SHENTEK

Mycoplasma DNA Extraction Kit (2G)

User Guide

Version: A/1
For Research Use Only
Product No.: 1509840
Reagents for 50 Extractions

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(IMPORTANT: Please read this document carefully before experiment.)

1. Product information

■ Product description

MycoSHENTEK® Mycoplasma DNA Extraction Kit (2G) is used for extraction of trace mycoplasma DNA from master cell banks, working cell banks, virus seed banks, and complex matrices such as 10^7 cells culture, 5% human albumin culture solution, or high-concentration plasmids, etc. It works with MycoSHENTEK® Mycoplasma Detection Kit (2G) for an integrated workflow from sample preparation to detection assay. Validated according to USP 63, EP 2.6.7 and JP XVIII for mycoplasma detection with a detection limit of 10 CFU/mL.

For sample volume that is less than 400 μL, test sample DNA can be extracted directly using Mycoplasma DNA Extraction Kit (2G). If it is necessary to increase the sample volume to achieve a higher detection sensitivity, the sample should be concentrated by centrifugation to a final volume of approximately 400 μL before using this extraction kit.

The kit is compatible with manual sample preparation, or with automated extraction using rHCDpurify instrument.

■ Kit contents and storage

WARNING: Please read the Material Safety Data Sheets (MSDS) and follow the handling instructions. Wear appropriate protective eyewear, mask, clothing and gloves.

Table 1. Kit component and storage

Reagent	Part No.	Quantity	Storage
Wash buffer A	NND015	15 mL × 1 bottle	room temperature
Binding solution	NND017	10 mL × 1 bottle	
Elution buffer	NND019	5 mL × 1 bottle	
Dilution buffer	NND022	5 mL × 1 bottle	
Lysis buffer	NND028	5 mL × 1 bottle	
Pretreatment buffer	NND002	1.25 mL × 4 tubes	2-8℃
Magnetic particles	NND033	750 μL × 2 tubes	
5M NaCl	NND040	500 μL × 1 tube	
Precipitation solution I	NND003	25 μL × 1 tube	-20℃
Precipitation solution II	NND004	500 μL × 1 tube	
Proteinase K	NND023	500 μL × 2 tubes	

The kit components can be stored at the appropriate conditions for up to 24 months.

Please check the expiration date on the labels.

■ Required materials not included in the kit

- ddH_2O
- Anhydrous Ethanol (AR)
- 100% Isopropanol(AR)
- PCR 8-well strip tubes with caps or 96-well plate with seals
- Nonstick, Low Retention tips 1000μL, 100μL, 10μL
- Nonstick, RNase-free centrifuge tubes 1.5mL, 2.0mL, 50mL

■ Related equipment

- Benchtop microcentrifuge
- Magnetic stand or rHCDpurify
- High-speed refrigerated centrifuge
- Vortex mixer
- Dry bath incubator

- Pipettors,1000μL, 100μL, 10μL
- Real-time PCR instrument
- Biosafety cabinet
- Microplate and microtube shaker

2. Methods

■ Experiment preparation

Before first use of the kit:

- Add 20mL of Anhydrous Ethanol to Wash buffer A.
- Prepare a 70% Anhydrous Ethanol buffer in a clean tube, label it "Wash buffer B".
- Store Wash buffer at room temperature.
- Centrifuge the Internal Control (IC) (2G) lyophilized powder (NNA035) at 16,000×g for 2 min and add 600 μL of DNA Diluent Buffer (NND001). Vortex to mix well and spin in a microcentrifuge, and repeat three times.

  Note: IC (NNA035) & DNA Diluent Buffer (NND001) are the components of Mycoplasma DNA Detection Kit (2G).

Before each use of the kit:

- Prepare 100% Isopropanol.
- Set the dry bath temperatures to 55°C (or 25°C) and 70°C.

  Note: If the Lysis Buffer or Binding Solution is cloudy or contains precipitates, heat at 37°C until it clears.
- Set a refrigerated centrifuge temperature to 2-8°C.
- Incubate the Magnetic particles (NND033) suspension at room temperature for 10 min, and vortex to mix well before use.
- Binding buffer preparation:

  1. Binding buffer preparation (for one sample): Add 200 μL of Binding solution (NND017), 5 μL of diluted Precipitation solution I (please see Binding buffer preparation - Step 2) and 9 μL of Precipitation solution II

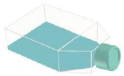
(NND004), and mix well.

