SICKLE IN AFRICA
BIOBANK
STANDARD OPERATING PROCEDURE

PROCEDURE ID:	**PROCEDURE NAME:** DNA EXTRACTION		
NAME	**POSITION**	**SIGNATURE**	**DATE**
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REVIEWED BY:			
APPROVED BY:			
ISSUED BY:	Quality Officer		
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DNA Extraction

PURPOSE

1.1 The purpose of this SickleInAfrica Biobank Standard Operating Procedure (SOP) is to outline standardized procedures to follow when extracting high quality genomic DNA from blood, saliva and other frozen tissue samples collected from participants with sickle cell disease. These are samples collected from participants that have been through the informed consent process and agreed to participate in the Biobank. Genomic studies often utilize nucleic acids (DNA and RNA) derived from these samples and high-quality samples are essential for downstream purposes.

1.2 When extracting and storing deoxyribonucleic acid (DNA) from blood and tissue samples, all efforts should be made to avoid contamination, prevent degradation, and preserve molecular integrity. This SOP does not describe detailed safety procedures for handling Human Biological Materials (HBM) or hazardous chemicals.

1.3 **Intended Use:** The SOP is intended to guide biorepository personnel including, laboratory scientists and technologists across the SickleInAfrica consortium in competently performing DNA extraction for biorepository material obtained from study participants in the various sites.

SCOPE

2.1 This SOP will be applied to the extraction of DNA from fresh and frozen blood samples, saliva, and tissue samples.

2.2 The procedures listed in this SOP pertain to all personnel who may be responsible for extracting DNA from blood and tissue.

2.3 It is highly recommended that all personnel handling the human biological materials (HBM) follow the Institutional safety guidelines.

ROLES & RESPONSIBILITIES

This SOP applies to all Biobank personnel that are responsible for extracting DNA from blood and tissue samples.

3.1 Managers, supervisors and chief scientists are responsible for making sure that laboratory technicians are properly trained, and the equipment and facility are maintained in good working order.

3.2 Laboratory personnel are responsible for reading and understanding this SOP and related documents and to perform these tasks in accordance with the SOPs. They are responsible for following clinical laboratory and tissue biobanking best practices.

3.3 The research assistant or tissue biobank officer together with the laboratory personnel are responsible for labelling tubes, extracting DNA from tissue and/or blood and storing samples. Furthermore, the biobank personnel listed should **record and document outcomes**.

3.4 All biorepository staff are responsible for recording and notifying the Supervisor/Investigator of any deviations from this procedure, which are not accounted for in study specific procedures.

ABBREVIATIONS & DEFINITIONS

Abbreviation	Definition
SOP	Standard Operating Procedure
TE Buffer	Commonly used buffer for dilution and storage of DNA. TE buffer is made of Tris-Chloride (10 mM) EDTA (1 mM) and has pH8.0.
PCR	Polymerase Chain Reaction
20% SDS	Sodium dodecyl sulfate
HBM	Human Biological Materials
CIA Solution	Mixture of chloroform and isoamyl alcohol in a ratio of 24: 1.
FFPE	Formalin-fixed paraffin-embedded.
DBS	Dry Blood Spot

RELATED DOCUMENTS TO ACCOMPANY THIS SOP

5.1 SOP that describes the procedure for processing and aliquoting blood and tissue

samples collected from study participants.

5.2 SOP that describes methods of DNA quantification and estimation of purity.

5.3 SOP that describes the standardised procedure for retrieving stored Biobank samples for distribution or processing.

5.4 Recommended operational procedures that describe the key principles regarding safety and waste disposal that a Biobank should adhere to meet the current best practice standards.

5.5 Feasible method of providing or communicating research results to participants in a timely and ethical manner (Respect for Persons).

