
Annex 4

NCP: Laboratory Testing Guideline

This technical guideline is formulated to guide disease control agencies and relevant institutions at all levels to carry out laboratory testing for NCP.

I. Specimen Collection

1. Target of collection

Suspected cases and clustered cases and others requiring diagnosis or differential diagnosis for novel coronavirus infection; or other environmental or biological substances that require further screening and testing.

2. Collection requirements

- 1) The novel coronavirus testing specimens shall be collected by qualified technicians who have received biosafety training (who have passed the training) and are equipped with the corresponding experimental skills. Personal protective equipment (PPE) requirements for sampling personnel are: N95 masks or masks with higher filtration efficiency, goggles, protective clothing, double-layer latex gloves and waterproof boot covers; the outer layer of the latex gloves shall be changed in a timely manner should sampling personnel touch patients' blood, body fluids, secretions etc.
- 2) Specimens of inpatient cases shall be collected by medical staff of the hospital where they are being treated.
- 3) Specimens of close contacts shall be collected by the designated local CDCs and medical institutions.
- 4) Multiple specimens may be collected in the course of the disease, depending on the need of laboratory testing.

3. Categories of specimens collected

Respiratory tract specimens in the acute phase (including upper or lower respiratory tract specimens) must be collected from each case; lower respiratory tract specimens shall be preferred for the collection from severe cases. Stool samples, whole blood samples and serum samples can be collected according to clinical needs.

Categories of specimen:

- 1) Upper respiratory tract specimens: including nasopharyngeal swabs, pharyngeal swabs etc.
- 2) Lower respiratory tract specimens: including deep-cough sputum, alveolar lavage fluids, bronchial lavage fluid and respiratory tract extracts.
- 3) Fecal specimens: Fecal samples are about 10 g (peanut size). If it is not convenient to collect fecal samples, an anal swab can be collected.
- 4) Blood specimens: One should, as much as possible, collect anticoagulated blood in the acute phase within 7 days after the onset of disease. 5 ml of blood is required for each collection. Vacuum tubes containing EDTA anticoagulant are recommended in blood collection.
- 5) Serum specimens: Both acute-phase and convalescent serum specimens should be collected as much as possible. The first serum specimen should be collected as soon as possible (preferably within

7 days after the onset of illness), and the second specimen should be collected during 3-4 weeks after the onset of illness. 5 ml of blood is required for each specimen and vacuum tubes without anticoagulant are recommended. Serum specimens are mainly used for measuring antibodies, rather than nucleic acid testing.

4. Methods of specimen collection and processing

1) Nasopharyngeal swab: The sampler gently holds the person's head with one hand, the swab in another, insert the swab via nostril to enter, slowly get deep along the bottom of the lower nasal canal. Because the nasal canal is curved, do not force too hard to avoid traumatic bleeding. When the tip of the swab reaches the posterior wall of the nasopharyngeal cavity, rotate gently once (pause for a moment in case of reflex cough), then slowly remove the swab and dip the swab tip into a tube containing 2-3ml virus preservation solution (or isotonic saline solution, tissue culture solution or phosphate buffer), discard the tail and tighten the cap.

2) Pharynx swab: the sampled person first gargles with normal saline, the sampler immerses the swabs in sterile saline (virus preservation solution is not allowed to avoid antibiotic allergies), holds the head of the sampled person up slightly, with one's mouth wide open, making a sound "ah" to expose the lateral pharyngeal tonsils, insert the swabs, stick across the tongue roots, and wipe both sides of the pharyngeal tonsils with pressure at least 3 times, then wipe on the upper and lower walls of the pharynx at for at least 3 times, and dip the swabs in a tube containing 2-3ml storage solution (or isotonic saline solution solution, tissue culture solution or phosphate buffer solution), , discard the tail and tighten the cap. The pharyngeal swabs can also be placed in the same tube together with the nasopharyngeal swab.

3) Nasopharyngeal or respiratory tract extract: Extract mucus from the nasopharynx or extract respiratory secretions from the trachea with a collector connected to a negative-pressure pump; insert the head of the collector into the nasal cavity or trachea, turn on the negative pressure, rotate and slowly withdraw the head of the collector, collect the extracted mucus, and rinse the collector once with 3 ml of sampling solution (a pediatric catheter connected to a 50-ml syringe may be used as an alternative to the collector).

