PrepSEQ™ Sample Preparation Kits for *Mycoplasma*, MMV, and Vesivirus

USER GUIDE

Manual sample preparation protocols for *Mycoplasma*, MMV, and Vesivirus detection

for use with:

PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit
PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit

Catalog Numbers 4452222 and 4443789
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ThermoFisher SCIENTIFIC

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Revision history: Pub. No. 4465957

Revision	Date	Description
C	30 January 2018	Update the following protocols: • 1-2-3 manual protocol for *Mycoplasma* and/or MMV detection • 3-in-1 manual protocol for *Mycoplasma*, MMV, and/or Vesivirus detection • Large-scale manual protocol for *Mycoplasma* detection
B	01 April 2015	Update storage temperature for the Magnetic Particles. Remove Cat. No. 4460627.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The PrepSEQ™ Sample Preparation Kits use Magnetic Particle-based separation technology for manual extraction of DNA and/or RNA from *Mycoplasma* cells or viral particles. A variety of starting material can be used, such as infected cell cultures or *Mycoplasma* liquid cultures. The kits described in this document are:

- PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit (Cat. No. 4452222)
- PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit (Cat. No. 4443789)

Kit applications

Organisms	Sample volume	For use with kit	Protocol
Mycoplasma and MMV	100 µL (up to 10^6 cells)	PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	Chapter 3, "1-2-3 manual protocol for Mycoplasma and/or MMV detection"
Mycoplasma, MMV, and Vesivirus	100 µL (up to 10^6 cells)	PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	Chapter 4, "3-in-1 manual protocol for Mycoplasma, MMV, and/or Vesivirus detection"
Mycoplasma	Up to 11 mL (up to 2×10^8 cells)	PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit	Chapter 5, "Large-scale manual protocol for Mycoplasma detection"

Contents and storage: PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit

Kit components may be shipped separately depending on the kit configuration and storage conditions.

Table 1 PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit (Cat. No. 4452222)

Contents	Amount[1]	Storage[2]
RNase Cocktail™ Enzyme Mix	2 × 1.0 mL	–25°C to –15°C
Box 1		
Lysis Buffer	2 × 50 mL	18°C to 25°C (room temperature)
Binding Solution Bottle(Isopropanol)[3]	1 empty bottle	
Wash Buffer Concentrate[4]	2 × 26 mL	
Elution Buffer	25 mL	
Proteinase K (PK) Buffer	50 mL	
Box 2		
Magnetic Particles	2 × 1.5 mL	18°C to 25°C (room temperature)
Box 3		
Proteinase K, 20 mg/mL	1.25 mL	–25°C to –15°C

[1] Contains reagents for 100 small-scale (100 µL) cell culture extractions.
[2] See the product label for the expiration date.
[3] Add 30 mL of 100% isopropanol to the empty bottle before use.
[4] Add 74 mL of 95% non-denatured ethanol before use.

Contents and storage: PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit

The PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit can be ordered as a standalone kit (Cat. No. 4443789), or as part of the MycoSEQ™ Mycoplasma Detection Kit (Cat. No. 4460626).

Kit components may be shipped separately depending on the kit configuration and storage conditions.

Table 2 PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit (Cat. No. 4443789)

Contents	Amount[1]	Storage[2]
Cell Fractionation Buffer	3 × 25 mL	2°C to 8°C
RNase Cocktail™ Enzyme Mix	2 × 1.0 mL	–25°C to –15°C
Box 1		
Lysis Buffer	2 × 50 mL	18°C to 25°C (room temperature)
Binding Solution Bottle(Isopropanol)[3]	1 empty bottle	
Wash Buffer Concentrate[4]	2 × 26 mL	
Elution Buffer	25 mL	
Proteinase K (PK) Buffer	50 mL	
Box 2		
Magnetic Particles	2 × 1.5 mL	18°C to 25°C (room temperature)
Box 3		
Proteinase K, 20 mg/mL	1.25 mL	–25°C to –15°C

[1] Contains reagents for 100 small-scale (100–2,000 µL) or 100 large-scale (2–10 mL) cell culture extractions.
[2] See the product label for the expiration date.
[3] Add 30 mL of 100% isopropanol to the empty bottle before use.
[4] Add 74 mL of 95% non-denatured ethanol before use.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 3 All protocols

Item	Source
Laboratory supplies	
Heat block with block inserts, for use with 2-mL tubes	MLS
Vortex-Genie™ 2T Mixer	VWR™ Scientific Industries (14216-188 or 14216-186)
Vortex Adapter-60, for use with the Vortex-Genie™ 2T Mixer	AM10014
16-position Magnetic Stand	12321D
Eppendorf™ PCR Clean Microcentrifuge Tubes (Safe-Lock, 2 mL, round-bottom)	VWR™ Scientific Industries (62111-754)
Benchtop microcentrifuge (13,000 × *g* or greater), for use with 2-mL tubes	MLS
(Optional) Fisher Scientific™ Mini Plate Spinner Centrifuge	14-100-143
Serological pipettes	MLS
Conical Tubes (15 mL)	AM12500
Nonstick, RNase-free Microfuge Tubes (1.5 mL)	AM12450
Reagents	
Isopropanol, 100%	MLS
Non-denatured ethanol, 95% **IMPORTANT!** Do not use denatured ethanol because it contains components that are not compatible with the protocol.	MLS
EDTA, 0.5 M	AM9260G

