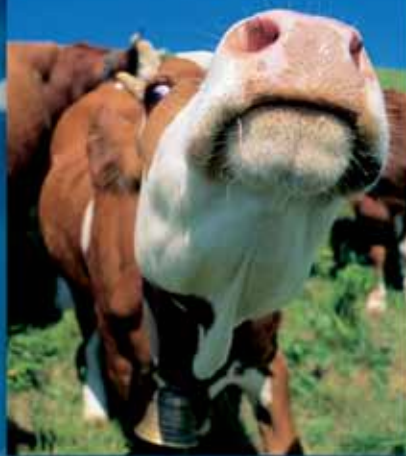


PathoProof® Mastitis Major-3 Kit

Now sold as
Thermo Scientific



F-914

Instruction manual

F-914S Sufficient for 50 tests*

F-914L Sufficient for 4 x 96 tests**

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* Sufficient for 50 reactions when at least 16 samples are analyzed per set

** Sufficient for 4 x 96 reactions when at least 20 samples are analyzed per set

1. Introduction

Finzymes' PathoProof® Mastitis Major-3 Kit is designed for accurate same-day identification of mastitis-causing bacteria from bovine milk using quantitative real-time polymerase chain reaction (PCR). PCR technology is used for amplifying DNA in a test tube which then enables further analysis of DNA. In PathoProof assay, real-time PCR detects the presence of bacterial DNA in a milk sample and identifies the bacterial species in question based 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 milk samples.

Real-time PCR has become the “gold standard” method for food pathogen testing and quality assurance. Based on this advanced technology, PathoProof Mastitis Major-3 Kit offers several advantages over the conventional bacterial culture method:

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

The PathoProof Mastitis Major-3 Kit identifies the following three contagious mastitis bacteria in a single PCR reaction:

- *Staphylococcus aureus*
- *Streptococcus agalactiae*
- *Mycoplasma bovis*

Norden Lab Mastitis Studio General Edition is a software application designed for interpreting, reporting and storing the results obtained using PathoProof Mastitis Major-3 Kit together with Stratagene Mx3005P® or Mx3000P® QPCR System. This software facilitates data analysis and is highly recommended as an integral part of the procedure for the PathoProof Mastitis Major-3 Kit.

2. Kit components and storage conditions

The PathoProof Mastitis Major-3 Kit contains all the necessary reagents for bacterial DNA extraction and real-time PCR. The F-914S reagent kit is sufficient for 50 tests*, and the F-914L kit for 4 x 96 tests**. The kits are stable for six months from the packaging date when stored and handled properly.

The F-914S kit uses a DNA extraction method by which the bacterial DNA is extracted from each milk sample one by one. The F-914S kit is suitable for laboratories handling small numbers of samples in each DNA extraction session.

The F-914L kit uses a 96-well-format DNA extraction method by which 96 samples can be processed simultaneously. The F-914L kit is designed for laboratories performing DNA extraction on large numbers of samples in each session.

The tables in Chapter 2.1 list the components included in the F-914S and F-914L kits. More detailed information on the components is found in Chapter 2.2.

2.1 Components included in the small and large kits

The F-914S kit consists of one QIAGEN® box and one Finnzymes box. The boxes contain the following components.

Component	F-914S	Storage Conditions	Box
QIAamp® Mini Spin Columns	50	RT	QIAGEN
Collection Tubes (2 ml)	150	RT	QIAGEN
Buffer AL ¹	12 ml	RT	QIAGEN
Buffer AW1 ¹ (concentrate)	19 ml	RT	QIAGEN
Buffer AW2 ² (concentrate)	13 ml	RT	QIAGEN
Buffer AE	12 ml	RT	QIAGEN
F-871S Lysis Solution 1	20 ml	RT	QIAGEN
F-872S Lysis Solution 2	4 x 1.3 ml	-20°C	Finnzymes
F-873S Proteinase K	1.4 ml	-20°C	Finnzymes
F-882 PathoProof Master Mix	1 x 1.1 ml	-20°C	Finnzymes
F-915S PathoProof Mastitis Major-3 Primer Mix	280 µl	-20°C. Protect from light.	Finnzymes
F-919 PathoProof Amplification Standard Major-3 & 4	150 µl	-20°C	Finnzymes

¹ Contains chaotropic salt. Not compatible with disinfecting agents containing bleach.

² Contains sodium azide as a preservative.

For more information, consult the material safety data sheets (MSDS) available from Finnzymes' website.

The F-914L kit consists of one QIAGEN® box and two Finnzymes boxes^{1,2}. The boxes contain the following components.

Component	F-914L	Storage Conditions	Box
QIAamp® 96 Plates	4	RT	QIAGEN
S-Blocks ³	2	RT	QIAGEN
Collection Microtubes (racked)	4 x 96	RT	QIAGEN
Elution Microtubes CL (racked)	4 x 96	RT	QIAGEN
Caps for Collection Microtubes	4 x 55	RT	QIAGEN
Caps for Elution Microtubes	1 x 50	RT	QIAGEN
AirPore Tape	25 sheets	RT	QIAGEN
Buffer AL ⁴	2 x 54 ml	RT	QIAGEN
Buffer AW1 ⁴ (concentrate)	95 ml	RT	QIAGEN
Buffer AW2 ⁵ (concentrate)	66 ml	RT	QIAGEN
Buffer AE	110 ml	RT	QIAGEN
F-871L Lysis Solution 1	165 ml	RT	QIAGEN
F-872L Lysis Solution 2	8 x 5 ml	-20°C	Finnzymes ¹
F-873L Proteinase K	2 x 6 ml	-20°C	Finnzymes ¹
F-882 PathoProof Master Mix	4 x 1.1 ml	-20°C	Finnzymes ²
F-915L PathoProof Mastitis Major-3 Primer Mix	2 x 1.1 ml	-20°C. Protect from light.	Finnzymes ²
F-919 PathoProof Amplification Standard Major-3 & 4	150 µl	-20°C	Finnzymes ²

¹ Finnzymes box with 10 bottles.

