Preparation and Storage of Specimens

Contents

I. *Mycobacterium tuberculous*

All specimens are to be handled as if they could have pathogens of infections. *Sputum, Urine, Blood, Tissue, and Cultured Specimens* can be used for this test and they are collected in an aseptic environment to prevent contamination. After collecting the specimens they can be kept for 24 hours at 2~8℃ without any pre-treatment(Keep refrigerated if the pre-treatment cannot be done within 1 hour) The pre-treated specimens can be kept for about 7 days at 2~8℃, and keep frozen below -15~-25℃for longer (within a month) storage.

1. Pre-treatment of Specimens.

Sputum	Urine	Blood	Tissue(Paraffin tissue block)	Solid cultured bacteria
① Mix the sputum specimen (1ml or more recommended) with the same amount of 4% NaOH (sputum:4% NaOH = 1:1), and vortex it for 1 minute, then leave for 30 minutes at the room temperature (15~30°C). ② Centrifuge at 3,000rpm for 10 minutes and discard the supernatant. ③ Add 1ml of the sterilized PBS(pH7.4) or sterilized distilled water, resuspend the pellets. ④ Transfer the solution to a new tube of 1.5ml, centrifuge at 3,000rpm for 10 minutes and remove the supernatant.	① Take 1ml of the urine in a 1.5ml microtube, centrifuge at13,000rpm for 10 minutes and discard the supernatant. ② Add 1ml of the sterilized PBS(pH7.4), vortex and centrifuge at 13,000rpm for 5 minutes. Discard the supernatant. * Repeat step ② two times for cleaning.	① Add 1ml of the sterilized distilled water to 1 ml of the EDTA treated blood, mix well with inversion and leave at the room temperature for 30 minutes. ② Centrifuge at 13,000rpm for 3 minutes, discard the supernatant. * Repeat ①~② one more time and clean it to remove the hemolyzed red blood cell and EDTA leaving the white precipitate.	① Add 1.2ml of xylene and vortex fast until the paraffin block is melted. ② Centrifuge at 13,000rpm for 5 minutes, remove the supernatant. ③ Add 1.2ml of 100% ethanol and vortex and mix slowly. ④ Centrifuge at 13,000rpm for 5 minutes, discard the supernatant. * Repeat ③~④. ⑤ Dry the pellet for 2 minutes. • Be careful not to be dried too much since it could reduce the DNA separation efficiency. • Live tissue: Without the pre-treatment, follow the DNA extraction process.	① Collect 1 loop of the solid cultured bacteria with a loop of 1㎕ diameter, mix it well with 100µl (200µl for a high density of bacteria) of the sterilized distilled water and vortex for 1 minute. ② Incubate at 100℃ for 10 minutes, centrifuge at 13,000rpm for 5 minutes. ③ Collect and use 2㎕ of the liquid at the top.

2. DNA extraction

For DNA extraction, DNA isolation kits that are commercially available may be used and follow the manufacturer's recommended protocol.

Using Eudipia DNA extraction kit, DNA Extraction Solution is to be used after the white precipitate is evenly dispersed and use vortex or tip to disperse them. In order not to collect the precipitate at the tip, cut off the end of the tip a little.

A. Extraction using Eudipia DNA extraction kit.

1. Remove any remaining liquid and add Chelex 100 Resin(Bio-Rad) of 50 μℓ (100 μℓ for a large number of specimens) and vortex for 1 minute.
2. Heat it up at 100℃ for 15 minutes, and do the centrifugal process at 13,000rpm for 10 minutes.
3. Collect and use 2 μℓ of the liquid at the top for the test.

B. Extraction using Chloroform and CTAB(N-cetyl-N,N,Ntrimethyl ammonium bromide)

1. Add 50 μℓ of lysozyme solution(10mg/ml) to the pellet, vortex and incubate at 37℃ for at least 1 hour.
2. Add 70 μℓ of 10% SDS and 5 μℓ of proteinase K(20mg/ml), vortex, and incubate at 65℃ for 1 hour.
3. Add 100 μℓ of 5M NaCl and 100 μℓ of CTAB/NaCl solution(2% CTAB, 100mM Tris.Cl(pH8.0), 20mM EDTA, 1.4M NaCl, 0.2% β-mercaptoethanol(added just before use)) solution, vortex until the liquid content becoming white; then incubate for 10 minutes at 65℃.
4. Add 750 μℓ of chloroform/isoamyl alcohol (24:1) , vortex for 10 seconds, and centrifuge at room temperature for 5 minutes, at 14,000 g.
5. Transfer the supernatant to a clean tube and mix with 0.6 volume of isopropanol, incubate at -20℃ for 30 minutes, and centrifuge for 15 minutes at 14,000 g.
6. Discard the supernatant, wash the pellet with 1 mL of 70% ethanol and centrifuge for 5 minutes at 14,000 g.
7. Resuspend the pellet in 20-30 μℓ of TE(pH8.0) or sterile H_2O.

