

## Standardized Protocols for Oral Biospecimen Collection and Storage: Experience from the Korea Oral Biobank Network

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### ABSTRACT

The Korea Oral Biobank Network (KOBN) was established in 2021 as a branch of the Korea Biobank Network under the Korea Centers for Disease Control and Prevention to provide infrastructure for the collection, management, storage, and utilization of human bioresources from the oral cavity and associated clinical data for basic research and clinical studies. To address the need for the unification of the biobanking process, the KOBN organized the concept review for all the processes. The KOBN established standard operating procedures for the collection, processing, and storage of oral samples. The importance of collecting high-quality bioresources to generate accurate and reproducible research results has always been emphasized. A standardized procedure is a basic prerequisite for implementing comprehensive quality management of biological resources and accurate data production.

**Keywords:** Collection, Korea biobank network, Korea centers for disease control and prevention, Korea oral biobank network, Oral cavity

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### Introduction

The Human Genome Project has opened the era of high-throughput genomics and shifted the paradigm to a holistic understanding of the genome of our planet. The Human Microbiome Project (HMP), the next largest step toward understanding microorganisms living with humans, has been facilitated by rapid advances in sequencing technologies [1]. Indeed, next-generation sequencing analyses have yielded a variety of omics data, affecting almost all aspects of biological research, including, but not limited to, biobanking [2, 3].

A biobank is defined as a well-organized storage system for human biological resources that includes related information, such as clinical data of body measurements, diseases, medications, nutrition, and genetic data from small-scale (e.g., genetics) to large-scale (e.g., genomics). The exponential increase of multi-omics big data necessitates the establishment of a dedicated repository for digital resources.

The oral cavity is the second-largest microbial habitat in humans after the intestine and constitutes a special environment consisting of hard tissue (the teeth and alveolar bone) and soft tissue (the gingiva and mucosa) [4]. Oral diseases such as dental caries, periodontitis, and mucosal diseases resemble other systemic diseases, but they are distinct entities independent of each other. The role of the oral microbiome in oral diseases and human health and disease is gaining traction as a result of the HMP, where the largest number of samples were collected from

the oral cavity for microbial species identification. However, in contrast to the well-established preservation strategies for blood, tissues, cells, and nucleic acids for medical research, the preservation of oral samples, such as teeth, mouthwash, saliva, dental plaques, gingival crevicular fluid (GCF), and gingival tissue, linked with radiological information and microbial genomic data, are fledgling fields of biopreservation.

With a dramatic increase in research interest in oral diseases and their relationship with systemic diseases, the importance of oral biobanks has become more prominent. As such, the Korea Oral Biobank Network (KOBN) aims to establish the standardization of biopreservation protocols of oral samples between oral biobank units participating in the National Biobank of Korea.