Table 4 Large-scale protocols

Item	Source
Laboratory supplies	
Refrigerated centrifuge (2–8°C, 16,000 × *g*), for use with 50-mL tubes	MLS
Conical Tubes (50 mL)	AM12502
Ice bucket	MLS

Item	Source
Reagents	
Cell Fractionation Buffer **Note:** This is in addition to the amount included in the PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit.	4403461
(Optional) For samples with high SYBR™ dye background: TURBO™ DNase (2 U/µL) (includes TURBO™ DNase and 10× Reaction Buffer)	AM2239
(Optional) RNase Cocktail™ Enzyme Mix **Note:** This is in addition to the amount included in the PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit.	AM2286

2 Lysate, Magnetic Particle, and positive control guidelines

Guidelines for preparing sample lysates that contain target DNA

Minimizing cellular DNA and/or RNA in the final extracted DNA is critical to *Mycoplasma* DNA detection. High amounts of cellular DNA and/or RNA cause PCR inhibition and high background of the SYBR™ Green I dye signal, reducing detection of low copy numbers of targets. Factors that affect levels of cellular DNA and/or RNA include:

- **Viability of cell culture sample**—Use fresh culture samples to increase the purity of your extracted target DNA. Avoid conditions such as long-term storage at 4°C (or freezing temperatures). Such temperatures cause increased death or lysis of cells, which contributes to additional background DNA in samples.
- In the large-scale protocols, when processing the mammalian cell pellet, keep it on ice and perform all processing steps at 4°C to avoid host cell nuclei lysis as much as possible. Room temperature increases lysis of nuclei and host DNA in the final extracted DNA, and causes PCR inhibition.
- In the large-scale protocols, if working with the mammalian cell pellet:
  - In some cases, the cell pellet is large and sticky and cannot be resuspended easily. Never vortex to resuspend the cells.
  - When transferring the cell culture supernatant, avoid touching the pellet, which contains nuclei and viscous material that may be generated from lysis of nuclei. If needed, use a P200 pipette to perform the transfer.
  - In the final transfer of the cell pellet supernatant, avoid contact with or transfer of the viscous material. If needed, recentrifuge the tube at 1000 × *g* for 3 minutes at 4°C, then very carefully transfer 300 µL (two 150-µL aliquots) with a P200 pipette.

Guidelines for working with Magnetic Particles

- Incubate the Magnetic Particle suspension at 37°C for a minimum of 10 minutes with intermittent vortexing at setting #7, or until the particles are completely suspended.
- When you place sample tubes into the Magnetic Stand, always orient the Magnetic Particles pellet toward the magnet.
- Except where noted, the Magnetic Particles capture of the DNA is complete after ~1 minute in the Magnetic Stand.
- When separating the liquid phase or eluate from the Magnetic Particles, do not disturb the Magnetic Particles. Magnetic Particles can inhibit PCR.
- During wash steps, it is not necessary to detach the Magnetic Particles from the tube wall. Particle adherence to the tube wall does not affect DNA recovery. Some samples cause the particles to adhere very firmly to the tube wall; for other samples, the particles form loose aggregates that readily detach during the vortex steps. The particles must disperse into a slurry during heating and vortexing in the elution step of the protocol.

(Recommended) Run extraction controls

IMPORTANT! Positive and/or negative extraction controls are primarily used during optimization or pre-validation testing. Extraction controls are not required, but we recommend that you run them.

The MycoSEQ™ Discriminatory Positive Control (DPC) provided with the MycoSEQ™ *Mycoplasma* Detection Kit is a multi-purpose control that can be used as an extraction positive control.

If you are running an extraction positive control, we recommend that you extract and analyze 1 replicate of the sample unspiked and 1 replicate of the sample spiked:

- **Sample 1, tube 1**—Test sample.
- **Sample 1, tube 2**—Test sample + DPC. Spike a volume of DPC to achieve 200 copies per PCR.

3 1-2-3 manual protocol for *Mycoplasma* and/or MMV detection

Use this protocol to process 100 µL of sample (up to 10^6 cells) for the detection of *Mycoplasma* and/or MMV.

Note: This protocol is typically recommended for in-process testing of *Mycoplasma* and/or MMV.

For use with kit

PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit

Prepare materials

1. Confirm that you have all kit components and other required materials.
2. *(Before first use of the kit)* Prepare the following reagents:
   - **Binding Solution**—Add 30 mL of 100% isopropanol to the empty Binding Solution bottle. Mark the bottle label to indicate that isopropanol has been added.
   - **Wash Buffer**—Add 74 mL of 95% non-denatured ethanol to the Wash Buffer Concentrate bottle, then mix well. Mark the bottle label to indicate that ethanol has been added.
3. Power on a heat block to 37°C.

4. Incubate the Magnetic Particles suspension at 37°C for a minimum of 10 minutes with intermittent vortexing at setting #7, or until the particles are completely suspended.

   Note: During extraction, when you place sample tubes into the Magnetic Stand, always orient the Magnetic Particles pellet toward the magnet.

Prepare test samples

1. Prepare each sample in a new 2-mL microcentrifuge tube:
   - $\leq 10^6$ total cells—Add 100 µL of sample to the tube.
   - $>10^6$ total cells—Spin the sample in a microcentrifuge at 500 × *g* for 2 minutes, then add 100 µL of supernatant to the tube.
2. ***(Optional)*** For an extraction positive control: Spike the appropriate amount of DPC to 100 µL of the prepared test sample to achieve 200 copies per PCR. Extraction controls can be dual- or triple-spiked with DPCs for each assay to conserve sample. You can also use one of the following in place of test samples: 1X PBS solution, Lysis Buffer, Cell Fractionation Buffer, or plain media.