4) Deep cough sputum: Ask the patient to cough deeply, and collect the sputum coughed up in a 50-ml screw-capped plastic tube containing 3 ml of sampling solution. If the sputum is not collected in the sampling solution, 2-3 ml of the sampling solution can be added into the tube before testing, or add sputum digestive reagents of equal volume of sputum.

Formula of storage fluid for sputum digestive reagents:

Ingredients	Per Bottle
Dithiothreitol	0.1g
Sodium chloride	0.78g
Phosphorus chloride	0.02g
Disodium hydrogen phosphate	0.112g
Potassium dihydrogen phosphate	0.02g
Water	7.5ml
pH 7.4 ± 0.2 (25° C)	

Dilute the storage solution to 100 ml with deionized water before use. Sputum can also be treated with a phosphate buffer containing 1 g/L of protease K in an equal volume of sputum.

5) Bronchial lavage fluid: Insert the head of the collector into the trachea (about 30cm deep) from the nostril or the tracheal insertion part, inject 5 ml of physiological saline, turn on the negative pressure, rotate the head of the collector and slowly withdraw it. Collect the extracted mucus and rinse the collector once with the sampling solution (a pediatric catheter connected to a 50-ml syringe may be used as an alternative to the collector).

6) Alveolar lavage fluid: After local anesthesia, insert a bronchoscope through the mouth or nose, pass through the pharynx into the branch of the right middle lobe or the lingular segment of the left lung, and insert the tip into the bronchial branch opening; slowly add sterilized physiological saline through the biopsy hole of the bronchoscope, with 30-50 ml of saline each time, 100-250 ml in total, 300 ml at most.

7) Fecal specimen: Take 1ml sample treatment solution, pick up a little sample about the size of a soybean and add it into the tube, gently blow for 3-5 times, set aside at room temperature for 10 minutes, centrifuge at 8,000rpm for 5 minutes, absorb the supernatant for detection.

Fecal specimen treatment solution can be prepared in-house by the laboratory: 1.211g tris, 8.5g sodium chloride, 1.1 g calcium chloride anhydrous or 1.47g calcium chloride containing crystalline water, dissolved into 800 ml deionized water, with the pH adjusted to 7.5 with concentrated hydrochloric acid and replenishing with deionized water to 1000 ml.

Stool suspensions can also be prepared using HANK'S solution or other isotonic saline solution, tissue culture solution or phosphate buffer solution. If the patient has diarrhea symptoms, collect 3-5 ml of stool specimen, gently blow and mix, centrifuge it at 8,000rpm for 5 minutes, absorb the supernatant to reserve for use.

8) Anal swab: Gently insert the disinfectant cotton swab into the anus for 3-5cm in depth, then gently rotate and pull out, immediately put the swab into a 15-ml screw-capped sampling tube containing 3-5ml virus preservation solution, discard the tail and tighten the tube cover.

9) Blood samples: it is recommended to use vacuum blood vessels containing EDTA anticoagulant to collect 5ml of blood samples. Nucleic acid extraction should be performed on whole blood or plasma according to the type of nucleic acid extraction reagent selected. For plasma separation, the whole blood should be centrifuged at 1,500 to 2,000 rpm for 10 minutes, and the supernatant will be collected in a sterile plastic tubes with screw cap.

10) Serum specimen: Collect a 5-ml blood specimen with a vacuum negative-pressure blood collection tube. Keep the specimen at room temperature for 30 minutes, centrifuge it at 1,500- 2,000 rpm for 10 minutes, and collect the serum in a sterile plastic tube with screw cap.

Other materials: To be collected in a standardized manner in accordance with design requirements.

5. Specimen packaging

Collected specimens shall be packaged separately in a biosafety cabinet of a BSL-2 laboratory.

1) All specimens should be placed in an airtight freeze-tolerant sample collection tube of appropriate size, with a screw cap and a gasket inside. The sample number, category, name and sampling date should be indicated on the outside of the container.

2) Specimens kept in an airtight container should be sealed in a plastic bag of appropriate size, with each bag containing one specimen. The specimen packaging requirements must meet the

corresponding standards of the *Technical Regulations for the Safe Transport of Dangerous Goods by Air*.