## Materials and Methods

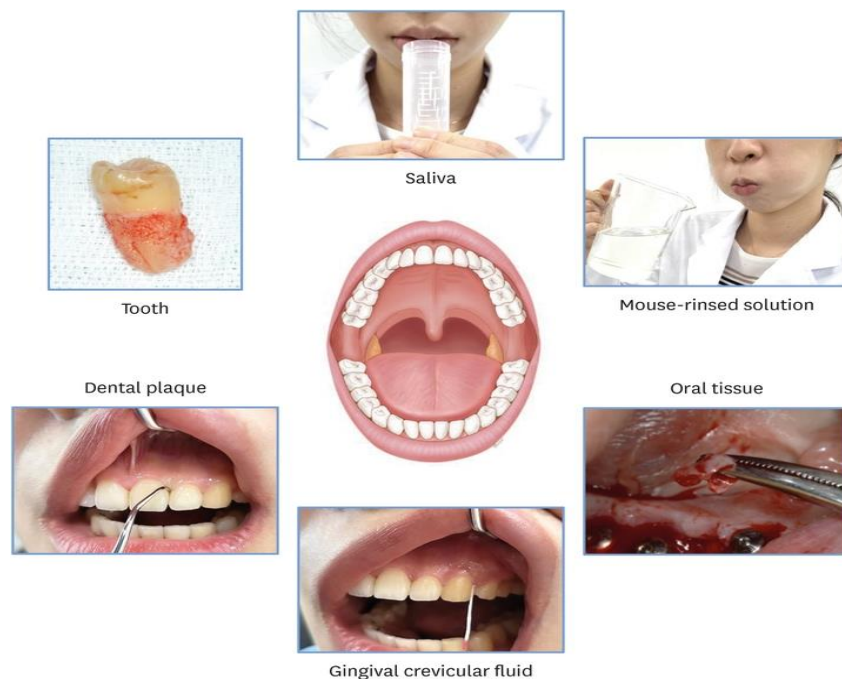
### *Consortium information*

In 2007, The Ministry of Health and Welfare and the Korea Centers for Disease Control and Prevention (KCDC) established the “Comprehensive Informatization and Management System for Health and Medical Biological Resources” to secure and utilize human resources at the national level. The Korea Biobank Network (KBN) includes biobank units located in university hospitals nationwide and was formed centering on the National Biobank of Korea of the KCDC. The National Biobank of Korea (NBK) is the fundamental infrastructure of the Korea Biobank Project (KBP), supporting collaborative subnetworks for specific disease-targeted future biobanking (n=10) and the biobank innovation consortia for biomedical and healthcare research (n=2). The NBK also supports the integration of the biobanking service platforms of the KBN. The first phase of the KBP started in 2008 and was completed in 2012. The fourth phase of the KBP started in 2021 when the KOBN was newly established as a unit participating in the KBP. The KOBN unit comprises Seoul National University Dental Hospital as the center of the unit, and Yonsei University Dental Hospital and Apple Tree Dental Hospital as cooperative banks of the unit.

## Results and Discussion

### *Collection, processing, and storage of orally derived bioresources*

Representative oral bioresources include teeth, saliva, mouth-rinse solution, dental plaque, GCF, and oral tissue (Figure 1, Table 1). All samples except oral tissue are transferred to the biobank within 4 hours of collection. Before being transferred to the laboratory, samples could be stored temporarily at 0°C–4°C in the clinic.



**Figure 1.** Representative oral-derived bioresources. Representative oral bioresources include teeth, saliva, mouth-rinse solution, dental plaque, gingival crevicular fluid, and oral tissue.

**Table 1.** Oral-derived bioresources; collection, processing, and storage

Specimen	Type of preservative	Short-term storage	Centrifuge	Processing time	Aliquot	Long-term storage	Sample specific data elements
Tooth	· 4% Chloramine T solution for hard tissue research	0–4°C	-	<4 hr	N/A	4°C or –80°C	-
	· Fresh frozen for research on cells, DNA/RNA, bacteria						
Saliva	· 80% glycerol solution	0–4°C	-	<4 hr	1.0 mL	–80°C	Date and time of participant last brushed teeth and refrained from oral hygiene, last alcohol, and smoking
Mouth-rinse solution	· 80% glycerol solution	0–4°C	4,000 rpm at 4°C for 30 min	<4 hr	1.0 mL	–80°C	-
Dental plaque	· PBS buffer	0–4°C	-	<4 hr	1.0 mL	–80°C	Sample amount
Gingival crevicular fluid	· Absorbed paper point itself	0–4°C	-	<4 hr	-	–80°C	-
Oral tissue	· Fixative or paraffin	0–4°C	-	<1 hr (not exceeding 3 hours)	50 mg	–80°C or liquid nitrogen	-

PBS: phosphate-buffered saline.

### Teeth

Teeth consist of hard and soft tissues. The hard tissues include enamel, dentin, and cementum, and the soft tissues include pulp, gingiva, and periodontal ligaments [5]. Teeth are used in research on dental materials, and stem cells derived from teeth are reportedly likely to differentiate into other tissues, which can be used in various studies such as tooth regeneration research [6].

- *Preparation materials*

The preparation materials comprise saline, 4% chloramine T solution, and a cryovial.

- *Collection and processing*

- After extraction, a tooth is stored in saline at 0°C–4°C at the clinic.
- After transfer to the laboratory, the tooth surface is washed with saline for at least 1 minute. During this procedure, care should be taken not to apply physical force to the tooth surface.

- *Storage*

- Teeth collected for hard tissue research are generally transferred to a dedicated vial containing 4% chloramine T solution, and stored at 0°C–4°C.
- Teeth collected for research on cells, DNA/RNA, or bacteria, among other purposes, are generally transferred to a dedicated vial and stored frozen at –80°C (ultra-low temperature).