Prepare sample lysate

For each sample tube:

1. Add 200 µL of Lysis Buffer, then vortex for ~5 seconds to mix.
2. Add the following volumes, then briefly vortex to mix:
   - 2 µL of 0.5 M EDTA
   - 18 µL of RNase Cocktail™ Enzyme Mix
   - 2 µL of Proteinase K
3. Incubate at 56°C for a minimum of 15 minutes.
4. Incubate at room temperature for 5 minutes.
5. Add 700 µL of Lysis Buffer.

Bind DNA

For each sample lysate tube:

1. Add 30 µL of Magnetic Particles.
2. Add 525 µL of Binding Solution, then immediately invert each tube to mix.

3. Using a vortex adaptor, vortex the tubes vertically at medium speed for 5 minutes to capture the nucleic acid.

   Note: To prevent accidental opening of the tubes, remove the tubes by pushing up from the bottom of the tubes.

4. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater).

5. Place in the Magnetic Stand for 5 minutes. See "Guidelines for working with Magnetic Particles" on page 11.

6. Aspirate the supernatant without disturbing the Magnetic Particles, then discard the supernatant.

Wash DNA

For each tube of Magnetic Particles pellet (bound DNA):

1. Add 300 µL of Wash Buffer, then vortex for ~5 seconds.

2. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater).

3. Place in the Magnetic Stand for 1 minute.

4. Aspirate the supernatant without disturbing the Magnetic Particles, then discard the supernatant.

5. Repeat step 1 through step 4.

   Set the timer for 5 minutes just before you remove the wash buffer for the second time. See step 7.

6. Use a P200 pipette to aspirate the residual supernatant from the bottom of the tube, then discard the supernatant.

7. With the cap open, air-dry the Magnetic Particles pellet at room temperature for ≤5 minutes to remove any residual ethanol.

   IMPORTANT! Set the timer for 5 minutes just before you remove the wash buffer for the second time. Do NOT dry longer than 5 minutes. Overdrying will make the pellets difficult to resuspend in Elution Buffer in the next steps.

   IMPORTANT! If you are using an aspirator or processing in a BSC (hood), no additional drying time is required. Aspirate and proceed; do not wait ≤5 minutes.

Elute DNA

For each sample:

1. Add 100 µL of Elution Buffer.
2. Vortex for ~10 seconds.
3. Incubate at 70°C for 7 minutes.
   Vortex 2–3 times during incubation to ensure complete resuspension of the Magnetic Particles. The total time may be 10–12 minutes.
4. Centrifuge for 5 minutes at top speed (13,000 × *g* or greater).
5. Place in the Magnetic Stand for 3 minutes.
6. Transfer the eluate to a non-stick 1.5-mL microcentrifuge tube.

Next steps

The extracted DNA is now ready for use in the appropriate PCR assay.

Note: If not used immediately, store the samples at 4°C for same-day use or at –20°C for longer storage.

4 3-in-1 manual protocol for *Mycoplasma*, MMV, and/or Vesivirus detection

Use this protocol to process 100 µL of sample (up to 10^6 cells) for the detection of *Mycoplasma*, MMV, and/or Vesivirus.

For use with kit

PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit

Prepare materials

1. Confirm that you have all kit components and other required materials.
2. *(Before first use of the kit)* Prepare the following reagents:
   - **Binding Solution**—Add 30 mL of 100% isopropanol to the empty Binding Solution bottle. Mark the bottle label to indicate that isopropanol has been added.
   - **Wash Buffer**—Add 74 mL of 95% non-denatured ethanol to the Wash Buffer Concentrate bottle, then mix well. Mark the bottle label to indicate that ethanol has been added.
3. Power on a heat block to 37°C.

4. Incubate the Magnetic Particles suspension at 37°C for a minimum of 10 minutes with intermittent vortexing at setting #7, or until the particles are completely suspended.

   Note: During extraction, when you place sample tubes into the Magnetic Stand, always orient the Magnetic Particles pellet toward the magnet.

Prepare test samples

1. Prepare each sample in a new 2-mL microcentrifuge tube:
   - ≤1 × 10^6 total cells—Add 100 µL of sample to the tube.
   - >1 × 10^6 total cells—Spin the sample in a microcentrifuge at 500 × *g* for 2 minutes, then add 100 µL of supernatant to the tube.
2. ***(Optional)*** For an extraction positive control: Spike the appropriate amount of DPC to 100 µL of the prepared test sample to achieve 200 copies per PCR. Extraction controls can be dual- or triple-spiked with DPCs for each assay to conserve sample. You can also use one of the following in place of test samples: 1X PBS solution, Lysis Buffer, Cell Fractionation Buffer, or plain media.

Prepare sample lysate

For each sample tube:

1. Add 500 µL of Lysis Buffer, then vortex for ~15 seconds to mix.
2. Incubate at 45°C for 10 minutes.
3. Vortex for ~10 seconds to mix.

Bind DNA

For each sample lysate tube:

1. Add 30 µL of Magnetic Particles.
2. Add 330 µL of Binding Solution, then immediately invert each tube to mix.
3. Using a vortex adaptor, vortex the tube vertically at medium speed for 5 minutes to capture the nucleic acid.