EQUIPMENT & MATERIALS

The reagents, materials and equipment included in the following list are recommendations only and alternative products as suitable may be substituted for the site-specific task or procedure:

	Reagents, Materials and Equipment
1	Labels and barcodes for tubes and vials, or barcoded vials
2	Appropriate volume tubes and racks; Eppendorf tubes.
3	Microcentrifuge
4	Vortex or Mixer
5	Appropriate kit/reagents, solutions, and buffers for DNA extraction (e.g., Proteinase K, TRIS saturated phenol, 20% SDS, TRIS Buffer (TBE), TRIS EDTA (TE) Buffer, pH 8.0, Phenol chloroform/isoamyl alcohol, Chloroform /isoamyl alcohol, NaCl), Isopropanol, 95% ethanol, 70% ethanol.
6	Hybridization oven at 56°C Low TE Buffer (10 mM Tris-HCl, 0.1 mM EDTA),
7	DNase/RNase-free 100 % Ethanol (molecular biology grade) Xylene Absorbent towels Spectrophotometer for DNA concentration
8	A shaking Heat Block like the Eppendorf Thermomixer or normal heating block or water bath. Hot water bath set at 55° C.
9	Mini-gel electrophoresis chamber
10	0.8% agarose gel
11	Rolling rack
12	Ethidium bromide
9	DNA molecular weight marker
10	DNA loading buffer
11	Gel photography system
12	NanoDrop 2000 UV-Vis/Nanodrop Spectrometer or an equivalent.
13	Micropipettes, single channel: 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl volumes Transfer pipettes

	Glass pipettes for transferring phenols and chloroforms (do not use polystyrene) Pipettors for large glass pipettes.
14	Incubator (35-37°C)
15	Absorbent towels
16	Safety glasses and disposable laboratory coat

Reagents: All reagents should be stored at 20-25°C unless noted otherwise. Manufacturer's expiration guidelines should be followed unless noted otherwise.

SAFETY

7.1 Use universal safety precautions when handling human biological samples and personal protective equipment (e.g., face mask with shield, gloves, lab coat or apron). Treat all human biospecimens as biohazardous materials.

7.2 Ethidium bromide is a carcinogen. Take universal precautions when handling and dispose of properly according to the Institutional disposing policies.

7.3 Ensure that chemicals, spent containers, and unused contents are disposed of in accordance with governmental safety standards. Similarly, sharps and wastes must be disposed of as per your Institutional Biohazard Waste and Sharps Management policy. All equipment must be sterile and standard sterile technique employed.

PROCEDURES/METHOD

This procedure is intended to ensure that DNA is extracted from blood, saliva and tissue samples in a safe and consistent manner while minimizing the risks of contamination and loss of molecular and structural integrity. Consistency in procedure is important for obtaining products with high integrity and quality and achieve comparable, reliable test results/outcomes.

8.1 General extraction considerations

8.1.1 Handle all biospecimens with care and caution. Treat all biospecimens as potentially infectious. Use gloves consistently and change them frequently.

8.1.2 Ensure to handle tubes in the same and correct order to minimize the possibly of sample mix up and cross contamination of samples. Keep tubes closed, whenever possible.

8.1.3 DNA is a weak acid and at 4ºC may be subject to acid hydrolysis. Therefore, DNA should be eluted in a suitable buffer, such as the TE buffer or similar for long term storage.

8.1.4 For long-term storage keep DNA at -20 ºC or -80ºC. Avoid subjecting the DNA to freeze/thaw cycles to prevent fragmentation of the genomic DNA.