### Saliva

Human saliva is a complex physiological fluid produced and released mostly by the paired parotid, submandibular, and sublingual salivary glands in the mouth [7]. Salivary secretion can be artificially stimulated by asking a subject to chew gum or paraffin, which may change the concentrations and compositions of saliva. Saliva consists of 98% water, electrolytes, mucus, metabolites, GCF, enzymes, antimicrobial agents, white blood cells, epithelial cells,

bacteria, and other microbial substances [8]. The composition, production flow rate, release site, and clearance of saliva differ between individuals, and saliva thus provides unique biological information about the host [8]. For microbiome research, approximately 0.25 mL of saliva can yield enough DNA for microbial profiling, which is reportedly biased toward tongue and palate communities [9]. Notably, the HMP has revealed that the microbiome profile of saliva in normal adult subjects has the highest median alpha diversities and the lowest beta diversities of operational taxonomic units [10]. Because of its high molecular and biological information content, high accessibility, non-invasive sampling, and ease of sampling methods, saliva has been proposed as a future diagnostic fluid that may reflect oral and systemic health status [11]. Different collection modalities can evidently yield inconsistent salivary biomarker metrics; however, including those pertaining to cortisol, amylase, C-reactive protein, and immunoglobulin A necessitates the standardization of sample collection, handling, and storage procedures.

- *Preparation materials*

The preparation materials comprise one 50-mL sterile conical polypropylene tube per sample (Falcon or a comparable brand), paraffin wax for stimulated saliva collection, 1.8-mL cryovial with external thread screw cap, ice (or refrigerator), and a timer.

- *Collection*

- *Passive drooling method*

- a. Collect saliva in the mouth for at least 1 minute.
    - b. Drool the saliva into the labeled 50-mL collection tube.
    - c. Repeat the process until 2–5 mL of saliva has been collected. This may take 5 to 10 minutes.
    - d. Record the time and duration of sample collection.

- *Stimulated method*

- a. Place 1.0–1.5 g of paraffin wax in the subject's mouth.
    - b. Instruct the subject to swallow any saliva in their mouth before chewing the wax.
    - c. Instruct the subject to chew the wax at a regular rate for 2 minutes until a sufficient amount of saliva is accumulated.
    - d. Drool the accumulated saliva into the labeled 50-mL collection tube.
    - e. Repeat the process until 2–5 mL of saliva has been collected. This may take 5 to 10 minutes.
    - f. Record the time and duration of sample collection.

- *Processing and storage*

- Transfer 1.0 mL of the sample into a 1.8-mL cryovial and barcode-label the vial. Record the amount of the last portion of the sample on the vial.
  - Add 40% (v/v) of 80% glycerol solution (0.4 mL for 1.0 mL sample) and mix thoroughly.
  - Briefly spin down the sample and store it at  $-80^{\circ}\text{C}$ .

### *Mouth-rinse solution*

The liquid obtained by mouth-washing with sterile water or saline solution is used as an alternative to saliva, especially in studies on oral metagenomics [12] and metabolomics [13]. The collection of mouth rinse solution is simpler than saliva collection and is more suitable for investigations involving a large number of subjects [12]. Notably, however, mouth rinse solution usually reflects all components of the oral cavity rather than the local microenvironment. Thus, such sampling should only be chosen if it is appropriate for the intended research purposes.

- *Preparation materials*

The preparation materials comprise one 50-mL sterile conical polypropylene tube per sample (Falcon or a comparable brand) filled with 12 mL saline solution or solutions containing cetylpyridium chloride, ethylene diamine tetraacetate, or other materials for specific purposes, a 15-mL sterile conical polypropylene tube (Falcon or a comparable brand), a 1.8-mL cryovial with external thread screw cap, swing bucket centrifuge, ice (or refrigerator), phosphate-buffered saline (PBS), and a timer.

- *Collection*

- Put 12 mL of saline solution in the participant's mouth.
  - Instruct the participant to swish 20 times in 10 seconds while maintaining a dental interlocking state, and not to gargle or tilt their head back during the process.

- Collect the mouth-rinse into the labeled 50-mL collection tube.
- *Processing and storage*
  - Centrifuge the sample using a swing bucket rotor (4,000 rpm for 30 minutes at 4°C).
  - Transfer the supernatant into a new 15-mL polypropylene tube and label it appropriately.
  - Dissolve the precipitate using 3.0 mL of PBS, transfer 1.0 mL of the sample into a 1.8-mL cryovial, and barcode-label the vial.
  - Add 40% (v/v) of 80% glycerol solution (0.4 mL for a 1.0-mL sample) and mix thoroughly.
  - Briefly spin down the sample and store it at –80°C.

#### *Dental plaque*

Dental plaque is a unique and dynamic biofilm composed of heterogeneous and poly-oral microorganisms that attach to teeth and surrounding tissues [14]. The collective of microorganisms colonizing the oral cavity is called the oral microbiome [15]. If dental plaque is not properly removed, oral diseases such as dental caries and periodontitis can be induced. Dental plaque is one of the crucial orally derived samples for research investigating relationships between oral bacteria and oral disease. The composition of dental plaque varies among individuals and can also reflect oral conditions [16]. In addition, multi-omics analyses such as genomics, metagenomics, and proteomics have characterized different stages of dental plaque formation and revealed interactions between hosts and pathogens [17].