2. Precipitation solution I dilution: According to the volume of binding buffer needed for the experiment, prepare the volume of Precipitation solution I (NND003) and dilute it with DNA diluent at a volume ratio of 1:99.

Note: Prepare the total volume of binding buffer appropriately more than necessary for experiment (the necessary volume is calculated based on the volume for one sample and total sample amount).

■ Sample pretreatment

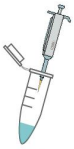
Test samples - Cell culture samples



① Cell culture samples

② Heat treatment
95 ℃ for 10 min, then cool to 2-8 ℃ for 20 min

③ Sample pretreatment
Add Pretreatment buffer to the sample (volume ratio 1:10), vortex and mix well, incubate at RT for 5 min



④ Debiris removal
Centrifuge for 10s at 7250 ×g aspirate the supernatant to a new centrifuge tube

⑤ Concentration
centrifuge at 18000 ×g for 30 min.remove the supernatant till the remaining at 100-150 μL,add 300 μL of Dilution buffer

⑥ Sample digestion
10 μL 5M NaCl
10 μL IC
20 μL Proteinase K
100 μL of Lysis buffer
55 ℃ for 60 min
（except for 5% human albumin samples at 25 ℃ for 60 min）

Test samples - Cell culture supernatant samples

① Cell culture supernatant samples

② Cell removal

Centrifuge for 5 min at 72 ×g
aspirate the supernatant to
a new centrifuge tube

③ Concentration

• Cell culture supernatant for 5% human albumin samples:
If sample volume ≤ 400 µL,concentration is not necessary;
If sample volume > 400 µL,spin at 18000 ×g for 30 min,
remove the supernatant till leftover volume ≤ 400 µL.
• High-concentration albumin samples:
Centrifuge at 18000 ×g for 30 min,
remove the supernatant till leftover volume ≤ 100 µL,
add 300 µL of Dilution buffer

④ Sample pretreatment

Add Pretreatment buffer to the sample
(volume ratio 1:10),
vortex and mix well,
incubate at RT for 5 min

⑤ Sample digestion

10 µL 5M NaCl
10 µL IC
20 µL Proteinase K
100 µL of Lysis buffer
55 ℃ for 60 min
（except for 5% human albumin samples at 25 ℃ for 60 min）

Test samples - Non-cellular samples

① Non-cellular samples

② Concentration

• Cell culture supernatant for 5% human albumin samples:
If sample volume ≤ 400 µL,concentration is not necessary;
If sample volume > 400 µL,spin at 18000 ×g for 30 min,
remove the supernatant till leftover volume ≤ 400 µL.
• High-concentration albumin samples:
Centrifuge at 18000 ×g for 30 min,
remove the supernatant till leftover volume ≤ 100 µL,
add 300 µL of Dilution buffer

③ Sample pretreatment

Add Pretreatment buffer
to the sample
(volume ratio 1:10),
vortex and mix well,
incubate at RT for 5 min

④ Sample digestion

10 µL 5M NaCl
10 µL IC
20 µL Proteinase K
100 µL of Lysis buffer
55 ℃ for 60 min
（except for 5% human albumin samples at 25 ℃ for 60 min）

For test samples (Please choose the appropriate procedure accordingly.)

- **Cell culture samples (≤ 107 cell/mL)**

1) Heat treatment

Incubate the samples at 95°C for 10 min and then cool the samples to 2-8°C for

20 min.

2) Sample pretreatment

After heat treatment, add Pretreatment buffer (NND002) to the sample at the volume ratio of 1:10, then vortex and mix well. Incubate at room temperature for 5 min.

3) Debris removal

Centrifuge for 10 seconds at 7250×g to precipitate the cell debris; after centrifugation, aspirate the supernatant to a new centrifuge tube with a 200 μL-volume pipette.