² Finnzymes box with 7 tubes.

³ Reusable. After use rinse thoroughly in tap water, incubate for 1 minute at room temperature in 0.4 M HCl, empty, and wash thoroughly with distilled water. Used S-Blocks can also be autoclaved after washing.

⁴ Contains chaotropic salt. Not compatible with disinfecting agents containing bleach.

⁵ Contains sodium azide as a preservative.

For more information, consult the material safety data sheets (MSDS) available from Finnzymes' website.

* Sufficient for 50 reactions when at least 16 samples are analyzed per set

** Sufficient for 4 x 96 reactions when at least 20 samples are analyzed per set

2.2 Description of the real-time PCR reagents

This Chapter describes the real-time PCR reagents included in the kits. All other kit components are reagents for extraction of bacterial DNA from milk.

- **F-882: PathoProof Master Mix.** PCR master mix in an optimized buffer containing $MgCl_2$, deoxynucleoside triphosphates and hot start DNA polymerase.
- **F-915: PathoProof Mastitis Major-3 Primer Mix.** PCR primer mix for PCR reaction, including oligonucleotides for identification of *Staph. aureus*, *Str. agalactiae*, *Mycoplasma bovis* and an Internal Amplification Control. Also includes Internal Amplification Control DNA.
- **F-919: PathoProof Amplification Standard Major-3 & 4.** Control DNA for all three bacterial targets of the assay for use as a positive control and for setting up appropriate threshold values for the real-time PCR instrument (see Norden Lab Mastitis Studio instruction manual for further information).

3. Materials needed but not supplied

The materials needed but not supplied with the PathoProof Mastitis Major-3 Kit are listed below. Most of the instruments and consumables needed are included in the PathoProof Starter Pack available from Finnzymes.

- Disposable powder-free gloves
- Ethanol (96–100 %)
- Pipettes
- Sterile pipette tips with filter
- PCR vessels compatible with the real-time PCR Instrument
- Optically clear PCR vessel caps or sealers compatible with the real-time PCR Instrument
- Two incubators (+37–55°C) **or** one fast-ramping incubator.
- Vortex mixer
- Microcentrifuge and appropriate tubes and strip tubes
- Plate centrifuge
- For the F-914L kit: Plate centrifuge Thermo Scientific Heraeus Multifuge X3 centrifuge
- Real-time PCR instrument: 7500 Fast Real-Time PCR System (Applied Biosystems), Stratagene Mx3005P® or Mx3000P® QPCR System (Agilent Technologies) or Chromo4™ Real-Time PCR Detection System (Bio-Rad Laboratories). Consult Finnzymes for compatible real-time PCR instrument software versions.

4. Sample material

The PathoProof Mastitis Major-3 Kit is intended for use with raw milk, frozen milk, or milk samples preserved with bronopol. If you wish to use the assay for other sample material (such as bacterial cultures, lyophilized bacteria or milk containing other preservatives, for example), please contact Finnzymes for instructions.

5. General guidelines

The following general guidelines should be followed throughout the PathoProof Mastitis Major-3 Kit protocol:

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

5.1 Avoiding carryover contamination

Due to their high sensitivity, real-time PCR assays 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 bacterial DNA.

The PathoProof Mastitis Major-3 Kit does not require opening of the real-time PCR vessels once a real-time PCR program has been started. While this assay 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: (1) DNA extraction (handling of milk samples or other samples containing bacteria); and (2) setup of the real-time PCR reactions.
- 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 plasticware.
- 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.

Figure 1. Schematic illustration of the PathoProof[®] Mastitis Major-3 Kit workflow.