- *Preparation materials*

The preparation materials comprise a micro Mini-Five Gracey curette, a 50-mL conical tube containing 5 mL of PBS, a cryovial, and cotton rolls.

- *Collection*

- Select the sample collection targets with a sufficient amount of dental plaque around the teeth or dental implants.
- Isolate the target area from the saliva and mucous membranes using a cotton roll, and dry it with soft air flow using a 3-way syringe.
- Scrape as much dental plaque as possible from the mesial surface of the target tooth or implant with a Gracey curette, and soak the plaque in 5 mL of PBS. If the amount of dental plaque is insufficient, additional plaque can be collected from the distal surface.
- The process is repeated for each target tooth.

- *Processing and storage*

- Dispense 1 mL of sample into each cryovial, and label each cryovial.
- Briefly spin down the samples and store them at –80°C.

#### *GCF*

GCF is an inflammatory exudate derived from periodontal tissue and found in the periodontal pocket between the teeth and gingiva [18]. It consists of locally produced substances such as inflammatory cells, serum, tissue destruction products, inflammatory mediators, and antibodies against oral bacteria [19]. In subjects with a healthy oral condition, the amount of GCF is small, but in the presence of inflammation, GCF flow increases and its composition begins to resemble that of inflammatory exudate. For this reason, GCF is widely used in the study of diagnosis, treatment, and prevention of periodontal diseases.

- *Preparation materials*

The preparation materials comprise a paper point (#20), a cryovial, and cotton rolls. The paper point can be changed to Periopaper at the request of the researcher.

- *Collection*

- After isolating the teeth, dry them with soft air-flow using a 3-way syringe.
- Insert 4 sterile paper points into the gingival pocket for 20 seconds and put them into a cryovial.

- *Processing and storage*

- Dispense 1 mL of sample into each cryovial, and label each cryovial.
- Briefly spin down the sample and store it at –80°C.

#### *Oral tissue*

Normal tissue or tissue with disease may be collected for banking purposes. Common sources of oral soft tissue include those harvested during tooth extraction procedures, oral cysts, tumors, and incised mucosal tissue. These may be collected from the maxilla, mandible, buccal mucosa, soft and hard palate, mouth floor, tongue, labial mucosa, salivary glands, oropharynx, maxillary sinus, and the temporomandibular joint. Because the soft tissue of the maxillofacial region is closely associated with the jaw and teeth, hard tissue including bone, cartilage, and tooth material are also potential specimens [20, 21]. Soft tissue sampled from or nearby the jaw may contain calcified particles.

It is challenging for a biobank to collect and preserve specimens in a way that satisfies the diverse preferences of researchers, each with different plans for analysis even for the same disease entities [22, 23]. These discrepancies may be reconciled through discreet and predetermined banking plans discussed with the sampling clinician and potential researcher. Because this is not always possible, tissues are usually collected and preserved to maintain appropriate levels of DNA, RNA, or protein even after long-term storage [24].

- *Preparation materials*

The preparation materials comprise ice (or a refrigerator), PBS, surgical blades, a pair of tweezers, surgical scissors, and cryovials.

- *Collection*

- All tissue samples must be treated as possible infection sources; thus, safety equipment and caution are required throughout the process.
- Tissue samples should be acquired from the sites that best represent their pathological or normal status.
- Avoid sampling from areas of necrosis, hemorrhage, ulceration, irrelevant inflammation, or sites that should be preserved for a pathologic diagnosis.
- It is recommended that where normal tissue is adjacent to diseased tissue, each should be sampled at least 1.0 cm from the gross disease border.
- In tumor specimens, paired sampling of the tumor and adjacent normal tissue is recommended if possible.
- Information on sampling conditions such as the clinical disease status, collection site, sample size and amount, and warm and cold ischemia time should be recorded.

- *Processing and storage*

Tissues can be either cryopreserved or preserved using fixatives and then embedded in paraffin. Cryopreservation can be done in  $-80^{\circ}\text{C}$  deep freezers or liquid nitrogen [25]. Tissues that are to be stored at  $-80^{\circ}\text{C}$  may be snap-frozen in liquid nitrogen before long-term storage. Protein degradation and enzymatic activity are suppressed under low temperatures. Proteome stability is known to remain intact for years under storage in deep freezers or liquid nitrogen [26]. Tissue DNA can be successfully preserved at temperatures of  $-80^{\circ}\text{C}$  or below for several years [27]. RNA is known to be relatively unstable compared to DNA during tissue handling, cold ischemia periods, and preservation, although there are conflicting opinions on the subject [28-30]. In tissue stored at  $-80^{\circ}\text{C}$  RNA degradation has been reported at 5 years [27, 31, 32], but in another study, tissue RNA remained intact after long-term storage for up to 12 years [33]. Liquid nitrogen can maintain tissue RNA stability for up to 11 years [34].