8.1.5 Care must be taken to avoid introducing nucleases and other contaminants to the samples:

a. Ensure that all reagents, solutions, and chemicals used are of analytical reagent grade and molecular biology grade i.e., use RNase and DNase free pipette tips and microcentrifuge tubes. Always use aerosol-barrier tips.
b. Autoclave reagents and tubes before any procedure and handle them in a laminar flow cabinet to minimize the risk of contamination with DNA from the operator.
c. Irradiate flow cabinets with UV light for 30 mins prior to use. Flow-through generated after each centrifugation step may contain hazardous materials. Dispose of appropriately.
d. Sterilize all surfaces (bench area) with bleach and 70% ethanol before use. Equipment and reagent containers can be rinsed with H_2O after use.
e. Avoid moistening the rim of the spin columns with pipette tips and avoid touching the column with the pipette tip.
f. Close the lids of the spin columns before placing the microcentrifuge.
g. Open only one spin column at a time to avoid creating aerosols.
h. Avoid cross-contamination after each vortexing step. Briefly centrifuge the tubes to remove droplets that may be on the lids of the tubes.

i. Take precautions not to introduce RNase or DNase into the sample during or after the purification procedure.

8.2 METHOD

There are various specialized techniques and sophisticated DNA extraction kits currently being used for the isolation and extraction of DNA from biospecimens. It is highly recommended that biobanks across the SickleInAfrica consortium have standardized practice and be aware of changes and improvements in best laboratory practice. In this SOP, two methods are outlined: Salt precipitation method (organic) & Extraction using Organic Solvents (nonorganic).

The method of salt/alcohol precipitation has the advantage of obtaining high purity DNA and use of solutions that are very safe and not hazardous to health. The phenol-chloroform method might be time-consuming but more cost-effective and DNA can be extracted more accurately than extracting the DNA using the DNA extraction kit. Additionally, this method gives a high yield, but traces of organic solvents often contaminate the sample. Furthermore, the entire process is performed in a chemical fume hood due to the toxic nature of chemicals.

8.2.1 Transport and collection of samples

Sample type & collection: 10mls of whole blood should be drawn from each study participant, with each 5mls placed in a separate EDTA [(10 µl/ml blood) of 10% solution of Potassium EDTA (K_2EDTA)] container to ensure adequate sample for storage and analysis. Aliquoting, labelling and storage of samples must be performed and handled according to the SOP.

Ensure that samples collected from the umbilical cord, peripheral blood, heel-prick) are stored and preserved at −20˚C freezer (medium-term) or −80˚C (long-term) until DNA extraction. Buffy coats obtained after centrifuging can be placed in 1.5 or 2ml safe-lock Eppendorf tubes and transported frozen.

Saliva samples collected in Oragene or Norgene kits should be transported to the laboratory at ambient temperature.

8.3 SALT PRECIPATION METHOD

8.2.2 Principle

Firstly, the cells are lysed with an anionic detergent that solubilizes the cell components and inhibits the action of intracellular nucleases. Next, proteins are denatured and

eliminated by **salt precipitation**. The DNA in solution is precipitated with isopropanol, washed with ethanol, and finally resuspended in a DNA-stabilizing buffer. This method is used to obtain DNA from the following sample types:

Peripheral blood: volumes from 3 ml to 20 ml (the amounts of the reagents for this method are scalable according to sample volume).

Peripheral blood mononuclear cells (PBMCs): PBMCs preparations have a volume that varies between 0.1 and 0.2 ml on average, and from 1 to 3x 107 cells can be processed.

Cells: the number of cells that can be processed depends on the cell type and on the way the cells were obtained (e.g., cell cultures or cells purified by flow cytometry). In any case, a broad range of cells can be processed, ranging from 1 x 106 to 50 x 106, as long as the amounts of reagent solutions are scaled up proportionally.

Saliva: 2 ml of saliva mixed with 2 ml of Oragene® or Norgene preservative can be processed.

Tissues: very variable amounts of tissue, from 5 mg to 1 g of fresh frozen tissue, can be processed. This DNA extraction technique can also be used to obtain DNA from tissue fixed in formalin and embedded in paraffin (FFPE: formalin-fixed paraffin-embedded).