4) Sample concentration

Spin the supernatant in a refrigerated centrifuge at 18,000×g for 30 min. Remove the supernatant and leave the remaining solution at around 100-150 μL; add 300 μL of Dilution buffer (NND022), vortex and mix well, then spin in a microcentrifuge for 3 seconds.

- **Non-cellular samples**

1. High-concentration plasmids

1) Sample concentration

Spin the sample in refrigerated centrifuge at 18,000×g for 30 min; Remove the supernatant and leave the remaining solution at around 100μL; add 300 μL of Dilution buffer (NND022).

2) Sample pretreatment

Add Pretreatment Buffer (NND002) to the sample at the volume ratio of 1:10, then vortex and mix well, incubate at room temperature for 5 min, and spin in microcentrifuge for 3 seconds.

2. 5% human albumin samples

1) Sample concentration

If sample volume ≤ 400 μL, concentration is not necessary. If sample volume > 400 μL, spin the sample in a refrigerated centrifuge at 18,000×g for 30 min,

remove the supernatant with a pipette keeping a leftover that is less than 400 μL.

2) Sample pretreatment

Add Pretreatment buffer (NND002) to the sample at the volume ratio of 1:10 (Pretreat buffer to sample), then vortex and mix well, incubate at room temperature for 5 min, and spin in a microcentrifuge for 3 seconds.

- **Cell culture supernatant samples**

1) Cell removal

Centrifuge for 5 min at 72×g to precipitate the cells; after centrifugation, aspirate the supernatant to a new centrifuge tube.

2) Sample concentration

If sample volume ≤ 400 μL, concentration is not necessary; If sample volume > 400 μL, spin the sample in a refrigerated centrifuge at 18,000×g for 30 min. Remove the supernatant with a pipette keeping a leftover that is less than 400 μL.

3) Sample pretreatment

Add Pretreatment Buffer (NND002) to the sample at the volume ratio of 1:10 (Pretreat buffer to sample), then vortex and mix well, incubate at room temperature for 5 min, and spin in microcentrifuge for 3 seconds.

For control samples:

- **Negative control samples (NCS)**

NCS evaluates whether there is cross-contamination or environmental contamination during sample extraction.

Add 100-400μL of Dilution buffer (NND022) (Take the volume of Dilution buffer same as the volume of the test sample), to the Pretreatment buffer (NND002) (Dilution buffer to Pretreatment buffer at the volume ratio of 10:1), vortex to mix well and incubate at room temperature for 5 min, spin 3 seconds in a microcentrifuge.

Note: If the sample matrix is added, follow the corresponding matrix pretreatment method in the procedure for test sample.

■ Samples digestion

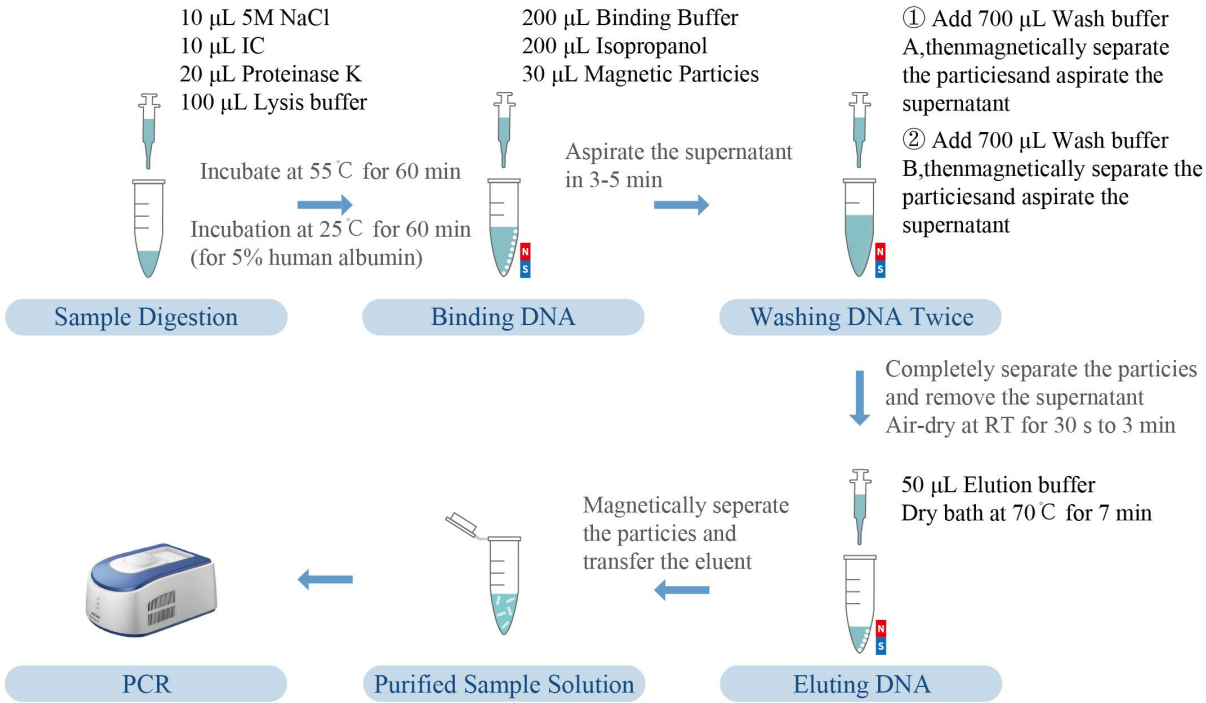
1. To each sample, add 10 µL of 5M NaCl and 10 µL of IC, vortex to mix well, then spin 3 seconds in a microcentrifuge.