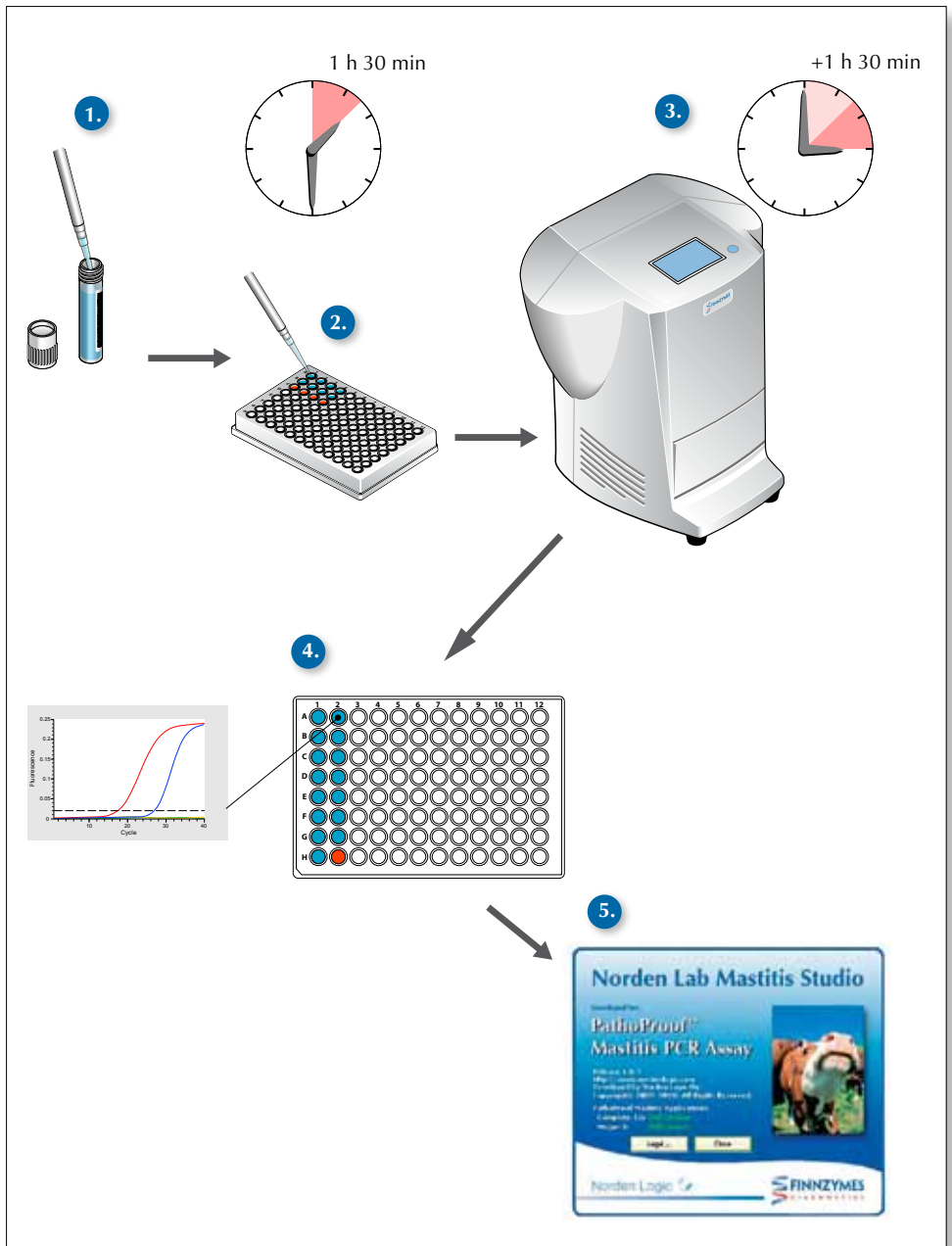


Figure 1. Schematic illustration of the PathoProof® Mastitis Major-3 Kit workflow.

1. DNA extraction. Bacterial DNA is extracted from raw or bronopol-preserved bovine milk samples. The F-914S kit is suitable for laboratories that process few samples per DNA extraction session; the F-914L kit is suitable for large numbers of samples per DNA extraction session.
2. Real-time PCR setup. Extracted bacterial DNA and a PCR solution are used to set up reactions in a 96-well PCR plate.
3. Real-time PCR. One PCR reaction is run for each sample. The reaction identifies three bacterial targets and contains an Internal Amplification Control (IAC) for verification of acceptable reaction conditions
4. The PathoProof Mastitis Major-3 Kit identifies three contagious mastitis bacterial species. Although a given milk sample is often positive for only one primary pathogen, the kit can identify all three targets simultaneously. In the example illustrated here, a milk sample analyzed in the well A2 is positive for *Mycoplasma bovis* (red curve). The blue curve represents the Internal Amplification Control.
5. The data obtained using Stratagene Mx3005P® or Mx3000P® QPCR System is interpreted, reported and databased with Norden Lab Mastitis Studio General Edition (see instructions in Norden Lab Mastitis Studio instruction manual).

6. Workflow of the assay

An overview of the PathoProof Mastitis Major-3 Kit laboratory workflow is presented in Figure 1 (page 10). This chapter contains instructions for performing DNA extraction, real-time PCR setup and run. For users of Stratagene Mx3005P® or Mx3000P® QPCR System, the instructions for performing results analysis using Norden Lab Mastitis Studio software are given in a separate Norden Lab Mastitis Studio instruction manual.

Norden Lab Mastitis Studio software's Major-3 application is not compatible with 7500 Fast Real-Time PCR System (Applied Biosystems) or Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories). More information for results analysis using those real-time PCR instruments can be found in Chapter 7.

Important:

When first time using the PathoProof Mastitis Major-3 Kit together with Stratagene Mx3005P® or Mx3000P® QPCR System and the Norden Lab Mastitis Studio software, it is necessary to calibrate the Norden Lab Mastitis Studio (see separate Norden Lab Mastitis Studio instruction manual). If you are not using the software, you must calibrate the real-time PCR instrument by adjusting its threshold values (see Section A). Note that calibration may need to be performed when qPCR plastic type changes or when the real-time PCR instrument has undergone maintenance. The calibration runs and the experiment runs must be performed using the same real-time PCR instrument, the same type of vessels and the same sealing method (sealers or caps).

6.1 DNA extraction

Things to do before starting

- Buffers AW1 and AW2 are supplied as concentrates. Before using them for the first time, add the appropriate amount of ethanol (96–100 %) as indicated on the bottles.
- Heat two incubators: one to 55°C and one to 37°C **or** use one fast-ramping incubator.
- Equilibrate Buffer AE to room temperature.
- If a precipitate has formed in Buffer AL, dissolve it by incubating at 55°C.

