select **Export**.

Make sure that the following check boxes have been selected:

- Sample Setup
- Amplification Data
- Open file(s) when export is complete
- Save current settings as the default

Make sure that the following check boxes have been unselected:

- Raw Data
- Results
- Multi component Data

Make sure that the file format is **.xls** and that the selection is **One File**.

Select **Start Export**.

- Save the generated excel file to the computer on which PathoProof Norden Lab Studio is installed.

Now follow the instructions in your PathoProof Norden Lab Studio Software manual in order to import the data into Norden Lab Studio.

5.3.2 Agilent Mx3005P qPCR System

PathoProof Mycoplasma-8 kit is only compatible with instrument that has the following filter sets:

- FAM
- CY5
- ROX
- HEX/JOE
- ATTO

If your Agilent instrument does not have these filter sets, please contact Thermo Fisher Scientific technical support: microbiology.techsupport.uk@thermofisher.com.

1. Open template file

- Open the template file PathoProof_Mycoplasma-8_template.mxp
- Switch the instrument's lamp on by clicking the lamp icon.

2. Name samples and load the plate

- Name the samples as needed (samples may be named after the run if preferred).
- Insert the PCR plate

3. Clear empty wells

Select the empty wells and click “Clear Selected Wells” in the panel at the right.

4. Verify correct settings in the template file

Before the first run and after instrument maintenance, verify that the Filter Gain Settings are as follows (from the Instrument selection -> Filter Set Gain Settings):

- 1x for CY5, 4x for ATTO, 1x for ROX, 2x for HEX/JOE and 4x for FAM

Verify that the plate setup has all the 16 targets and IACs active, and that filter sets CY5, ROX, HEX/JOE, FAM and ATTO have a mark in the check boxes at right side of the screen (under the Collect fluorescence data).

Verify that the settings for Thermal Profile Setup are as follows:

Segment 1

- 10 min. at 95 °C

Segment 2 (40 cycles)

- 95 °C 5 seconds
- 60 °C 60 seconds, Endpoint read (End symbol on Step 2)

5. Save the run

Save the file in a separate location and with different file name for easy identification and to ensure, that the Pathoproof Mycoplasma-8 template file is not over written.

6. Start the run

Start the run by clicking the **Start run** -button in the Thermal Profile Setup.

7. Export raw data for the PathoProof software

After the real-time PCR run:

- Click the Analysis button, then the Results tab.
- Select File menu from the top left.
- Select Export Chart → Export Chart Data to Text file → Format 1 -- Vertically Grouped by Plot....
- Copy the resulting files to the computer on which PathoProof Norden Lab Studio is installed.

Now follow the instructions in your PathoProof Norden Lab Studio Software manual in order to import the data into PathoProof Norden Lab Studio.

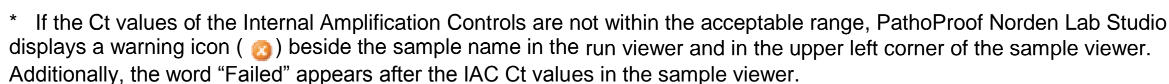
6. Troubleshooting

Possible problems in real-time PCR are presented first, followed by problems in DNA extraction.

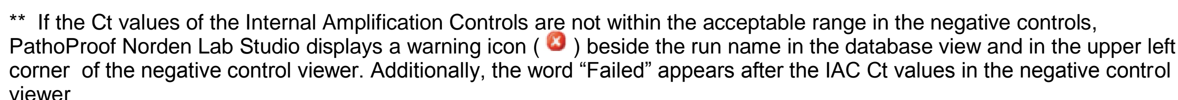
6.1 Real-time PCR

This table presents explanations for the following problems: IAC Ct values are not within acceptable range, positive target amplification signal in negative control, acceptable IAC Ct in all samples but no positive results, false positive signal read in most of the samples

