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

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

Catalog Numbers 4452222 and 4443789

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the *PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus User Guide* (Pub. No. 4465957). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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	"1-2-3 manual protocol for Mycoplasma and/or MMV detection" on page 2
Mycoplasma, MMV, and Vesivirus	100 µL (up to 10^6 cells)	PrepSEQ™ 1-2-3 Nucleic Acid Extraction Kit	"3-in-1 manual protocol for Mycoplasma, MMV, and/or Vesivirus detection" on page 4
Mycoplasma	Up to 11 mL (up to 2×10^8 cells)	PrepSEQ™ *Mycoplasma* Nucleic Acid Extraction Kit	"Large-scale manual protocol for Mycoplasma detection—Option 1: Direct sample testing" on page 5 *OR* "Large-scale manual protocol for Mycoplasma detection—Option 2: Process pooled cell culture media and mammalian cells" on page 6

Before you begin

Before you begin, review the *PrepSEQ™ Sample Preparation Kits for Mycoplasma, MMV, and Vesivirus User Guide* (Pub. No. 4465957) for required materials and procedural guidelines.

Note: This Quick Reference provides brief procedures for sample preparation. For detailed procedures, see the User Guide.

Prepare materials

1. *(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.
2. Power on a heat block to 37°C.
3. 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.
4. *(Large-scale protocols only)* Power on the refrigerated centrifuge to allow it to cool to 2–8°C before use.

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.

1 Prepare test samples

a. Prepare each sample in a new 2-mL microcentrifuge tube:
   - ≤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.

b. ***(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.

2 Prepare sample lysate

For each sample tube:

a. Add 200 µL of Lysis Buffer, then vortex for ~5 seconds to mix.

b. Add 2 µL of 0.5 M EDTA, 18 µL of RNase Cocktail™ Enzyme Mix, and 2 µL of Proteinase K, then briefly vortex to mix.

c. Incubate at 56°C for a minimum of 15 minutes.

d. Incubate at room temperature for 5 minutes.

e. Add 700 µL of Lysis Buffer.

3 Bind DNA

For each sample lysate tube:

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

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

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

d. Place in the Magnetic Stand for 5 minutes.

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

4 Wash DNA

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

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

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

c. Place in the Magnetic Stand for 1 minute.

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

e. Repeat step 4a through step 4d.

   Set the timer for 5 minutes just before you remove the wash buffer for the second time. See step 4g.

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

g. 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.

5 Elute DNA

For each sample:

a. Add 100 µL of Elution Buffer.

b. Vortex for ~10 seconds.

c. 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.

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

e. Place in the Magnetic Stand for 3 minutes.

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

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.

1 Prepare test samples

a. 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.

b. ***(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.

2 Prepare sample lysate

For each sample tube:

a. Add 500 µL of Lysis Buffer, then vortex for ~15 seconds to mix.

b. Incubate at 45°C for 10 minutes.

c. Vortex for ~10 seconds to mix.

3 Bind DNA

For each sample lysate tube:

a. Add 30 µL of Magnetic Particles.

b. Add 330 µL of Binding Solution, then immediately invert each tube to mix.

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

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

e. Place in the Magnetic Stand for 5 minutes.

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

4 Wash DNA

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

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

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

c. Place in the Magnetic Stand for 1 minute.

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

e. Repeat step 4a through step 4d.

   Set the timer for 5 minutes just before you remove the wash buffer for the second time. See step 4g.

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

4 Wash DNA *(continued)*

g. 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.

5 Elute DNA

For each sample:

a. Add 100 µL of Elution Buffer.

b. Vortex for ~10 seconds.

c. 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.

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

e. Place in the Magnetic Stand for 3 minutes.

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

Large-scale manual protocol for *Mycoplasma* detection—Option 1: Direct sample testing

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

1 Prepare test samples

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

- ≤10^8 cells—Add 11 mL of sample to the tube.
- >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.

2 Separate mammalian cells from cell culture media

a. Centrifuge each tube at 1,000 × *g* for 5 minutes at 4°C to pellet the mammalian cells.

b. Transfer 10 mL of the supernatant to a new 50-mL conical tube, then place on ice.

The supernatant contains free *Mycoplasma*.

c. Discard the mammalian cell pellet.

