

Standard Operating Procedure: Collection and Processing of Biological Samples for the Identification of *Pneumocystis jirovecii*

This SOP standardizes how participating centers collect, label, store and ship biological samples so that *Pneumocystis jirovecii* results are comparable across the registry of COST Action CA23142 (Delve-into-Pneumocystis).

1. Purpose and scope

The purpose of this procedure is to obtain respiratory and serum samples of homogeneous quality from every registry subject. These samples support the detection, quantification and genotyping of *P. jirovecii* in multicenter studies.

The SOP covers three study populations:

- **Chronic pulmonary disease:** subjects with chronic lung disease (for example COPD or asthma) assessed for *Pneumocystis* colonization.
- ***Pneumocystis* pneumonia (PcP):** subjects with suspected or confirmed PcP.
- **General population:** subjects without known pulmonary disease, used as a reference group.

It describes subject enrolment, the clinical parameters recorded in the online registry, sample coding, collection, primary processing, aliquoting and storage. Downstream laboratory assays are defined in the harmonized protocols of Working Group 2 and are referred to here only where they determine how a sample must be handled.

2. Subject enrolment, ethics and biosafety

A subject is considered enrolled once he or she has signed the informed consent. No study procedure, including sampling, may take place before that point.

1. A physician explains the study to each subject individually.
2. Legally effective written informed consent is obtained from each subject before any study procedure.
3. The investigator records the subject's clinical data, including medical history and the compulsory and optional parameters below, in the online registry.
4. Each center keeps the link between the case number and the subject's identity in its own confidential file; only the pseudonymized case number is entered in the registry and written on samples.

Each center is responsible for local ethics committee approval and for compliance with the General Data Protection Regulation (GDPR). All samples are handled as potentially infectious, following universally recommended biosafety precautions, and all procedures are carried out under sterile conditions.

Clinical parameters recorded in the registry

Parameter	Status	Specification
Chest radiograph	Compulsory	Need not be repeated if performed within the previous 4 weeks and the subject has no exacerbation of pulmonary disease at assessment
Haematology	Compulsory	White cells, neutrophil and lymphocyte counts
Biochemistry	Compulsory	Serum creatinine only
Arterial blood gases	Optional	Arterial blood gas tensions
CD4+ and CD8+ cell counts	Optional	Fluorescence-activated cell sorting (FACS)
HIV serology	Optional	Seropositivity requires a positive screening test and a positive confirmatory test (Western blot, or a combination of competitive and non-competitive ELISA)
Spirometry	Optional	FEV1 (% predicted), FEV1/VC (% predicted) and FEV1/FVC (%)
Sputum staining and culture	Optional	Routine microbiology of sputum

3. Samples by study population

Every subject provides a serum sample; the respiratory sample depends on the study population (Figure 1).

Every subject gives serum; the respiratory sample depends on the group

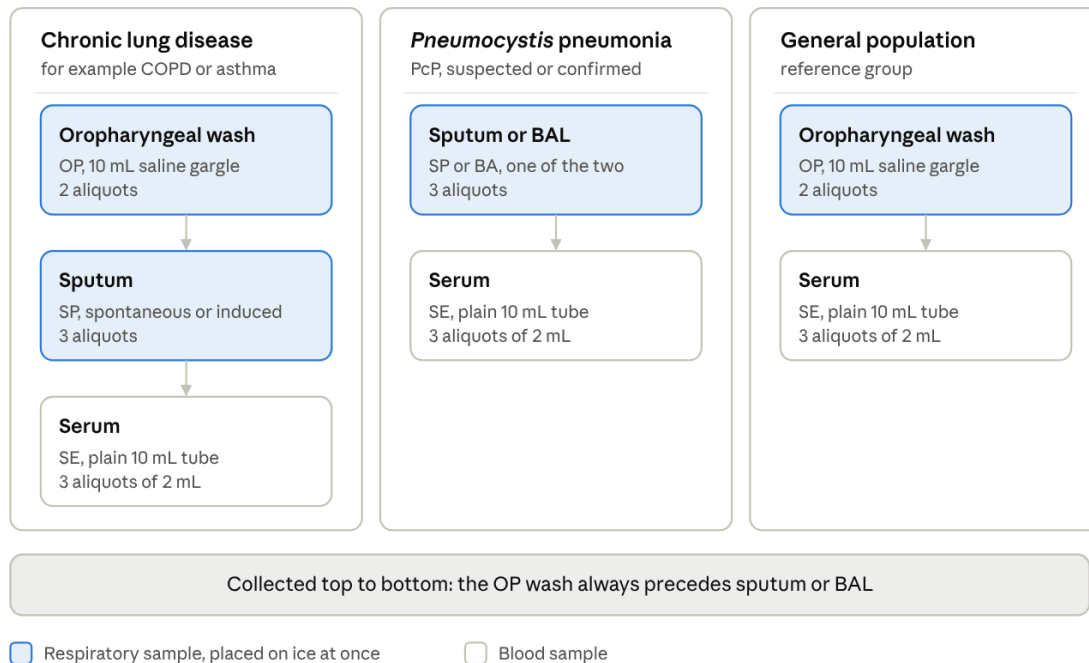


Figure 1 · samples collected per study population, in collection order

Subjects with chronic pulmonary disease give an oropharyngeal wash, a sputum sample and serum. PcP subjects give sputum or BAL, plus serum. The general population gives oropharyngeal wash and serum.