Problem	Possible explanation(s)	Recommended action
The Ct values of the Internal Amplification Controls are not within the acceptable range in samples* and in negative controls**.	Missing reagents in the PCR setup.	Repeat the real-time PCR
	Incorrect volume of master mix and/or primer mix in wells.	Make sure that the correct amounts of primer mix and master mix are added into the correct wells.
Unacceptable IAC amplification signals for all samples* but acceptable IAC signals for the Negative controls.	PCR inhibitors originating from the DNA extraction are present in the test samples.	Refer to Chapter 5.1 "DNA extraction".
Unacceptable IAC amplification signals for all parallel reactions of one sample* but acceptable IAC signals in the other samples and the Negative controls.	Missing reagents in the wells of the sample or incorrect volume of reagent.	Repeat the real-time PCR for the sample.
	The PCR inhibitor concentration of the sample is too high.	Dilute the extracted DNA sample (use 1:5 and 1:10 dilutions for example) and repeat the real-time PCR. See Chapter 5.1 "DNA extraction”.
Unacceptable IAC amplification signals for one reaction of one sample* but acceptable IAC amplification signals for the other parallel reactions of the sample, the other samples and the Negative controls.	Missing reagents in the well concerned or incorrect volume of reagent.	Repeat all parallel real-time PCR reactions of the sample.
	Bacterial DNA is present in a high quantity (+++) in the sample (confirm the presence of bacterial DNA by inspecting the amplification curves).	No action required.
	PCR inhibitors originating from the DNA extraction are present in the test samples at such high concentration that they inhibit the amplification of less robust IAC (the higher the inhibitor concentration, the more IACs will fail starting from primer mix 1)	Dilute the extracted DNA sample (use 1:5 and 1:10 dilutions for example) and repeat the real-time PCR for all parallel reactions of that sample. See Chapter 5.1 “DNA extraction”.
Unacceptable IAC amplification signals** for one reaction of Negative control or all parallel reactions of Negative control but acceptable IAC amplification signals in sample wells.	Missing reagents in the wells of the negative control.	No action required because the IACs in the samples are acceptable
	Incorrect volume of master mix and/or primer mix in wells.	
Positive bacterial target amplification signals in wells for Negative control.	A carryover contamination may have occurred in the laboratory.	Take precautions detailed in Section 4.1 to minimize contaminations.
Acceptable IAC amplification signals in all wells, but all targets negative.	Failed DNA extraction.	Refer to Chapter 5.1 “DNA extraction”. Include PathoProof Amplification Standard and/or a positive DNA extraction control, such as a milk sample previously tested positive with PathoProof kit.
	There were no bacteria present in the samples.	
Amplification signal is not smooth and/or is linear	Adhesive seal or heat seal was used in closing PCR plate. Heating protocol used in PathoProof will stretch the seal on well, which may result in abnormal signal reads.	Refer to section 4.3 in PathoProof Norden Lab Studio IFU to interpret and exclude false positive signal reads from sample. To avoid false positive signal reads, use optically clear caps



* If the Ct values of the Internal Amplification Controls are not within the acceptable range, PathoProof Norden Lab Studio displays a warning icon ( ) beside the sample name in the run viewer and in the upper left corner of the sample viewer. Additionally, the word “Failed” appears after the IAC Ct values in the sample viewer.



** If the Ct values of the Internal Amplification Controls are not within the acceptable range in the negative controls, PathoProof Norden Lab Studio displays a warning icon ( ) beside the run name in the database view and in the upper left corner of the negative control viewer. Additionally, the word “Failed” appears after the IAC Ct values in the negative control viewer

6.2 DNA extraction

This table presents explanations for the following problems: little or no DNA was obtained from the milk samples, or the extracted DNA is contaminated with inhibitory substances.

Possible explanation(s)	Recommended action
Inefficient lysis due to reduced Proteinase K activity.	Be sure to store the Proteinase K solution at -20 °C or, when in use, on ice. Always be sure to prepare fresh mixes of Proteinase K with Lysis Solution 1 and with Buffer AL. Repeat the DNA purification procedure with a new sample.
Inefficient cell lysis due to insufficient mixing of the sample with Buffer AL.	Repeat the DNA purification procedure with a new sample. Be sure to mix the sample and Buffer AL immediately and thoroughly by pulse-vortexing.
No ethanol or incorrect kind of ethanol added to the lysate before loading onto the column or the 96 column well plates.	Repeat the purification procedure with a new sample. Use 96–100% ethanol. Do not use denatured ethanol, isopropanol or lower percentage ethanol.
Buffer AW1 or AW2 prepared incorrectly.	Repeat the purification procedure with a new sample. Ensure that the Buffer AW1 and AW2 concentrates were diluted with the correct volumes of 96–100% ethanol as indicated on the bottles. Do not use denatured alcohol, isopropanol or lower percentage ethanol.
Buffers AW1 and AW2 used in the wrong order.	Ensure that Buffers AW1 and AW2 are used in the correct order in the protocol. Repeat the purification procedure with a new sample.
Residual Buffer AW2 in the eluate.	Ensure that the spin column or the 96 column well plate does not come into contact with the filtrate prier to elution.
	Before DNA elution, ensure that the 96 column well plate is centrifuged without the Microporous tape sheet in order to allow ethanol to evaporate from the sample.
Mini spin column or 96 column well plate not incubated at room temperature (15–25 °C) for 1 min before elution.	After the addition of Buffer AE, the spin column or the 96 column well plate should be incubated at room temperature for at least 1 min.
DNA not eluted efficiently.	To increase elution efficiency, add Buffer AE onto the center of the spin column or the 96 column well plate and incubate for 5 min at room temperature before centrifugation.

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6.6 Permissible use

This product is intended to be used for the purpose of detection and/or analysis of microorganisms in milk for quality assurance and quality control purposes (Food Testing Applications), as well as for identification, enumeration, or count of microorganisms in raw material sample, process control sample, or finished product sample of an industrial process for the purpose of detecting the presence, absence or amount either of a contaminant or of an intended component (Industrial Microbiology Applications).

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Contact Information:
+44 (0) 1256 841144
microbiology.techsupport.uk@thermofisher.com
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