   Note: To prevent accidental opening of the tubes, remove the tubes by pushing up from the bottom of the tubes.
4. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater).

5. Place in the Magnetic Stand for 5 minutes.

6. Aspirate the supernatant without disturbing the Magnetic Particles, then discard the supernatant.

Wash DNA

For each tube of Magnetic Particles pellet (bound DNA):

1. Add 300 µL of Wash Buffer, then vortex for ~5 seconds.

2. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater).

3. Place in the Magnetic Stand for 1 minute.

4. Aspirate the supernatant without disturbing the Magnetic Particles, then discard the supernatant.

5. Repeat step 1 through step 4.

   Set the timer for 5 minutes just before you remove the wash buffer for the second time. See step 7.

6. Use a P200 pipette to aspirate the residual supernatant from the bottom of the tube, then discard the supernatant.

7. With the cap open, air-dry the Magnetic Particles pellet at room temperature for ≤5 minutes to remove any residual ethanol.

   IMPORTANT! Set the timer for 5 minutes just before you remove the wash buffer for the second time. Do NOT dry longer than 5 minutes. Overdrying will make the pellets difficult to resuspend in Elution Buffer in the next steps.

   IMPORTANT! If you are using an aspirator or processing in a BSC (hood), no additional drying time is required. Aspirate and proceed; do not wait ≤5 minutes.

Elute DNA

For each sample:

1. Add 100 µL of Elution Buffer.

2. Vortex for ~10 seconds.

3. Incubate at 70°C for 5 minutes.

   Vortex 2–3 times during incubation to ensure complete resuspension of the Magnetic Particles. The total time may be 10–12 minutes.

4. Centrifuge for 5 minutes at top speed (13,000 × *g* or greater).

5. Place in the Magnetic Stand for 3 minutes.

6. Transfer the eluate to a non-stick 1.5-mL microcentrifuge tube.

Next steps

The extracted DNA is now ready for use in the appropriate PCR assay.

Note: If not used immediately, store the samples at 4°C for same-day use or at –20°C for longer storage.

5 Large-scale manual protocol for *Mycoplasma* detection

Use this protocol to process up to 11 mL of sample (up to 10^8 cells) for the detection of *Mycoplasma*.

This protocol includes two options for sample preparation:

- "Option 1: Direct sample testing" on page 21
- "Option 2: Process pooled cell culture media and mammalian cells" on page 23

For use with kit

PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit

Prepare materials

1. Confirm that you have all kit components and other required materials.
2. *(Before first use of the kit)* Prepare the following reagents:
   - **Binding Solution**—Add 30 mL of 100% isopropanol to the empty Binding Solution bottle. Mark the bottle label to indicate that isopropanol has been added.
   - **Wash Buffer**—Add 74 mL of 95% non-denatured ethanol to the Wash Buffer Concentrate bottle, then mix well. Mark the bottle label to indicate that ethanol has been added.
3. Power on a heat block to 37°C.

4. Incubate the Magnetic Particles suspension at 37°C for a minimum of 10 minutes with intermittent vortexing at setting #7, or until the particles are completely suspended.

   Note: During extraction, when you place tubes into the Magnetic Stand, always orient the Magnetic Particles pellet toward the magnet.

5. Power on the refrigerated centrifuge to allow it to cool to 2–8°C before use.

Prepare test samples

Prepare each sample in a new 50-mL conical tube:

- $\leq 10^8$ cells—Add 11 mL of sample to the tube.

  Note: Add 11 mL of sample at this step; you will process a final volume of 10 mL.

- $>10^8$ cells—Add 15 mL of sample to the tube, centrifuge at 1,000 × *g* for 5 minutes to pellet the cells, then transfer 11 mL of supernatant to a new 50-mL conical tube.