(Ref: Honore´-Bouakline, S., Vincensini, J.P., Giacuzzo, V., Lagrange,P.H. and Herrmann, J.L. (2003) Rapid diagnosis of extra-pulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction.J Clin Microbiol41, 2323–2329.)

C. Extraction using Phenol:Chloroform:isoamyl alcohol.

1. Add 100 μℓ of lysozyme solution(10mg/ml) to the pellet,vortex and incubate at 37℃ for at least 1 hour.
2. Add 140 μℓ of 10% SDS and 5 μℓ of proteinase K(20mg/ml), vortex and incubate at 65℃ for 1 hour.
3. Add 250 μℓ of phenol:chloroform:isoamyl alcohol(25:24:1), vortex for 10 seconds and centrifuge at room temperature for 5 minutes at 14,000 g.
4. Transfer the supernatant to a new tube ,add 250 μℓ of chloroform/isoamyl alcohol (24:1) , vortex for 10 seconds and centrifuge at room temperature for 5 minutes, at 14,000 g.
5. Transfer the supernatant to a new tube, add 25 μℓ 3M sodium acetate(pH6.0) and 400 μL absolute ethanol. Mix by inversion, store at -80℃ for 20 minutes.
6. Centrifuge for 15 minutes at 14,000 g. Discard the supernatant and wash the pellet with 1 mL of 70% ethanol and centrifuge for 5 minutes at 14,000 g.
7. Dry the pellet and resuspend the pellet in 20-30 μℓ of TE(pH8.0) or sterile H_2O.

(Ref:A. Ani, S.Okpe, M. Akambi, Comparison of a DNA based PCR method with conventional methods for the detection of M. tuberculosis in Jos, Nigeria, J. Infect. Dev. Ctries. 3(2009)470-475)

D. Extraction using Chelex 100 resin, Nonidet P-40

1. Add 100 μℓ of Chelex solution(5% Chelex-100, 1% Nonidet P-40, 1% Tween 20) to a pellet.
2. Mix and incubate 100℃ for 30 minutes.
3. Centrifuge for 15 minutes at 14,000 g , transfer the supernatant to a new tube and use 2 μℓ of the liquid at the top for the test.

(Ref:Van Der Zanden AGM, Hoeilmann FGC, Weltevred EF, Schouls LM, Van Embden JDA: Simultaneous detection and strains differentiation of Mycobacterium tuberculosis complex in paraffin wax embedded tissues and stained microscopic preparation. J Clin Pathol 1998, 51(4):209–214. 9.da Saúde M: Manual Nacional de Vigilância Laboratorial da tuberculose e outras Micobactérias. Brasília: Ministério da Saúde; 2009.)

II. Atypical Pneumonia(*Mycoplasma pneumoniae, Legionella pneumophila, Chlamydophila pneumoniae*)

1. Preparation and Storage of Specimens.

Nasopharynx swab is used for this test. Specimens are collected in an aseptic environment to prevent contamination. Since the levels of DNA is directly proportional to number of initial cells, be careful to manage the swab to sterile. For a stable condition, store the clinical specimen at instructed temperature. After collecting the specimens, it can be kept for 24 hours at 0~8℃ The specimens can be kept for about 7 days at 0~8℃, and keep frozen below -15~-25℃ for longer (within a month) storage. (If storing the samples for a long period or repeat freezing and thawing, the sensitivity of products may be decreased.)
All specimens are to be handled as if they are infectious.

2. DNA Extraction of Nasopharynx Swab.

*****Verified DNA isolation kit is recommended. A potential PCR obstruction on a clinical swab can be detected using 4X Reaction Mixture. Follow the instruction accordance of DNA isolation kit.

III. Flu & RSV

1. Preparation and Storage of Specimens.

Preparation and Storage of Specimens * All specimens are considered to be potent to transfer infectious agents.

1) Nasopharyngeal swab is used for this test.
2) Specimens are collected in an aseptic environment to prevent contamination.
3) Be careful to manage the swab to be sterile, the levels of RNA is directly proportional to the amount of initial cells.
4) To maintain stable condition, store the clinical specimen at instructed temperature.
5) Keep the collecting specimens for 24 hours at 2~8 ℃ without any pre-treatment (Keep refrigerated if the pre-treatment cannot be done within 1 hour).

2. RNA Extraction of Nasopharynx Swab.

* RNA extraction was performed using a commercially available RNA isolation kit (QIAGEN). User should verify their own RNA extraction reagent.