4. Collection procedures

When several respiratory samples are taken from one subject, the oropharyngeal wash is always collected first, before sputum or bronchoalveolar lavage. Respiratory samples are placed on ice immediately after collection and kept on ice until they reach the laboratory.

4.1 Oropharyngeal wash (OP)

1. Ask the subject to rinse the mouth.
2. Give 10 mL of sterile normal saline (0.9% NaCl) and ask the subject to gargle for 30 to 60 seconds.
3. Collect the full volume in a sterile container.
4. Place the container on ice immediately, if possible, and send it to the laboratory.

4.2 Spontaneous or induced sputum (SP)

1. Collect the sputum immediately after the OP sample, in a sterile container.
2. Use induced sputum when the subject cannot expectorate spontaneously, following the center's standard induction procedure.
3. Place the sample on ice immediately and send it to the laboratory.

4.3 Bronchoalveolar lavage (BAL, code BA)

1. Perform BAL only when clinically indicated, in subjects with suspected PcP.
2. Follow the technical recommendations and guidelines for BAL of the European Society of Pneumology Task Group (Klech and Pohl, 1989; reference 1).
3. Send the BAL fluid to the laboratory without delay; it is processed as soon as possible after collection (section 6.3).

4.4 Serum (SE)

1. Draw venous blood into a plain (no additive) 10 mL tube.
2. Allow the blood to clot and send the tube to the laboratory for separation (section 6.4).

5. Sample coding, labelling and storage

Every tube carries a single code, **Case number / Sample**, written clearly and legibly on the tube itself. For example, the first sputum aliquot from the fourth subject enrolled in Seville is coded **SEV/04/SP1**.

- **Case number:** the center abbreviation allocated by the consortium, followed by a correlative number allocated by the center (one per subject). The fourth subject in Seville is SEV/04.
- **Sample:** the sample-type code, followed by a correlative number (one per aliquot).

One code per tube: centre, subject, sample type and aliquot

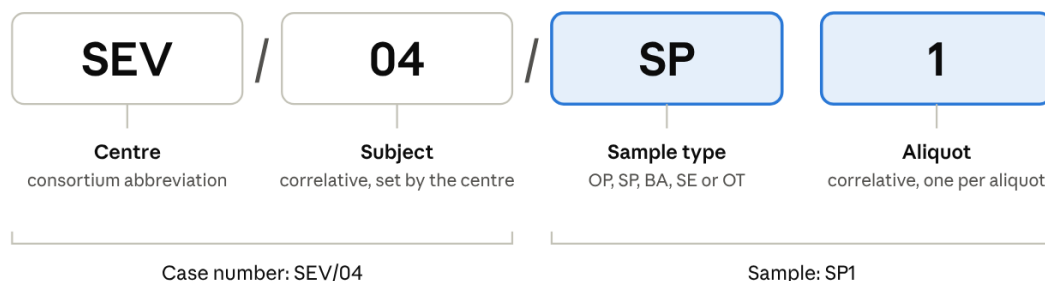


Figure 3 · Example of structure of the sample code (example SEV/04/SP1)

The case number identifies the subject; the sample part identifies the tube.

Centre	Abbreviation
Seville	SEV
Wroclaw	WRO
Brest	BRE
Lisbon	LIS
Guadalajara	GUA

Centers joining the registry later receive their abbreviation from the WG1 coordinating team before their first enrolment.

Sample type	Code
Oropharyngeal wash	OP
Sputum or induced sputum	SP
Bronchoalveolar lavage	BA
Serum	SE
Other	OT

Storage

- Aliquots for molecular techniques and internal control are stored in screw-capped 2 mL cryotubes at -20°C , as soon as possible after sampling.
- Serum is never heat-inactivated.
- Each center logs the date and time of collection and of freezing for every aliquot in the registry.

6. Laboratory processing and aliquoting

Each sample is mixed and divided into aliquots on arrival. Each aliquot has a fixed purpose: staining, DNA extraction for detection and typing, or internal control. Aliquots for staining are processed fresh; all other aliquots are frozen at -20°C .

6.1 Oropharyngeal wash: 2 aliquots

1. Mix the OP sample and divide it into two aliquots of 5 mL each (OP1, OP2).
2. Centrifuge both aliquots at 2,900 g for 10 min.
3. Discard the supernatant and keep the pellet (about 0.5 mL).
4. Transfer each pellet into a screw-capped 2 mL cryotube and freeze it at -20°C .

Aliquot	Use
OP1	DNA extraction (detection and typing)
OP2	Internal control

6.2 Sputum or induced sputum: 3 aliquots

1. Mix the sample and divide it into three aliquots (SP1, SP2, SP3).
2. Process SP1 for staining methods.
3. Freeze SP2 and SP3 in screw-capped 2 mL cryotubes at -20°C as soon as possible.