Below, separate instructions for DNA extraction are presented for the F-914S and F-914L kits.

DNA extraction for the F-914S kit

1. Prepare a fresh mix containing the following volumes per sample: 7 µl Proteinase K and 350 µl Lysis Solution 1. Calculate 1–2 extra reactions to ensure sufficient volume.

2. Vortex the milk samples thoroughly. Add 350 μ l of Lysis Solution 1 / Proteinase K mix and 350 μ l 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 (only reagents) in each DNA extraction.
3. Vortex briefly and incubate at 55°C for 5 min.
4. Centrifuge for 5 min at 5000 x g (~7500 rpm).
5. Remove the supernatant by pipetting. The presence of fat on top of the supernatant following centrifugation is normal. Remove the fat by pipetting. Residual fat on top of the pellet is normal and does not need to be removed.
6. Resuspend the pellet in 100 μ l Lysis Solution 2 by pipetting.
7. Incubate at 37°C for 10 min.
8. Prepare a fresh mix containing the following volumes per sample: 20 μ l Proteinase K and 200 μ l Buffer AL. Calculate 1–2 extra reactions to ensure sufficient volume. Add 220 μ l of the mix to the reaction vessel. Mix by vortexing for 5–10 s.
9. Incubate at 55°C for 10 min. Centrifuge for a few seconds.
10. Add 200 μ l ethanol (96–100 %) to the sample, and mix by pulse-vortexing for 15 s. 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 since this may result in reduced DNA yields. After mixing, briefly centrifuge the reaction vessel to remove drops from inside the caps.
11. Remove the possible viscous clots from this mixture with a pipettor. Then, carefully apply the supernatant to the QIAamp® Mini 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 x g (~14 000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
12. Carefully open the QIAamp Mini spin column and add 500 μ l Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 20 000 x g (~14 000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
13. Carefully open the QIAamp Mini spin column and add 500 μ l 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 QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 100 μ l 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.

15. If the purified DNA is to be stored, storage at -20°C is recommended.

Proceed to Chapter 6.2 for real-time PCR setup.

DNA extraction for the F-914L kit

Centrifugation of QIAamp 96 plates is performed in the Heraeus Multifuge X3 plate centrifuge at 6300 rpm (6168 x g).

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 pipettor, calculate some extra to ensure sufficient volume.
2. Vortex the milk samples thoroughly. Add 350 µl of Lysis Solution 1 / Proteinase K mix and 350 µl milk into racked Collection Microtubes. Avoid pipetting milk clots to the reaction vessels. In addition to the milk samples, include at least one negative control (only reagents) 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 6000 rpm.
5. Remove the supernatant by pipetting. The presence of fat on top of the supernatant following centrifugation is normal. Remove the fat by pipetting. Residual fat 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. 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 pipettor, calculate some extra 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 s. 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 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 15 s. 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 QIAamp 96 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 with a pipettor. Then, carefully apply the supernatant to the QIAamp 96 plate. Take care not to wet the rims of the wells to avoid aerosol formation during centrifugation.
13. Seal the QIAamp 96 plate with an AirPore Tape sheet. Load the S-Block and QIAamp 96 plate onto the carrier, then place the carrier in the rotor bucket. Centrifuge at 6000 rpm for 4 min.
14. Remove the tape. Carefully add 500 µl Buffer AW1 to each well. Seal the QIAamp 96 plate with a new AirPore Tape sheet. Centrifuge at 6000 rpm for 4 min.
15. 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 QIAamp 96 plate on top of a rack of Elution Microtubes (provided). To elute the DNA, add 100 µl Buffer AE to each well. Seal the QIAamp 96 plate with a new AirPore tape sheet and incubate for 1 min at room temperature. Centrifuge at 6000 rpm for 4 min. Seal the wells of the microtubes for storage using the caps for Elution Microtubes provided.
17. If the purified DNA is to be stored, storage at -20°C is recommended.

Proceed to Chapter 6.2 for real-time PCR setup.

6.2 Real-time PCR setup

An overview of the PathoProof Mastitis Major-3 Kit laboratory workflow is presented in Figure 1 (page 10). Figure 2 (page 17) illustrates the sample order in the real-time PCR setup necessary for PathoProof Mastitis Major-3 Kit. Throughout the procedure, follow the general guidelines detailed in Chapter 5.

Important points before starting

- Use at least one negative (no template) PCR control for the PCR reaction in each real-time PCR run.
- If all the samples are expected to be negative for bacteria, it is advisable to include the PathoProof Amplification Standard Major-3 & 4 and/or a positive

DNA extraction control (such as a milk sample previously tested positive with PathoProof Mastitis Major-3 Kit) in each real-time PCR run.

- Note that the experiment runs must be performed using the same real-time PCR instrument, the same type of vessels and the same sealing method (sealers or caps) that were used for the calibration runs (see Norden Lab Mastitis Studio instruction manual).

Vortex the thawed PathoProof Master Mix and PathoProof Mastitis Major-3 Primer Mix briefly and spin down.

1. Prepare a PCR solution by combining PathoProof Master Mix and PathoProof Mastitis Major-3 Primer Mix in a microcentrifuge tube. Use one of the formula sets below to calculate the volumes of the PCR solutions.