Formalin-fixed paraffin-embedded (FFPE) sections have traditionally been used to identify the histologic features of tissues and can be used for sequencing and spatial analyses of protein (via immunohistochemistry) and mRNA/miRNA (via *in situ* hybridization). Acquiring high-quality RNA directly from FFPE tissue is challenging due to nucleic acid degradation [35-37]. FFPE sections are inadequate for protein extraction and proteomics analysis due to chemical cross-linking and protein modification in the tissue during formalin fixation [25, 38-40]. Tissues are usually fixed in 10% formalin before paraffin embedding, but several alternative fixatives are commercially available [25]. The fixation time depends on the size of the tissue sample. Formalin permeates tissue at an approximate rate of 1 mm/hour. For sufficient fixation, the amount of fixative used should be 20 times that of the tissue [25]. Paraffin blocks should be stored in a cool place that is never exposed to direct sunlight.

- Tissues must be transferred from the operating room to the biobank as quickly as possible. The cold ischemia time must be kept to a minimum; within an hour is recommended, and it should not exceed 3 hours.
- If the tissue processing step is delayed, the sample should be temporarily stored at  $0^{\circ}\text{C}$ – $4^{\circ}\text{C}$  in PBS, or under similar conditions.
- Tissue should be processed in a biologic safety cabinet to protect staff from infectious agents.

- Tissues are cut into samples weighing approximately 50 mg and placed into cryovials.
- The tissues should be cryopreserved immediately at  $-80^{\circ}\text{C}$  or in liquid nitrogen. Isopropanol-filled containers may be required to achieve slow freezing at a rate of  $-1^{\circ}\text{C}/\text{min}$ .
- All cryovials must be properly labeled before storage.

*Quality control of long-term storage bioresources*

- *Basic principles of storage and inspection*

To keep the quality of collected human bioresources as constant as possible, the KOBN periodically checks the quality of the stored bioresources and related information, and the functioning of the bioresource storage facilities, equipment, and systems. Quality control is performed on stored bioresources twice a year. Samples are selected randomly, and if the number of bioresources stored during the period is low, it is conducted when 3% of the storage resources exceed 1 person. To improve the reliability of long-term stored bioresources, whether information such as storage location and input or output amounts is consistent between the bioresource, and information system is periodically checked. Regular quality assessment tests are conducted to evaluate whether denaturation or microbial contamination of the contents of stored bioresources has occurred. If damage or contamination of a stored bioresource is detected, it is discarded.

- *Quality control method*

All samples are checked by a visual inspection. A bacterial smear test is performed in the tooth storage solution, and DNA purity and concentration tests are conducted on saliva, mouth-rinse solution, dental plaque, GCF, and soft tissue samples (Table 2).

**Table 2.** Quality control of long-term storage bioresources

Bioresource	Inspection method
Tooth	1. Visual inspection
	2. Bacterial smear test
Saliva	DNA purity and concentration test against oral microbiome
Mouth-rinse solution	
Dental plaque	
Gingival crevicular fluid	
Oral soft tissue	1. H&E staining
	2. DNA purity and concentration test against host

H&E: hematoxylin and eosin.

The KOBN’s first collaborative effort has been the establishment of an annotated repository and the collection of high-quality orally derived bioresources, including teeth, saliva, mouth-rinse solution, dental plaque, GCF, and oral tissue. For this process, standard operating procedures (SOPs) were required for the integration of work routines within the KOBN and the provision of consistent bioresources to researchers. If there is no special request from researchers, sampling is conducted according to the relevant SOP, and if there is a request from researchers sampling is conducted accordingly. Due to the lack of human bioresource banks specializing in oral disease samples or orally derived resources, researchers had difficulty researching human-derived materials in the field of dentistry. Dental hospital-based biobanks are currently in their infancy, and to provide customized research support to researchers it is necessary to collect standardized bioresources, store them at appropriate locations, and maintain their quality. To achieve this, it is important to cooperate with clinicians who donate resources, along with the KOBN and researchers who conduct practical translational research. It is expected that the activities of

the KOBN based in dental hospitals and local dental clinics, and the expansion of dental hospitals participating in the network, will enhance intermediary dentistry research.

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**Conflict of interest:** None

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**Ethics statement:** None

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