8.2.3 Step-wise Procedure

Ensure that materials and equipment are ready before starting the procedure:

Step 1. Add lysis solution to the sample. Ensure an efficient cell lysis and proceed to the next step if the sample has a homogeneous aspect. If cell bodies are observed in the solution, incubation at 37ºC or 65ºC is recommended to achieve complete homogenization of the sample. Samples are stable in lysis solution for at least 2 years at room temperature; they can be stored in the dark and the process can be continued later.

Step 2. To obtain an RNA-free sample, add RNase and incubate at 37ºC for 15- 45 min. Note that this step is not necessary for peripheral blood samples, since in these samples contamination with RNA is virtually undetectable. This is highly recommended for other samples such as cells, saliva or tissues. The amount of RNase required is proportional to the type and amount of the initial/starting sample.

Step 3. Add saline solution to precipitate the cytoplasmic and nuclear proteins of the sample. Vigorously vortex the sample for 20-30 seconds. Next, centrifuge the sample at the speed and time needed to ensure complete precipitation of the proteins. The precipitated proteins are seen at the bottom of the tube as a brown pellet. The

supernatant must be clear and without particulate matter or brownish traces; if this is not the case, the sample must be incubated for 5 minutes on ice and centrifuged again.

Step 4. The supernatant containing the DNA in solution is transferred to a fresh tube with isopropanol. The sample is mixed with isopropanol by gently inverting the tube approximately 50 times. In this step the appearance of the DNA strand of is seen. However, observation of this strand will depend on the amount of DNA sample processed.

Step 5. Centrifuge the sample to precipitate the DNA at the bottom of the tube. The DNA is seen as a whitish precipitate.

Step 6. Decant the supernatant carefully and place the tube with the DNA precipitate in an inverted position on a clean piece of absorbent paper to remove as much of the remaining isopropanol as possible.

Step 7. Add Ethanol to 70%, cap the tube and invert gently several times to wash the DNA pellet.

Step 8. Centrifuge the sample and remove the ethanol by decanting or with a pipette tip (very carefully, because the DNA may come off the bottom of the tube). Ensure that the DNA precipitate remains visible at the bottom of the tube.

Step 9. Excess ethanol is removed by placing the tube upside down on a clean piece of absorbing paper or by leaving the tube to air dry for a few minutes until no more traces of ethanol are observed. However, it is important not to let the pellet become too dry because this makes subsequent resuspension difficult.

Step 10. Hydrate the DNA with an adequate buffered solution, with TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA), or with sterile water.

Step 11. It is recommended to incubate for about 1 hour at 65ºC to facilitate resuspension of the DNA. Thereafter, the DNA in solution is stirred at room temperature until checking with a pipette tip verifies that the DNA is fully resuspended without lumps or viscous debris in the solution.

Note: The amount of the different solutions used in this protocol may vary depending on the type and amount of starting sample. Because different kits based on the salt precipitation method are used, it is **recommended to follow the specific instructions of the kit regarding the quantities of reagents, incubation times, and centrifugation times** and velocities specifically indicated for each type of sample.

8.2.4 Expected Yield

Various protocols have documented the expected yield from whole blood, buccal cells, cells, buffy coat, tissue and body fluids.

1. For a simple salting out procedure, a yield of approximately **25 µg DNA/ ml** whole blood is expected.
2. The Gentra Puregene Handbook for purification of archive-quality DNA from human whole blood, bone marrow, buffy coat, buccal cells, body fluids, cultured cells, tissue, etc. documented an expected yield of **25 to 50 µg DNA/ ml** blood
3. from whole blood, with an average of about 35 µg DNA/ ml blood. The average yield is around 55 µg DNA /ml saliva in saliva samples.
3. According to the Qiagen Genomic DNA Handbook, the expected yield is in the range of **25-35 µg DNA/ ml** blood.
4. In the Flexigene® DNA Handbook for the purification of DNA from human whole blood, buffy coat, etc. the average yield is about **37 µg DNA/ ml** blood

5. Using Oragene DNA Saliva 2 Extraction-AGF: the average yield is approximately **140 µg DNA/ ml** saliva.

8.2.5 Sample Quality

Purity: The purity of DNA obtained with the salt precipitation method is very high with values of **A260/A280 ratio of > 1.8 for all sample types**. In most cases, the A260/A230 ratio can be > 1.5 and can even approach 2.0 (including fixed and paraffin-embedded tissue samples). However, in saliva samples, a ratio of < 1.5 can be observed without influencing the integrity and functionality of the sample.