Formula set for < 20 samples

PCR solution:

- Volume of PathoProof Master Mix = $(N + 2) \times 10 \mu\text{l}$
- Volume of PathoProof Mastitis Major-3 Primer Mix = $(N + 2) \times 5 \mu\text{l}$

N = Number of samples

Including one or several of the following controls:

- Negative PCR control (**necessary in every run**)
- PathoProof Amplification Standard Major-3 & 4 (positive PCR control)
- Negative DNA extraction control
- Positive DNA extraction control

The formulas provide excess volume to compensate for volume loss due to reagent pipetting.

Formula set for ≥ 20 samples

PCR solution:

- Volume of PathoProof Master Mix = $N \times 11 \mu\text{l}$
- Volume of PathoProof Mastitis Major-3 Primer Mix = $N \times 5.5 \mu\text{l}$

N = Number of samples

Including one or several of the following controls:

- Negative PCR control (**necessary in every run**)
- PathoProof Amplification Standard Major-3 & 4 (positive PCR control)
- Negative DNA extraction control
- Positive DNA extraction control

The formulas provide excess volume to compensate for volume loss due to reagent pipetting.

2. Vortex the PCR solution briefly and spin down.
3. Prepare a 96-well PCR plate. Always prepare the plate as illustrated in Figure 2. For example, allocate wells A1–G2 for samples 1–15. Fill the wells of the plate in the direction A → H, i.e. first down, then right, then down. **Add 15 µl of the PCR solution into the wells.** If the PathoProof Amplification Standard Major-3 & 4* is included, add 15 µl of the PCR solution into the well allocated for the PathoProof Amplification Standard Major-3 & 4. Always allocate the last well to be pipetted for a negative (no template) PCR control (see Figure 2).
4. **Add 5 µl of the eluate from the DNA extraction protocol (Chapter 6.1) into the wells allocated for the samples.** If the PathoProof Amplification Standard Major-3 & 4 is included, add 5 µl of the standard into the well allocated for it. In the last well allocated for the negative (no template) control, add 5 µl of sterile water or the Elution Buffer (AE) (Figure 2).

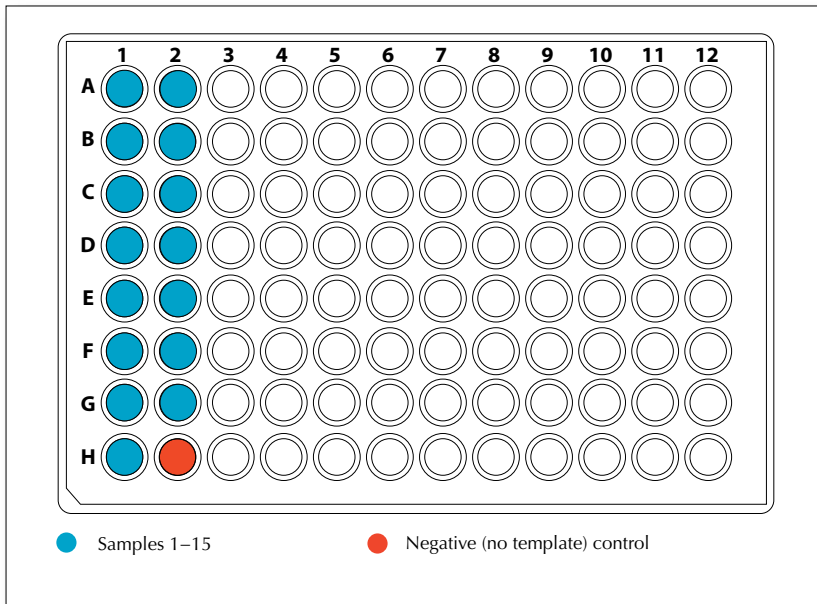


Figure 2. Illustration of a 96-well real-time PCR plate and the sample order necessary for PathoProof[®] Mastitis Major-3 Kit.

* The PathoProof Amplification Standard Major-3 & 4 contains DNA for all three bacterial targets of the PathoProof Mastitis Major-3 Kit. It can be used as a positive control in the real-time PCR. It is also used for the calibration of the Norden Lab Mastitis Studio General Edition software-

5. Close the 96-well PCR plate with a compatible optically clear sealer or optically clear caps and spin the plate down with a plate centrifuge (3000 rpm, 5 s).
6. Place the 96-well plate in a real-time PCR instrument and start a real-time PCR program following the instrument-specific instructions in Chapter 6.3.

6.3 Real-Time PCR instrument settings and run

The PathoProof Mastitis Major-3 Kit is compatible with the following real-time PCR instruments: 7500 Fast Real-Time PCR System (Applied Biosystems), Chromo4™ Real-Time PCR Detection System (Bio-Rad Laboratories) and Stratagene Mx3005P® or Mx3000P® QPCR System (Agilent Technologies). Download ready-made instrument-specific template files from <http://diagnostics.finnzymes.fi/>. Save the templates on the computer connected to the real-time PCR instrument and follow the guidelines below.

Applied Biosystems 7500 Fast Real-Time PCR System

Instructions for Sequence Detection Software v1.4

To start a run, open the template file (PathoProof_Mastitis_Major-3_template.sdt). Name the samples as needed (samples may be named after the run if preferred) and mark empty wells by selecting “Omit Well” in the Well Inspector.