Option 1: Direct sample testing

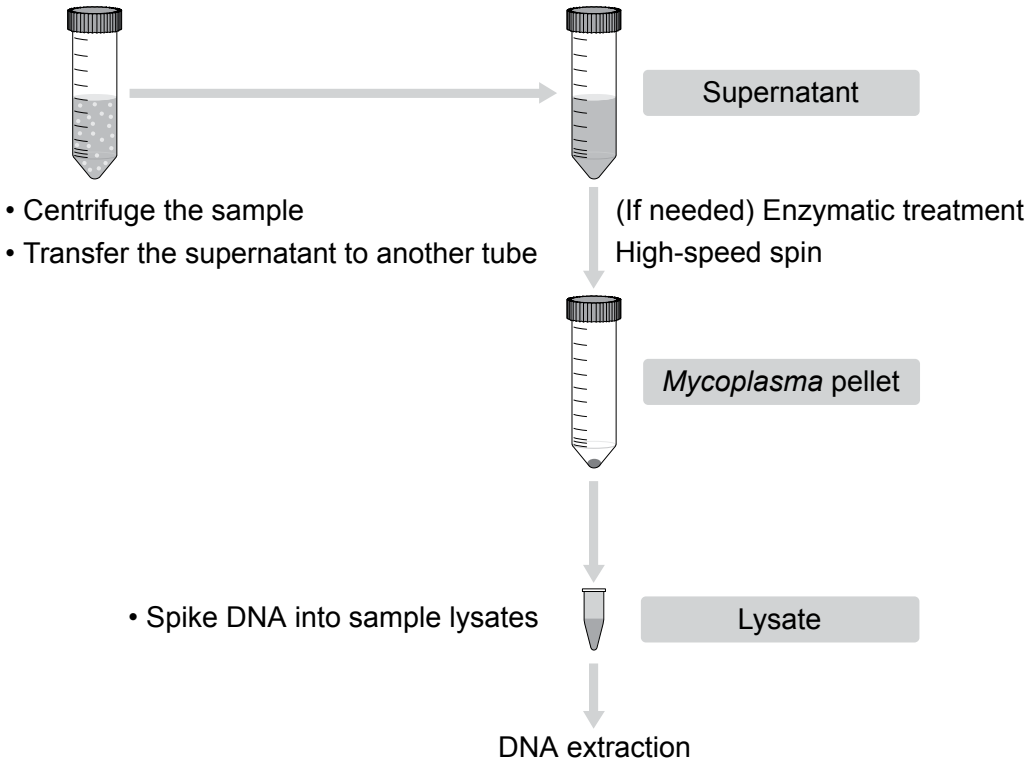

Figure 1 Option 1: Direct sample testing

Separate mammalian cells from cell culture media

1. Obtain the 11-mL samples from "Prepare test samples" on page 21.
2. Centrifuge each tube at 1,000 × *g* for 5 minutes at 4°C to pellet the mammalian cells.
3. Transfer 10 mL of the supernatant to a new 50-mL conical tube, then place on ice. The supernatant contains free *Mycoplasma*.
4. Discard the mammalian cell pellet.

Treat with RNase and DNase

If the samples have high SYBR™ Green I dye background because of excess cellular nucleic acid in the supernatant, perform RNase treatment.

IMPORTANT! For some high-density samples, both RNase and DNase treatments are needed. In this case, perform the DNase treatment before the RNase treatment.

Treat with DNase

1. Add the following TURBO™ DNase (2 U/µL) components, then gently vortex to mix:
   - 450 µL of 10× Reaction Buffer
   - 90 µL of TURBO™ DNase
2. Incubate at 37°C for 30 minutes.

Treat with RNase

1. Add the following components, gently vortex to mix, then briefly spin:
   - 180 µL of 0.5 M EDTA
   - 225 µL of RNase Cocktail™ Enzyme Mix
   - 150 µL of Proteinase K

   Note: Alternatively, you can prepare a stock mix of EDTA, Proteinase K, and RNase Cocktail™ Enzyme Mix, then add 555 µL of stock mix to each sample.
2. Incubate at 56°C for a minimum of 30 minutes to digest the cellular RNA and proteins.

Process the supernatant to obtain resuspended *Mycoplasma*

1. Centrifuge the supernatant at 16,000 × *g* for 30 minutes at 4°C to pellet the *Mycoplasma*.
2. Aspirate and discard the supernatant without disturbing the *Mycoplasma* pellet. Do not decant the liquid and do NOT touch the pellet.
3. Add 300 µL of Lysis Buffer, then mix thoroughly by vortexing to resuspend the *Mycoplasma* pellet.

   If the pellet is difficult to dislodge, vigorously agitate the tube.
4. Transfer the resuspended pellet to a 2-mL microcentrifuge tube.

Treat the resuspended *Mycoplasma*

Separately process the resuspended *Mycoplasma* in the 2-mL microcentrifuge tube.

1. Add the following volumes, then briefly vortex to mix:
   - 2 µL of 0.5 M EDTA
   - 18 µL of RNase Cocktail™ Enzyme Mix
   - 5 µL of Proteinase K
2. *(Optional)* Spike with Discriminatory Positive Control (DPC) for optimization or with *Mycoplasma* DNA for lot release validation.
3. ***(Optional)*** For an extraction positive control: Spike the appropriate amount of DPC to 300 µL of the prepared test sample to achieve 200 copies per PCR. Extraction controls can be dual- or triple-spiked with DPCs for each assay to conserve sample. You can also use one of the following in place of test samples: 1X PBS solution, Lysis Buffer, Cell Fractionation Buffer, or plain media.
4. Incubate at 56°C for 15 minutes to digest the cellular RNA. Vortex twice during incubation.
5. Add 700 µL of Lysis Buffer.

Next steps

Proceed directly to "Extract the DNA" on page 26.