6.3 Bronchoalveolar lavage: 3 aliquots

1. Centrifuge the lavage at about 900 g for routine cytological and parasitological examination.
2. Re-centrifuge the remaining fluid at about 1,000 g as soon as possible, preferably immediately (about 3,000 rpm, depending on the rotor).
3. Divide the resulting material into three aliquots (BA1, BA2, BA3).
4. Process BA1 for staining methods.
5. Freeze BA2 and BA3 in 2 mL cryotubes at –20 °C as soon as possible.

Aliquot (SP or BA)	Use
1	Staining methods
2	DNA extraction (detection and typing)
3	Internal control

6.4 Serum: 3 aliquots of 2 mL

1. Centrifuge the clotted blood to separate the serum.
2. Divide the serum into three screw-capped 2 mL cryotubes (SE1, SE2, SE3).
3. Freeze them at –20 °C. Do not heat-inactivate.

Figure 2 summarizes the processing of each sample type.

Every sample ends as coded aliquots, frozen except those for staining

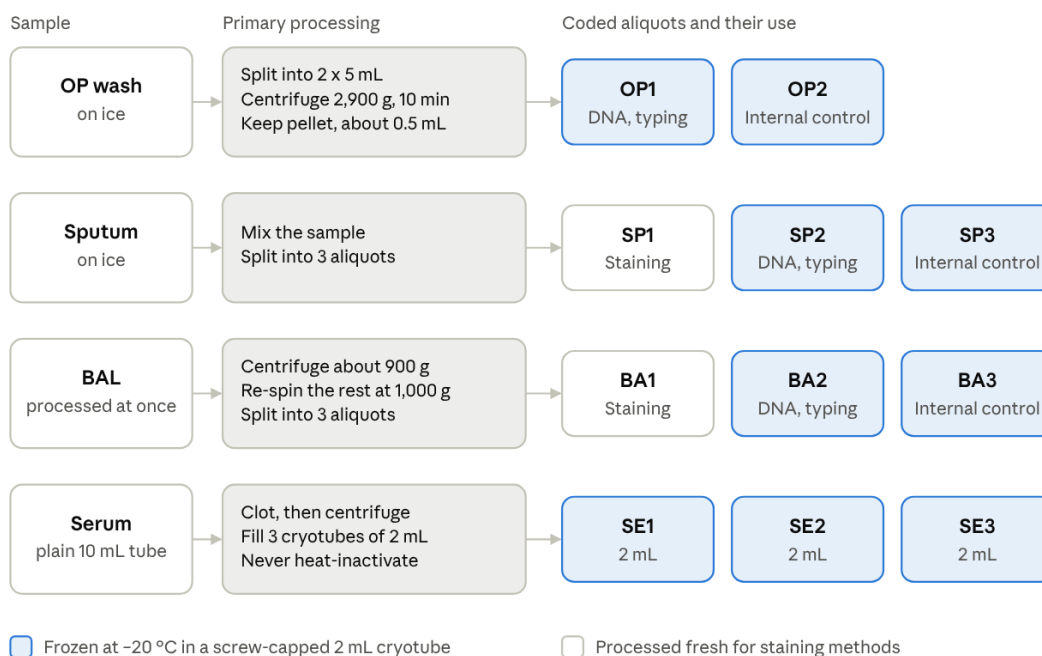


Figure 2 · primary processing and aliquoting by sample type

Only SP1 and BA1 are processed fresh for staining; every other aliquot is frozen at -20°C as soon as possible.

7. Quality control and data recording

The internal-control aliquot of each sample (OP2, SP3, BA3, and one serum aliquot) is kept untouched at -20°C . It allows any result to be confirmed, or re-tested centrally, without a new sampling.

- **Traceability:** for every aliquot, the registry records the sample code, sample type, date and time of collection, date and time of freezing, and storage location (freezer, rack, box, position).
- **Cold chain:** respiratory samples stay on ice from collection to processing. Any break in the cold chain, delay or freeze-thaw cycle is recorded as a deviation.
- **Sample order:** if the OP wash could not be collected before sputum or BAL, this is recorded, as it may affect the comparability of OP results.
- **Shipment to reference laboratories:** frozen aliquots are shipped on dry ice, with a sample manifest listing every code, under the material transfer arrangements agreed within the Action.
- **Deviations:** any departure from this SOP is recorded in the registry with its reason; the sample is not discarded unless WG2 advises it.

8. References and related documents

1. Klech H, Pohl W. [Technical recommendations and guidelines for bronchoalveolar lavage \(BAL\). Report of the European Society of Pneumology Task Group.](#) *Eur Respir J.* 1989;2(6):561–85. PMID 2663535 (via PubMed).
2. COST Action CA23142 Memorandum of Understanding, Delve-into-Pneumocystis.
3. WG2 harmonized laboratory protocols for *P. jirovecii* detection and typing (in preparation).