Verify that the settings are now as follows:

- For all wells that are in use:
 - FAM, VIC, ROX and CY5 dye filters are defined
- Thermal Cycler Protocol:
 1. Stage 1 (Reps 1)
 - a. 10 min at 95°C
 2. Stage 2 (Reps 40)
 - a. 5 s at 95°C
 - b. 1 min at 60°C
 3. Stage 3 (Reps 1)
 - a. 5 s at 25°C
- Sample Volume: 20 µl
- Run Mode: Fast 7500
- Data Collection: during Stage 2, Step 2b (60.0°C @ 1 min)

Save the file in .sds format. The instrument should now be connected to the software. Start the run by clicking the Start button.

After the real-time PCR run, analyze the run data according to the following instructions.

- Choose the “Results” menu.
- Choose “Amplification plot” and mark all the wells.
- Click the right mouse button and choose “Graph Settings..”. Change now the Y-Axis from logarithmic to linear in the “Post Run Settings” and press “OK”.
- Adjust the threshold values received from the calibration run (see "Calibration without Norden Lab Mastitis Studio software" in Section A) for each of the three targets by changing the “Auto Ct” to “Manual Ct” and then selecting one target at a time from the “Detector” menu.
- Check that the Internal Amplification Control has amplified acceptably in every reaction. IAC should receive a Ct value within a range 25.6–31.6. If the IAC has failed (Ct outside the range) and the sample is negative for the bacterial targets, the eluted DNA should be diluted for example 1:10 into sterile water and the PCR protocol should be performed again.
- For reporting the results, go to “Report” interleaf and then go to “File”, “Export” and “Results”.
- Quantities for positive results are shown in Table 1.
- Refer to Chapter 7 when inspecting results.

Table 1. Approximate Ct ranges for each bacterial target and for each quantity class.

Target	+++	++	+
<i>Staphylococcus aureus</i>	< 24	24–30	30–37
<i>Streptococcus agalactiae</i>	< 24	24–32	32–37
<i>Mycoplasma bovis</i>	< 22	22–32	32–37

Bio-Rad Chromo4™ Real-Time PCR Detection System

Instructions for Opticon Monitor 3.00

To start a run, open the template file from the main window (Master File -> Open -> PathoProof_template.mast). Name the samples as needed (samples may be named after the run if preferred). Indicate empty wells by choosing "Empty" in the Plate Setup Section.

Verify that the settings are now as follows:

Plate Setup

- Plate type: clear
- For all wells that are in use:
 - FAM, VIC, TXR and CY5 dye filters are defined
 - All reactions are defined as sample (red)
 - Data is collected from all four channels

Protocol Setup

- Temperature Control: Sample Calculation
- Lid Settings: Constant 100°C, Shutoff < 30°C
- Reaction Volume: 20 µl
- PCR protocol:
 1. 10 min at 95°C
 2. 5 s at 95°C
 3. 60 s at 60°C
 4. Plate read
 5. Goto line 2 for 39 more times
 6. 5 s at 25°C

After completing the Plate Setup and Protocol Setup, save the data as a .tad file, insert the PCR plate and press the Run button to start the run. After the real-time PCR run, analyze the run data according to the following instructions.

- Go to the "Quantitation" menu and set up the threshold values received from the calibration run (see "Calibration without Norden Lab Mastitis Studio software" in Section A).
- Check that the Internal Amplification Control has amplified acceptably in every reaction. IAC should receive a Ct value within a range 25.6–31.6. If the IAC has failed (Ct outside the range) and the sample is negative for the bacterial targets, the eluted DNA should be diluted for example 1:10 into sterile water and the PCR protocol should be performed again.

- For reporting results, go to “Calculations” interleaf and then press “Copy the Clipboard” button on the upper right corner. Use “Paste” feature to add the information into an excel sheet.
- Quantities for positive results are shown in Table 1 (page 19).
- Refer to Chapter 7 when inspecting results.

Stratagene Mx3005P® or Mx3000P® QPCR System*

Instructions for MxPro™ - Mx3005P v4.10

*The following Stratagene Mx3005P or Mx3000P QPCR System instrument filters are compatible with the PathoProof Mastitis Major-3 Kit: FAM/SYBR Green, HEX/JOE, ROX/Texas Red and CY5. If your Stratagene instrument does not have these filters, please contact Finnzymes.

To start a run, open the template file (File -> Open -> PathoProof_template.mxp). Switch the instrument's lamp on by clicking the lamp icon.

Verify that the settings are now as follows:

- Gain settings: 1x for CY5, 1x for ROX, 2x for HEX/JOE and 4x for FAM (Instrument -> Filter Set Gain Settings)
- Thermal Profile:
 1. Segment 1 (1 cycle)
 - a. 10 min at 95°C
 2. Segment 2 (40 cycles)
 - a. 5 s at 95°C
 - b. 60 s at 60°C, Endpoint read (End symbol on Step 2b)

Verify that the lamp status field in the lower right corner of the window indicates “Lamp Ready” (green) or “Warm-Up”. Insert the PCR plate and name the samples as needed (samples may be named after the run if preferred). Mark the empty wells and click “Clear Selected Wells” in the panel at the right. Save the run file in .mxp format and click “Run” to start the run.

After the real-time PCR run, save the run data and the plate setup as one file.

- Click the “Analysis” button, then the “Results” tab.
- Go to the “File” menu, choose “Export Chart Data to Text file” and “Format 1 – Vertically Grouped by Plot...”.
- Copy the resulting files to the computer on which Norden Lab Mastitis Studio is installed.

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

7. Interpretation of the results

This Chapter gives instructions on the important parameters that should be inspected for each sample.