Option 2: Process pooled cell culture media and mammalian cells

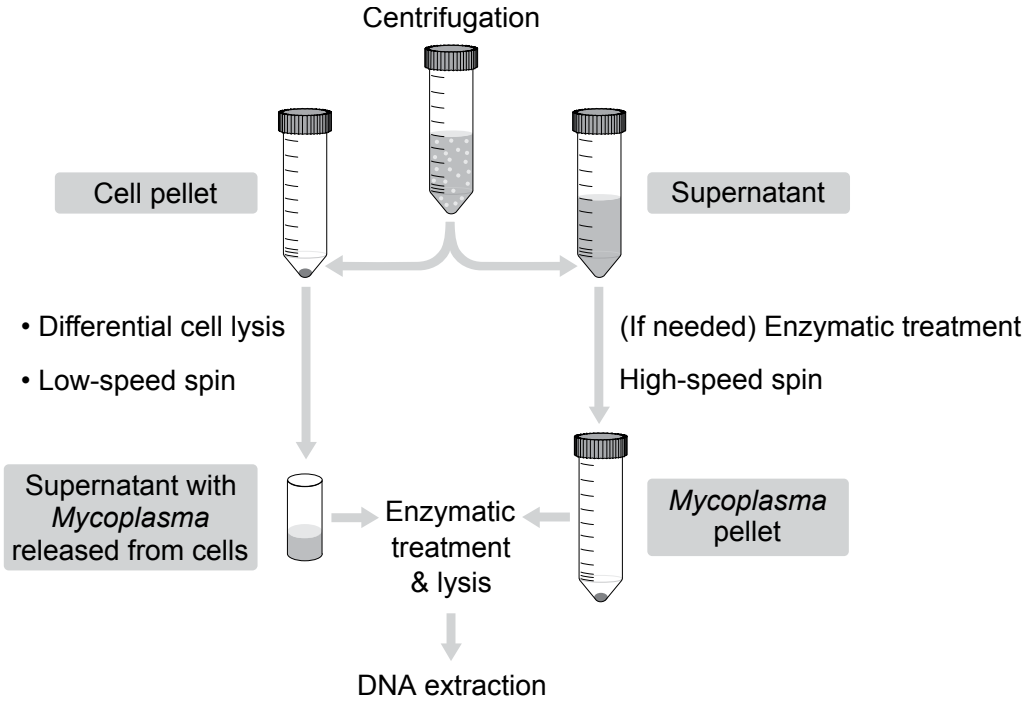

Figure 2 Option 2: Process pooled cell culture media and mammalian cells

Separate mammalian cells from cell culture media

1. Obtain the 11-mL samples from "Prepare test samples" on page 21.
2. Centrifuge each tube at 1,000 × *g* for 5 minutes at 4°C to pellet the mammalian cells.
3. Transfer 10 mL of the supernatant to a new 50-mL conical tube, then place on ice. The supernatant contains free *Mycoplasma*.
4. Remove residual supernatant from the mammalian cell pellet, then place the cell pellet on ice.

Treat with RNase and DNase

If the samples have high SYBR™ Green I dye background because of excess cellular nucleic acid in the supernatant, perform RNase treatment.

IMPORTANT! For some high-density samples, both RNase and DNase treatments are needed. In this case, perform the DNase treatment before the RNase treatment.

Treat with DNase

1. Add the following TURBO™ DNase (2 U/µL) components, then gently vortex to mix:
   - 450 µL of 10× Reaction Buffer
   - 90 µL of TURBO™ DNase
2. Incubate at 37°C for 30 minutes.

Treat with RNase

1. Add the following components, gently vortex to mix, then briefly spin:
   - 180 µL of 0.5 M EDTA
   - 225 µL of RNase Cocktail™ Enzyme Mix
   - 150 µL of Proteinase K

   Note: Alternatively, you can prepare a stock mix of EDTA, Proteinase K, and RNase Cocktail™ Enzyme Mix, then add 555 µL of stock mix to each sample.
2. Incubate at 56°C for a minimum of 30 minutes to digest the cellular RNA and proteins.

Process the supernatant to obtain the *Mycoplasma* pellet

1. Centrifuge the supernatant at 16,000 × *g* for 30 minutes at 4°C to pellet the *Mycoplasma*.
2. Carefully remove and discard the supernatant; retain the *Mycoplasma* pellet for use in the next section.

   IMPORTANT! Do not decant the liquid and do NOT touch the pellet. Use a P200 pipette to remove the last of the supernatant.
3. Place the 50-mL tube containing the *Mycoplasma* pellet on ice.

Process the mammalian cell pellet to obtain free *Mycoplasma* and combine with the *Mycoplasma* pellet

Perform this procedure during the 30-minute centrifugation step in the previous section.

1. Add 550 µL of ice-cold Cell Fractionation Buffer to the mammalian cell pellet. Gently vortex or pipet up and down several times with a P1000 pipette to completely resuspend the mammalian cells.

   If the pellet is difficult to dislodge, vigorously agitate the tube.

2. Transfer the mammalian cell suspension to a 2-mL microcentrifuge tube, then place on ice for 5 minutes.

3. Centrifuge the 2-mL tube at 1,500 × *g* for 10 minutes at 4°C to pellet the cellular membranes and nuclei.

4. Carefully transfer 300 µL (two 150-µL aliquots) of the Cell Fractionation Buffer supernatant (mammalian cell lysate) to the *Mycoplasma* pellet obtained in the previous section. Avoid the pellet and viscous material.

5. Resuspend the *Mycoplasma* pellet in the supernatant by pipetting up and down or by vortexing on medium speed.

6. Transfer the resuspended *Mycoplasma* pellet to a new 2-mL microcentrifuge tube.

Treat the resuspended *Mycoplasma*

Separately process the resuspended *Mycoplasma* in the 2-mL microcentrifuge tube.

1. Add the following volumes, then briefly vortex to mix:
   - 2 µL of 0.5 M EDTA
   - 18 µL of RNase Cocktail™ Enzyme Mix
   - 5 µL of Proteinase K

2. *(Optional)* Spike with Discriminatory Positive Control (DPC) for optimization or with *Mycoplasma* DNA for lot release validation.

3. ***(Optional)*** For an extraction positive control: Spike the appropriate amount of DPC to 300 µL of the prepared test sample to achieve 200 copies per PCR. Extraction controls can be dual- or triple-spiked with DPCs for each assay to conserve sample. You can also use one of the following in place of test samples: 1X PBS solution, Lysis Buffer, Cell Fractionation Buffer, or plain media.