3) Prior to transportation, external specimens shall undergo the three-layer packaging applicable to Category A and Category B infectious substances based on the categories of the specimens.

6. Specimen preservation

Specimens for virus isolation and nucleic acid detection purposes should be tested as soon as possible. Specimens to be tested within 24 hours can be stored at 4 °C; those that cannot be tested within 24 hours should be stored at -70 °C or below (specimens may be temporarily stored in -20 °C refrigerators in the absence of -70 °C storage condition). Serum can be stored at 4 °C for 3 days and below -20 °C for a longer period. A special depot or cabinet is required to store specimens separately. Repeated freeze-thaw cycles during specimen transportation should be avoided.

7. Specimen submission and examination

Collected specimens should be sent to laboratories as soon as possible. Dry ice and other refrigeration methods are recommended for the preservation of specimens to be transported over long distances.

1) Submission of specimens

Specimens of cluster cases in each province (autonomous region, municipality directly under the central government) shall be submitted to the National Institute for Viral Disease Control and Prevention (NIVDC) under China CDC for testing and review, with the specimen submission form attached (see Appendix).

2) Pathogen and specimen transportation

2.1) Domestic transport

Novel coronavirus strains or other potentially infectious biological substances are subject to the packaging instructions for Category A substances assigned to UN2814, and the PI 602 of the *Technical Instructions For The Safe Transport of Dangerous Goods by Air (Doc 9284)* issued by ICAO; environmental samples, assigned to UN3373, shall be transported in Category B packaging in accordance with the PI 650, *Doc 9284*; one may refer to the aforementioned standards for specimens to be transported in other modes of transportation.

A *Permit of Transport* is required for the transportation of the novel coronavirus strains or other potentially infectious substances, according to the *Transport Regulations on the Highly Pathogenic Microorganism (Virus) Strains and Specimens that are Pathogenic to Humans (Order No. 45, former Ministry of Health)*.

2.2) International transport

Standard packaging shall be applied to novel coronavirus strains or samples to be transported internationally, with relevant procedures handled in accordance with the *Provisions on the Administration of the Health Quarantine of Entry/ Exit Special Articles* as well as relevant national and international requirements.

2.3) Management of strains and samples

Novel coronavirus strains and samples should be managed by designated personnel, with the source, category, quantity and registration number of the strains and samples recorded accurately. Effective

measures should be adopted to ensure the security of the strains and samples. Efforts should be made to prevent the misuse, malicious use, theft, robbery, loss, and leakage of the strains and samples.

II Laboratory testing of the novel coronavirus

The conventional testing method for novel coronavirus infection is real-time fluorescence-based RT-PCR assays. Any test of the novel coronavirus must be performed in a laboratory with appropriate conditions by personnel trained in relevant technical safety skills. The nucleic acid detection method introduced in this guideline mainly targets at open reading frame 1ab (ORF 1ab) and nucleocapsid protein (N) in the novel coronavirus genome.

To confirm a case as positive in the laboratory, one of the following criteria shall be met:

1. The real-time fluorescence-based RT-PCR assay of the novel coronavirus in the same specimen shows that the two targets, ORF1ab and Protein N, are both positive. In case of the result showing positive for one target, then samples shall be re-collected for another test. If it is still positive for a single target, it is determined to be positive.
2. The real-time fluorescence-based RT-PCR assay of two types of specimens show one single target positive at the same time, or one target positive in two samples of the same type, it could be determined as positive.

Negative nucleic acid results cannot rule out novel coronavirus infections. Factors leading to false negatives shall be precluded, including: poor qualities of samples, for instance the respiratory tract samples in the oropharynx and other parts; samples collected too early or too late; samples that are improperly stored, transported or processed; technical reasons such as virus mutations, PCR inhibition, etc.

III. Real-Time fluorescence-based RT-PCR assay of the novel coronavirus nucleic acid

1. Purpose

To standardize the procedure of testing the novel coronavirus nucleic acid with the real-time fluorescence-based RT-PCR assay, ensuring correct and reliable test results.

2. Scope

Applicable to the real-time fluorescent RT-PCR assay of the novel coronavirus nucleic acid.