2. To each sample, add 20 µL of Proteinase K (NND023) and 100 µL of Lysis buffer (NND028), vortex to mix well and spin 3 seconds in a microcentrifuge. Then incubate all samples including the control samples at 55°C for 60 min, except for 5% human albumin samples with the corresponding control samples at 25°C for 60 min.

Note: For complete digestion, vortex to mix well after around 30-min incubation, then continue incubation. After sample digestion, the following DNA extraction experiment should be performed as soon as possible.

■ Mycoplasma DNA extraction

1. Manual procedure



Binding DNA

1. Incubate the Magnetic particle (NND033) at room temperature for 10 min, and vortex to mix well before use.

2. Take the sample from the dry bath, and spin it for 3 seconds , and add 200 μL Binding buffer, vortex to mix well and spin it for 3 seconds .

3. Add 200 μL of Isopropanol and 30 μL of Magnetic particles (NND033) suspension. Vortex the tubes vertically at medium speed for 5 minutes to bind the nucleic acids. Spin for 10 seconds in a microcentrifuge and place the tubes in the magnetic stand.

4. Until the solution is clear and the particles are completely separated, aspirate the supernatant without disturbing the particles, and discard the supernatant.

Note:

The time for complete clear solution and particle separation is about 3-5 minutes. During the removal of the supernatant, avoid removing the magnetic particles with the supernatant. During extraction, when the sample tubes are placed into the magnetic stand, always orient the Magnetic particle pellet toward the magnet.

Washing DNA

1. Take each tube from the Magnetic stand, and add 700μL of Wash buffer A. Vortex for 10 seconds to mix well and spin for 10 seconds in a microcentrifuge, then place the tubes in the magnetic stand. Aspirate the supernatant without disturbing the magnetic particles, then discard the supernatant to complete the first washing.

2. Then take each tube again from the Magnetic stand, and add 700μL of Wash buffer B. Vortex for 40 seconds and spin for 10 seconds, then place the tubes in the magnetic stand. Aspirate the supernatant without disturbing the magnetic particles, then discard the supernatant to complete the second washing.

3. To remove supernatant completely, spin in a microcentrifuge for 10s and place each tube in the Magnetic stand again. Wait until the particles completely separate, use a 10 μL volume pipette to carefully remove the residual supernatant.

4. With the cap open, air-dry the Magnetic Particles pellet at room temperature for 30s - 3min to remove any residual ethanol.

Note: When removing the supernatant, avoid removing the Magnetic particles

together with the supernatant.

The drying time depends on the specific environment. It could be shorter in higher temperature or lower humidity condition, while slightly longer in lower temperature or higher humidity condition..