Checklist for inspecting results

For each sample and run, check the following parameters:

- Internal Amplification Controls (IAC)
- Amplification curves of the bacterial targets
- Negative Controls and their IAC

7.1 Cycle threshold values

The results of the PathoProof Mastitis Major-3 Kit are based on Cycle threshold (Ct) values obtained for the amplification curves of the bacterial targets. The Ct value represents the number of cycles required to reach a particular threshold fluorescence signal level. The fewer cycles it takes to obtain a detectable fluorescence level, the greater the amount of bacterial DNA in the milk sample. The cut-off Ct value of the PathoProof Mastitis Major-3 Kit is 37. This means that if the Ct value obtained for a bacterial target is lower than 37, the sample is positive. All other results (Ct >37) are negative or contain bacteria in very low quantity.

7.2 Internal Amplification Control

An Internal Amplification Control (IAC) is included in the assay's Primer Mix. The purpose of the IAC is to confirm, for each sample and PCR reaction, that the reaction conditions were acceptable for the identification of the bacterial targets.

If the Ct values of the Internal Amplification Control is not within the acceptable range, refer to Chapter 9 for recommended action.

7.3 Real-time PCR amplification curves

The amplification curves of true positive targets are exponential. Figure 3.A shows an example of ideal amplification curves, and Figure 3.B shows an example of an abnormal curve that does not represent true amplification.

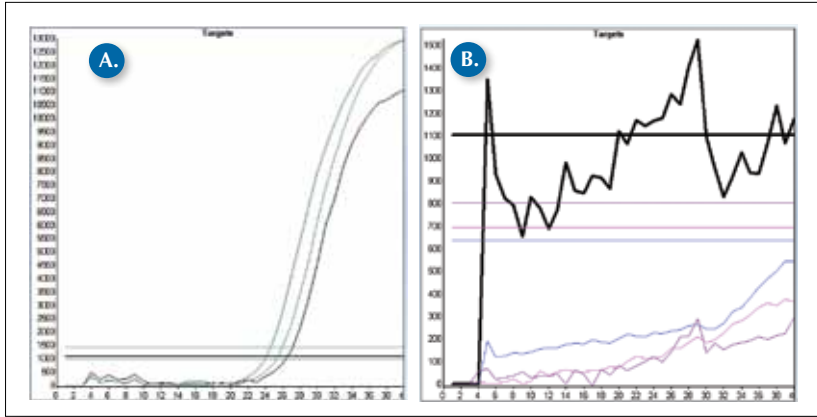


Figure 3. A. Example of ideal amplification curves. B. Example of abnormal curves that do not represent true amplification.

7.4 Negative (no template) controls

If the Ct value of the target in the Negative controls is less than 3 cycles away from the Ct of the same target in a sample, that target in that sample fails to qualify as positive and must be disregarded in the analysis. It is recommended to re-run these samples.

8. Results categories

Each bacterial target is assigned to one of five results categories depending on the Ct value. The table below describes these categories.

Results category in the report	Interpretation
-	Bacterial DNA not detected.
+/-	Bacterial DNA detected in very low quantity.
+	Bacterial DNA detected in low quantity.
++	Bacterial DNA detected in intermediate quantity.
+++	Bacterial DNA detected in high quantity.

9. Troubleshooting

Because the possible problems are only detected after the whole PathoProof Mastitis Major-3 Kit protocol has been completed, problems in real-time PCR are presented first, followed by problems in DNA extraction.


9.1 Real-time PCR


Problem	Possible explanation(s)	Recommended action
The Ct value of the Internal Amplification Control is not within the acceptable range in samples* and in negative controls**.	Missing reagents in the PCR setup	Repeat the real-time PCR, checking the concentration of all reagents.
	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 signal for the Negative controls.	PCR inhibitors originating from the DNA extraction are present in the test samples.	Refer to Chapter 9.2, “DNA extraction”.
Unacceptable IAC amplification signals for one sample* but acceptable IAC signals in the other samples and the Negative controls.	Missing reagents in the wells of the sample.	Repeat the real-time PCR for the sample.
	The PCR inhibitor concentration of the sample is too high.	Dilute the eluate from the DNA extraction protocol (use 1:5 and 1:10 dilutions, for example) and repeat the real-time PCR. See Chapter 9.2, “DNA extraction”.
	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.
Unacceptable IAC amplification signals for Negative control** but acceptable IAC amplification signals in sample wells.	Missing reagents in the well 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.	No action required because the IACs in the samples are acceptable.***
Positive bacterial target amplification signals in wells for Negative control.	A carryover contamination may have occurred in the laboratory.	Take precautions detailed in Chapter 5.1 to minimize contaminations.
Acceptable IAC amplification signals in all samples*, but all samples are negative for all bacterial targets.	Failed DNA extraction.	Refer to Chapter 9.2, “DNA extraction”. Include PathoProof Amplification Standard and/or a positive DNA extraction control, such as a milk sample previously tested positive with PathoProof Mastitis Major-3 Kit.
	There were no bacteria present in the samples.	

9.2 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 inhibiting 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 QIAamp Mini column or the QIAamp 96 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 QIAamp Mini column or the QIAamp 96 Plate does not come into contact with the filtrate prior to elution. Before DNA elution, ensure that the QIAamp 96 Plate is centrifuged without the AirPore Tape sheet in order to allow ethanol evaporate from the sample.
QIAamp Mini column or QIAamp 96 Plate not incubated at room temperature (15–25°C) for 1 min before elution	After the addition of Buffer AE, the QIAamp Mini column or the QIAamp 96 Plate should be incubated at room temperature for at least 1 min.
DNA not eluted efficiently	To increase elution efficiency, pipet Buffer AE onto the center of the QIAamp Mini column or the QIAamp 96 Plate and incubate for 5 min at room temperature before centrifugation.