3. Responsibilities

Testing personnel: testing the samples in accordance with this guideline.

Reviewing personnel: reviewing whether the test operations are standardized and whether test results are accurate or not.

Department head: comprehensively managing the department and reviewing test reports.

4. Sample reception and preparation

Reviewing the name, sex, age, number, and test items of the sample to be tested; indicate any abnormalities of the testing samples; testing samples shall be stored in a -70 °C refrigerator.

5. Test items

- (1) Novel coronavirus nucleic acid assay (real-time fluorescence-based RT-PCR assay)

Primers and probes targeting the ORF1ab and N gene regions of the novel coronavirus are recommended.

Target 1 (ORF1ab):

Forward primer (F): CCCTGTGGGTTTTACTTAA

Reverse primer (R): ACGATTGTGCATCAGCTGA

Fluorescent probe (P): 5'-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3'

Target 2 (N):

Forward primer (F): GGGGAAGTTCTCTGCTAGAAT

Reverse primer (R): CAGACATTTTGCTCTCAAGCTG

Fluorescent probe (P): 5'-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3'

For nucleic acid extraction and real-time fluorescence-based RT-PCR reaction system and reaction conditions, refer to kit instructions of the manufacturers concerned.

(2) Judgment of results

Negative: no Ct value or Ct value \geq 40.

Positive: Ct value $<$ 37.

Gray zone: Repeated experiments are recommended should Ct value range between 37 and 40. If the Ct value reads $<$ 40 and the amplification curve has obvious peaks, the sample should be considered being tested positive, otherwise it should be considered as negative.

Note: If a commercial kit is used, the instructions provided by the manufacturer shall prevail.

IV. Biosafety requirements for pathogen experiments

According to the biological features, epidemiological characteristics, clinical data and other available information concerning the novel coronavirus, the pathogen shall be temporally managed as Category B pathogens and microorganisms based on its hazards. Specific requirements are listed as follows:

1. Viral culture

Viral culture refers to operations such as virus isolation, culture, titration, neutralization test, purification of live virus and its protein, lyophilization of virus, and recombination test to produce live virus. The above operations should be performed in a biosafety cabinet of a BSL-3 laboratory. When viral medium is used to extract nucleic acid, the addition of lysing agent or inactivating agent must be performed under the same level of laboratory and protective conditions as viral culture. Laboratories shall report to the National Health Commission for approval and obtain relevant qualifications before carrying out the corresponding activities.

2. Animal infection experiment

Animal infection experiment refers to operations such as infecting animals with live viruses, sampling of infected animals, processing and testing of infectious samples, special test for infected animals, disposal of infected animal excrement, etc., which should be performed in a biosafety cabinet of a BSL-3 laboratory. Laboratories shall report to the National Health Commission for approval and obtain relevant qualifications before carrying out the corresponding activities.

3. Operation of uncultured infectious substances

The operation of uncultured infectious substances refers to viral antigen detection, serological testing, nucleic acid extraction, biochemical analysis, inactivation of clinical samples and other operations performed on uncultured infectious substances before inactivation through a reliable method. The operation should be performed in a BSL-2 laboratory, with personal protective equipment subject to BSL-3 laboratory protection requirements.

4. Operation of inactivated substances

After reliable inactivation of infectious substances or live viruses, operations such as nucleic acid testing, antigen testing, serological testing and biochemical analysis should be performed in a BSL-2 laboratory. Molecular cloning and other operations not involving live pathogenic viruses may be carried out in a BSL-1 laboratory.

Translation organized by WHO China Office

Annex

**Novel coronavirus testing
Specimen Submission Form**

Specimen submission unit (seal): _____ Submission date: _____ year _____ month _____ day Submitted by: _____

No.	Specimen type	Name	Sex	Age	Date of onset	Date of clinical visit	Sampling date	From a clustered outbreak or not	Testing date	Real-time fluorescent RT-PCR		Gene sequence homology*		Note
										Reagent manufacturer	Target gene	First generation	Deep sequencing	

Gene sequence homology * is not a required option; it Indicates completion of the specific target gene sequence / whole genome sequence and its homology with the novel coronavirus. For the column of “from a clustered break or not”, fill in “yes” or “no”.