4. Incubate at 56°C for 15 minutes to digest the cellular RNA. Vortex twice during incubation.

5. Add 700 µL of Lysis Buffer.

Next steps

Proceed directly to “Extract the DNA“ on page 26.

Extract the DNA

Bind DNA

For each sample lysate tube:

1. Add 30 µL of Magnetic Particles.
2. Add 525 µL of Binding Solution, then immediately invert each tube to mix.
3. Using a vortex adaptor, vortex the tubes vertically at medium speed for 5 minutes to capture the nucleic acid.

   Note: To prevent accidental opening of the tubes, remove the tubes by pushing up from the bottom of the tubes.
4. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater).
5. Place in the Magnetic Stand for 5 minutes. See "Guidelines for working with Magnetic Particles" on page 11.
6. Aspirate the supernatant without disturbing the Magnetic Particles, then discard the supernatant.

Wash DNA

For each tube of Magnetic Particles pellet (bound DNA):

1. Add 300 µL of Wash Buffer, then vortex for ~5 seconds.
2. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater).
3. Place in the Magnetic Stand for 1 minute.
4. Aspirate the supernatant without disturbing the Magnetic Particles, then discard the supernatant.
5. Repeat step 1 through step 4.

   Set the timer for 5 minutes just before you remove the wash buffer for the second time. See step 7.
6. Use a P200 pipette to aspirate the residual supernatant from the bottom of the tube, then discard the supernatant.
7. With the cap open, air-dry the Magnetic Particles pellet at room temperature for ≤5 minutes to remove any residual ethanol.

   IMPORTANT! Set the timer for 5 minutes just before you remove the wash buffer for the second time. Do NOT dry longer than 5 minutes. Overdrying will make the pellets difficult to resuspend in Elution Buffer in the next steps.

   IMPORTANT! If you are using an aspirator or processing in a BSC (hood), no additional drying time is required. Aspirate and proceed; do not wait ≤5 minutes.

Elute DNA

For each sample:

1. Add 100 µL of Elution Buffer.
2. Vortex for ~10 seconds.
3. Incubate at 70°C for 7 minutes.
   Vortex 2–3 times during incubation to ensure complete resuspension of the Magnetic Particles. The total time may be 10–12 minutes.
4. Centrifuge for 5 minutes at top speed (13,000 × *g* or greater).
5. Place in the Magnetic Stand for 3 minutes.
6. Transfer the eluate to a non-stick 1.5-mL microcentrifuge tube.

Next steps

The extracted DNA is now ready for use in the appropriate PCR assay.

Note: If not used immediately, store the samples at 4°C for same-day use or at –20°C for longer storage.

A

Troubleshooting

Troubleshooting

Observation	Possible cause	Action
Poor extraction efficiency (low yields)	Overdrying the magnetic pellet after the final wash step.	Start the timer before removing the Wash Buffer from the first tube. Do not dry longer than 5 minutes. If you are using aspiration or a Biological Safety Cabinet, no additional drying time is needed.
	Magnetic Particles are attached too tightly to the tube wall during the elution.	Place the tube in the benchtop microcentrifuge with the Magnetic Particles pellet oriented toward the center. Spin the tube for 30 seconds to detach the Magnetic Particles into the Elution Buffer.
	Magnetic Particles are difficult to resuspend during the elution.	Incubate the pellets at 70°C for 7 minutes. Vortex the tubes three times during incubation to help resuspension.
PCR inhibition (Figure 3) or high background signal (Figure 4)	Excess mammalian cell DNA in the sample.	For some high-density samples, both RNase and DNase treatments are needed. In this case, perform the DNase treatment before the RNase treatment. See "Treat with RNase and DNase" on page 22.
		Prewash samples that contain PCR inhibitors: 1. Add 300 µL of a 3:2 mixture of 95% non-denatured ethanol and Lysis Buffer. Invert the tubes 3 times to mix. 2. Spin in a microcentrifuge: Press the short spin button for 15 seconds, or until the microcentrifuge reaches top speed (13,000 × *g* or greater). **IMPORTANT!** Do not expose samples to the wash solution in this step for longer than 3 minutes. 3. Place the tubes in the Magnetic Stand for 1 minute, then aspirate and discard the liquid.
		Contact your local Field Applications Specialist or Sales Representative.