Eluting DNA

1. Add 50 μL Elution buffer to each sample, vortex for 5 seconds and incubate at 70℃ for 7 min. Vortex 2-3 times during incubation to ensure complete resuspension of the magnetic particles.

Note: Vortex to shake the magnetic particles and eluent to the bottom of the tube. If centrifuge is needed to spin the particles and eluent from the cap to the bottom of the tube, vortex it again to mix well.

2. After incubation, quickly spin the tube for 1 min, and then place the tubes in the magnetic stand. Wait until the particles completely separate, carefully transfer the eluate to a new nonstick centrifuge tube.

3. Quickly spin the tube for 10 seconds, and then place it in the magnetic stand again. Carefully transfer the eluent to a nonstick 1.5mL centrifuge tube and label the corresponding sample name.

Note: Transfer the eluate completely and avoid leaving any residuals behind.

2. Automated procedure with rHCDpurify

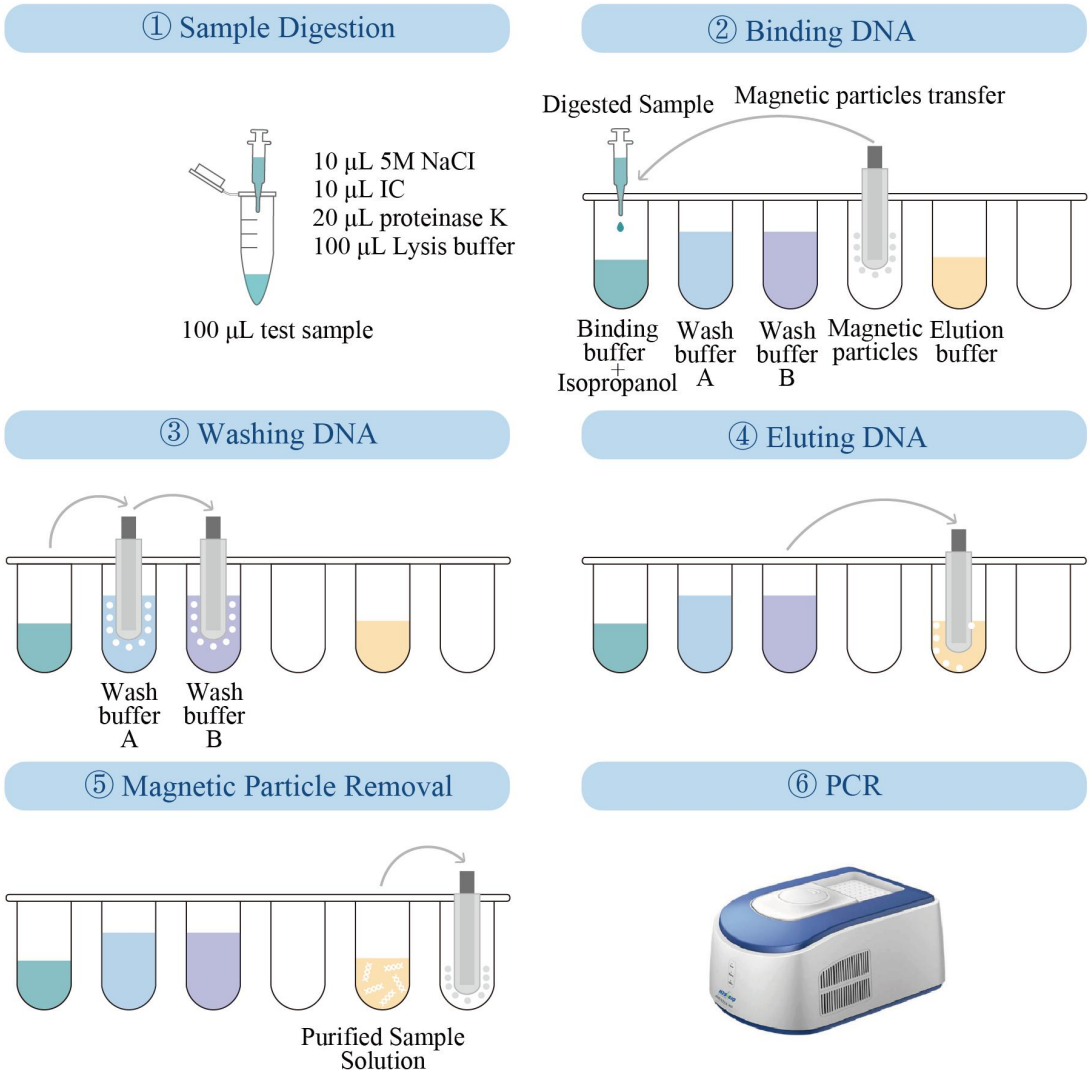


Plate preparation

Add the corresponding solution to each well according to the following 96 deep-well plate layout:

Table 2. Example of 96-well Plate layout

	Group 1					Group 2					
1	2	3	4	5	6	7	8	9	10	11	12
S1											
S2											
S3											
S4											
S5											
S6											
S7											
S8						NCS					

Column 1 or 7: Binding buffer 200 µL/well, Isopropanol 200 µL/well, the digested sample solutions

Column 2 or 8: Wash buffer A, 700 µL/well

Column 3 or 9: Wash buffer B, 700 µL/well

Column 4 or 10: Magnetic particles, 30 µL/well

Column 5 or 11: Elution buffer, 65 µL/well

Note: The samples should be added after all reagents are transferred.

Program Processing

Start the program before plate preparation:

Power on—self-test—↵/II.

1. Use 75% alcohol wipes to clean the insider walls.
2. ▶—UV—↵/II—▶15min—↵/II.

After plate is prepared, continue with the following steps:

3. Put the 96-deep well plate into the instrument and insert the plastic sleeve into the corresponding position.
4. Run—↵/II—Myco-2G—↵/II, run about 43 min.
5. After extraction, immediately take out the deep-well plate, and transfer each purified sample solution to a nonstick 1.5mL centrifuge tube.

Precautions

1. It is recommended to separate molecular laboratory spaces for reagent preparation area (negative control sample preparation, PCR reagent preparation, negative test control preparation), sample preparation area (sample preparations), amplification area, etc. Each area clearly marked with a fixed sign and has separate sets of equipment and supplies to avoid intermixing. Experimental reagents, test samples, and PCR amplification products should be stored separately and not in the same storage place. Eliminate every unnecessary walk in the experimental area to reduce the contamination risks.

2. Ensure that the ambient environment temperature not lower than 22°C during the experiment begins.

3. During the experiment, choose suitable gloves and change them regularly. Also use different lab coats, masks, hair covers and gloves in different experimental areas to avoid cross contamination.

4. Centrifuge the reagent tubes before opening to avoid aerosol production or liquid splashing, as well as to avoid contamination to the gloves or pipettes. The liquid on the caps or walls should be spun down to the bottom of the tube.

5. Used tips and liquid waste must be disinfected, and then discarded in a designated place, and if necessary, shipped off-site.

6. After PCR amplification, wear disposable gloves to take the PCR tube or plate out, and check whether the caps or seals are tightly closed, and whether the walls are cracked. Ensure that the reaction mixture does not leak. Discard it in the designated place, and the caps or seals should not be removed.

7. Place the tubes in the magnetic stand with the pellet against the magnet, and rotate the tubes slowly during the process to accelerate the magnetic particle aggregation.

8. During DNA washing and elution, centrifugation should be performed right after vortex to ensure that no magnetic particles or liquid on the tube caps or walls.

9. Do not over dry the pellet when removing the residual ethanol, over drying will make the pellets difficult to resuspend in the Elution buffer in next step.

10. Please perform DNA detection assay on the same day after DNA extraction to ensure the accurate results.

11. Before rHCDpurify program starts, ensure that the PCR plate and plastic sleeves are loaded appropriately.

12. Before and after rHCDpurify program running, UV sterilization of the machine is required for at least 15 min, and use 75% ethanol wipes to clean the insider walls. The minimum interval between two extractions is 30 min.

13. After rHCDpurify program, immediately transfer each sample solution to a new centrifuge tube. Condensed water may appear on the walls of Row 5th or 11th wells, which does not affect the DNA extraction, just simply transfer the bottom eluate to a new tube and guarantee more than 40μL as required for the assay.

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Support & Contact

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