* If the Ct values of the Internal Amplification Controls are not within the acceptable range, Norden Lab Mastitis Studio displays the 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, Norden Lab Mastitis Studio displays the 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.

*** However, it is possible but unlikely that reagents were contaminated and it could not have been detected because of failed reaction conditions.

A. Calibration without Norden Lab Mastitis Studio software

In order to obtain consistent performance, it is necessary to calibrate the real-time PCR instrument by adjusting its threshold values when using PathoProof Mastitis Major-3 Kit for the first time. Calibration may also need to be performed when qPCR plastic type changes or when the real-time PCR instrument has undergone maintenance.

Calibration PCR protocol is given in Section A1. Instructions for instrument-specific real-time settings, performing a run and handling files are provided in Section A2. Please complete the calibration before you start processing your samples. Note that the calibration runs and the experiment runs must be performed using the same real-time PCR instrument, the same type of vessels and the same sealing method (sealers or caps).

A1. Real-time PCR setup

Make sure that all the reagents are thoroughly thawed. Vortex the PathoProof Master Mix and PathoProof Primer Mix briefly and spin down.

1. Prepare a Calibration PCR solution by combining PathoProof Master Mix, PathoProof Mastitis Major-3 Primer Mix and PathoProof Amplification Standard Major-3 & 4 in a microcentrifuge tube.

Calibration PCR solution:

- 35 μ l PathoProof Master Mix
- 17.5 μ l PathoProof Mastitis Major-3 Primer Mix
- 17.5 μ l PathoProof Amplification Standard Major-3 & 4

The formula provides excess volume to compensate for volume loss due to reagent pipetting.

2. Vortex the PCR solution briefly and spin down.
3. Prepare a 96-well PCR plate by dispensing 20 μ l of Calibration PCR solution into its respective wells A1, B1 and C1.
4. Close the 96-well PCR plate with a compatible optically clear sealer or optically clear caps and spin the plate down with a plate centrifuge (3000 rpm, 5 s). Note that the experiment runs must be performed using the same real-time PCR instrument, the same type of vessels and the same sealing method.
5. Place the 96-well plate in a real-time PCR instrument and start the PCR program using the instrument-specific settings given in Section B3.

A2. Instrument-specific settings and instructions for run and file handling

Download a ready-made instrument-specific calibration template file from <http://diagnostics.finnzymes.fi/>. Save the template on the computer connected to the real-time PCR instrument. Below are separate instructions for each real-time PCR instrument.

A2.1 Applied Biosystems 7500 Fast Real-Time PCR System

Instructions for Sequence Detection Software v1.4

In the real-time PCR instrument software, open the calibration template file (Calibration_template.sdt).

Verify that the settings are now as follows:

- Thermal Cycler Protocol:
 1. Stage 1 (Reps 1)
 - a. 10 min at 95°C
 2. Stage 2 (Reps 40)
 - a. 5 s at 95°C
 - b. 1 min at 60°C
 3. Stage 3 (Reps 1)
 - a. 5 s at 25°C
- Sample Volume: 20 µl
- Run Mode: Fast 7500
- Data Collection: During Stage 2, Step 2b (60.0°C @ 1 min)

Save the file in .sds format. The instrument should now be connected to the software. Start the run by clicking the Start button.

After the calibration real-time PCR run, save the calibration data file as follows:

- Open the **Results** menu.
- Choose **Amplification plot** and click **Analyze**.
- Go to the **File** menu, choose **Export** and then **Delta Rn**.
- Save the file in .csv format.

Send the calibration data file (.sds) to Finnzymes' techsupport (finnzymes.techsupport@thermofisher.com) and use the provided threshold values for the following runs.

B2.2 Bio-Rad Chromo4™ Real-Time PCR Detection System

Instructions for Opticon Monitor 3.00

In the real-time PCR instrument software, open the calibration template file (Calibration_template.mxp).

Verify that the settings are now as follows:

Plate Setup

- Plate type: clear
- For all wells that are in use:
 - FAM, VIC, TXR and CY5 dye filters are defined
 - All reactions are defined as sample (red)
 - Data is collected from all four channels

Protocol Setup

- Temperature Control: Sample Calculation
- Lid Settings: Constant 100°C, Shutoff < 30°C
- Reaction Volume: 20 µl
- PCR protocol:
 1. 10 min at 95°C
 2. 5 s at 95°C
 3. 60 s at 60°C
 4. Plate read
 5. Goto line 2 for 39 more times
 6. 5 s at 25°C

After completing the Plate Setup and Protocol Setup, save the data as a .tad file, insert the PCR plate and press the Run button to start the run.

After the calibration run, save the calibration data file as follows:

- Go to the **Quantitation** menu and choose **Export**.
- Clear the **Interleaf Multiple Dyes** box and activate all the wells by clicking with the left mouse button.
- Save the file in .csv format.
- Copy this calibration data file to the computer on which Norden Lab Mastitis Studio is installed.

Send the calibration data file (.sds) to Finnzymes' techsupport (finnzymes.techsupport@thermofisher.com) and use the provided threshold values for the following runs.

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