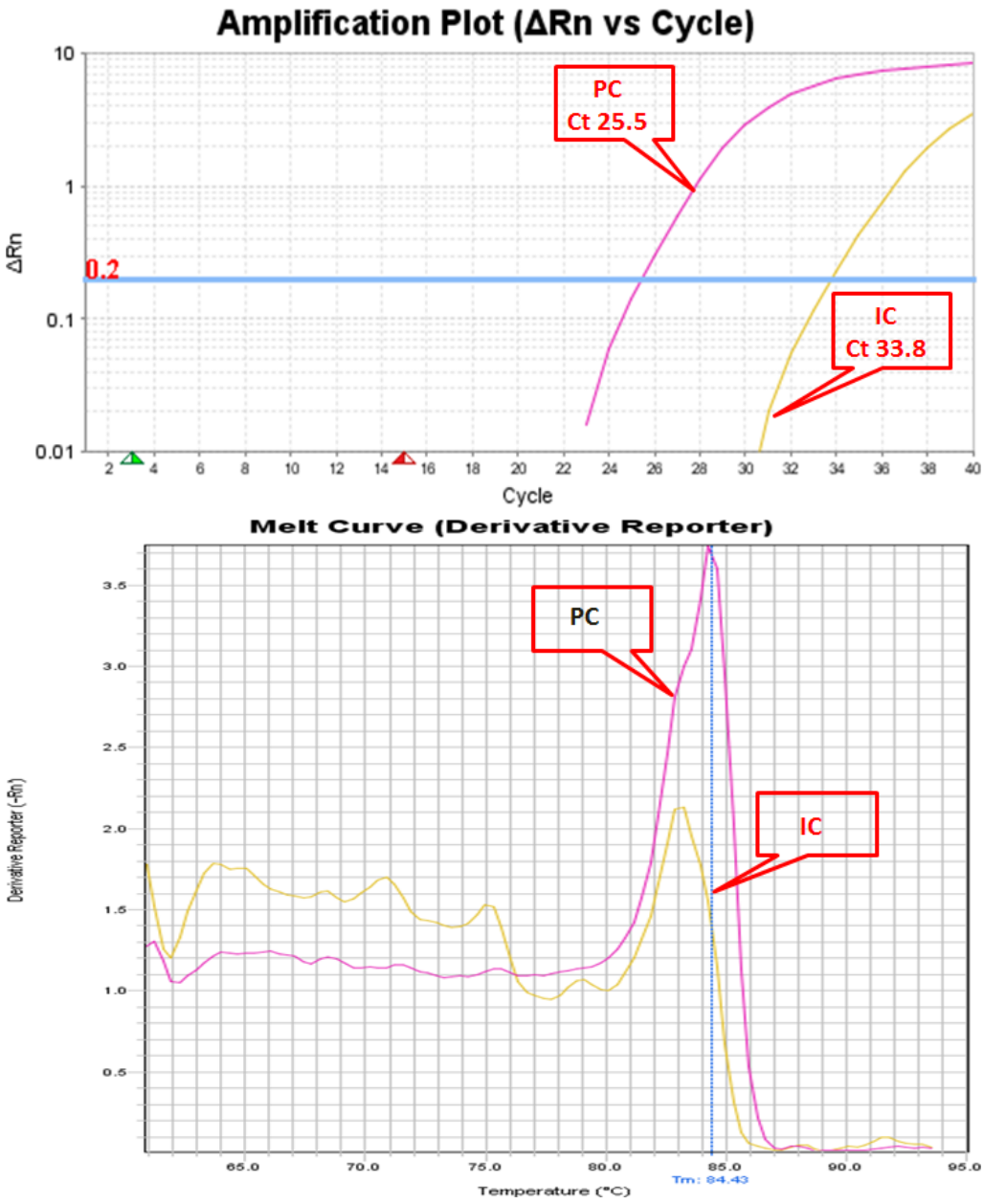

Figure 3 PCR inhibition; $\Delta C_T > 2$

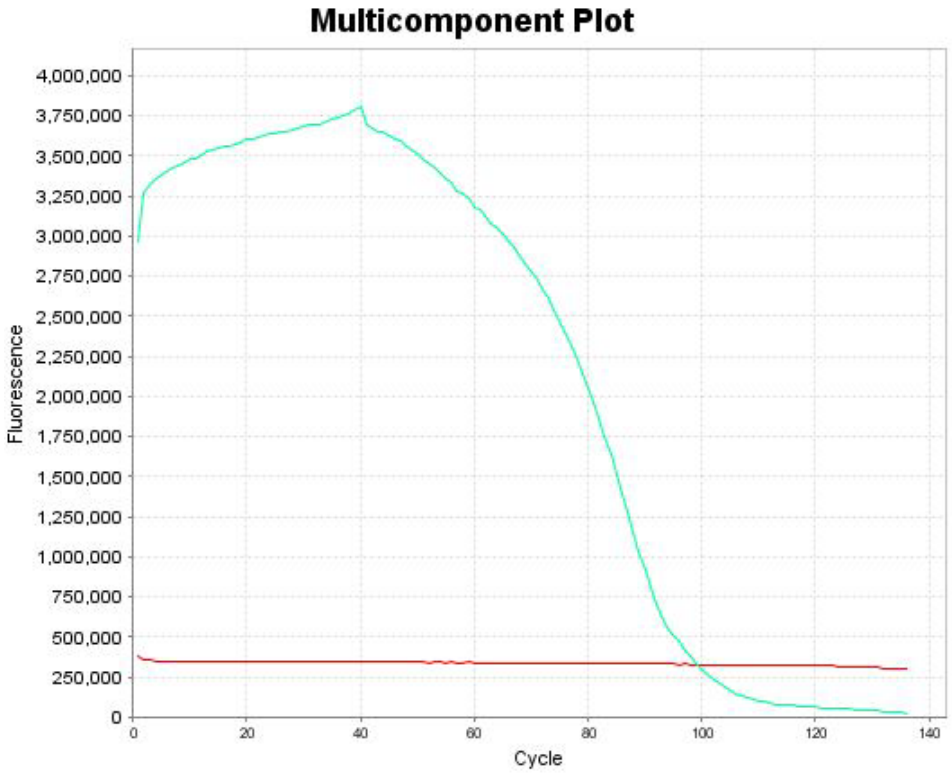

Figure 4 High background signal

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Publication number
PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus Quick Reference	4465875

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  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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