3 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.

a. To treat with DNase:

1. Add 450 µL of 10× Reaction Buffer and 90 µL of TURBO™ DNase, then gently vortex to mix.
2. Incubate at 37°C for 30 minutes.

3 Treat with RNase and DNase *(continued)*

b. To treat with RNase:
   1. Add 180 µL of 0.5 M EDTA, 225 µL of RNase Cocktail™ Enzyme Mix, and 150 µL of Proteinase K, gently vortex to mix, then briefly spin.
   2. Incubate at 56°C for a minimum of 30 minutes to digest the cellular RNA and proteins.

4 Process the supernatant to obtain resuspended *Mycoplasma*

a. Centrifuge the supernatant at 16,000 × *g* for 30 minutes at 4°C to pellet the *Mycoplasma*.

b. Aspirate and discard the supernatant without disturbing the *Mycoplasma* pellet. Do not decant the liquid and do NOT touch the pellet.

c. 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.

d. Transfer the resuspended pellet to a 2-mL microcentrifuge tube.

5 Treat the resuspended *Mycoplasma*

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

a. Add 2 µL of 0.5 M EDTA, 18 µL of RNase Cocktail™ Enzyme Mix, and 5 µL of Proteinase K, then briefly vortex to mix.

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

c. ***(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.

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

e. Add 700 µL of Lysis Buffer.

Proceed directly to "Large-scale protocol for Mycoplasma detection—Options 1 and 2: Extract the DNA" on page 8.

Large-scale manual protocol for *Mycoplasma* detection—Option 2: Process pooled cell culture media and mammalian cells

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

1 Prepare test samples

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

- ≤10^8 cells—Add 11 mL of sample to the tube.
- >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.

2 Separate mammalian cells from cell culture media

a. Centrifuge each tube at 1,000 × *g* for 5 minutes at 4°C to pellet the mammalian cells.

b. Transfer 10 mL of the supernatant to a new 50-mL conical tube, then place on ice.

   The supernatant contains free *Mycoplasma*.

c. Remove residual supernatant from the mammalian cell pellet, then place the cell pellet on ice.

3 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.

a. To treat with DNase:
   1. Add 450 µL of 10× Reaction Buffer and 90 µL of TURBO™ DNase, then gently vortex to mix.
   2. Incubate at 37°C for 30 minutes.

b. To treat with RNase:
   1. Add 180 µL of 0.5 M EDTA, 225 µL of RNase Cocktail™ Enzyme Mix, and 150 µL of Proteinase K, gently vortex to mix, then briefly spin.
   2. Incubate at 56°C for a minimum of 30 minutes to digest the cellular RNA and proteins.

4 Process the supernatant to obtain the *Mycoplasma* pellet

a. Centrifuge the supernatant at 16,000 × *g* for 30 minutes at 4°C to pellet the *Mycoplasma*.

b. 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.

c. Place the 50-mL tube containing the *Mycoplasma* pellet on ice.

5 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.

a. 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.

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

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

d. 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.

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

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

6 Treat the resuspended *Mycoplasma*

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

a. Add 2 µL of 0.5 M EDTA, 18 µL of RNase Cocktail™ Enzyme Mix, and 5 µL of Proteinase K, then briefly vortex to mix.

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

c. ***(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.

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

e. Add 700 µL of Lysis Buffer.

Proceed directly to "Large-scale protocol for Mycoplasma detection—Options 1 and 2: Extract the DNA" on page 8.

Large-scale protocol for *Mycoplasma* detection—Options 1 and 2: Extract the DNA

1 Bind DNA

For each sample lysate tube:

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

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

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

d. Place in the Magnetic Stand for 5 minutes.

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

2 Wash DNA

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

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

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

c. Place in the Magnetic Stand for 1 minute.

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

e. Repeat step 2a through step 2d.

   Set the timer for 5 minutes just before you remove the wash buffer for the second time. See step 2g.

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

2 Wash DNA *(continued)*

g. 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.

3 Elute DNA

For each sample:

a. Add 100 µL of Elution Buffer.

b. Vortex for ~10 seconds.

c. 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.

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

e. Place in the Magnetic Stand for 3 minutes.

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

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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.
A	August 2011	New document.