Integrity: DNA samples obtained using salt precipitation are samples of very high integrity, and usually a single defined band is seen on agarose gels. Some exceptions are: 1) saliva samples; a slight smear can be seen in the lane, together with a predominant defined band at the top of the gel, and 2) DNA samples from fixed and paraffin-embedded tissues, in which a pronounced smear is observed on the agarose gel.

Functionality: In most of the protocols used, the samples allow amplification of a large DNA fragment (> 17 kb). In DNA samples obtained with the method described by Miller et al., (1988) (salt precipitation), amplification of 8.4 kb fragments obtained allows good DNA sequencing.

8.3 DNA EXTRACTION USING ORGANIC SOLVENTS

8.3.1 Principle

This protocol allows purification of DNA by the addition of phenol and chloroform, resulting in the appearance of two phases: an upper aqueous phase containing the nucleic acids and an organic phase containing proteins solubilized in phenol and lipids dissolved in chloroform. For purifying DNA, phenol should have a pH≈7-8. Subsequently the DNA is precipitated from the aqueous phase with isopropanol or absolute ethanol and washed with 70% ethanol to remove salts and small organic molecules that may still be present

in the sample. Finally, the DNA is resuspended in an appropriate buffer (TE buffer). This method can be used to collect DNA from different sample types, including blood and frozen tissue.

8.4.2 Step-wise Procedure

Step 1. The cells of the sample are lysed. Lysis is dependent on the type of sample to be processed. Before adding phenol, lysis must be complete, and the resulting lysate must be homogeneous.

Step 2. One volume of phenol is added to a volume of the lysate and the contents mixed by inverting the tube for 20 seconds.

Step 3. The sample is centrifuged at 12,000xg for 3 minutes. After centrifugation two phases are observed: the upper aqueous phase and the organic phase. The interphase between the two is a whitish layer whose thickness will decrease as the sample becomes cleaner and contains fewer contaminating proteins.

Step 4. The aqueous phase containing the DNA is transferred to a clean tube taking care not to touch the interphase and the organic phase; these are discarded. An equal volume of a 1:1 phenol/CIA mixture is added to the aqueous phase. The mixture is homogenized by stirring.

Step 5. The sample is centrifuged again at 12,000xg for 3 minutes. The two phases are formed again but a considerable reduction of the interphase must be apparent. Steps 4 and 5 must be repeated as long as the interphase remains visible.

Step 6. The aqueous phase is transferred to a new tube and mixed with one volume of chloroform (chloroform removes phenol residues that may have remained in the sample). The aqueous phase and the chloroform are mixed by inversion for about 20 seconds.

Step 7. Centrifuge at 12,000xg for 3 minutes. Precipitate the aqueous phase in a clean tube with 2 volumes of isopropanol. Incubation of the mixture for 5-10 minutes is optional but may improve the precipitation of DNA.

Step 8. Centrifuge at 12,000xg for 10 minutes to pellet the DNA. Discard the isopropanol. The DNA pellet should be visible at the bottom of the tube.

Step 9. Wash the precipitate with 500 µl of 70% ethanol. The sample is centrifuged again at 12,000xg for 10-15 minutes. Optionally, a second wash can be done with ethanol to maximize sample purification.

Step 10. Remove the ethanol and leave the DNA precipitate to dry. Finally, the DNA is resuspended in an appropriate volume of TE buffer or distilled water.

Step 11. Analyze extracted DNA quality by 0.8% agarose gel electrophoresis.

Step 12. Photograph gel and save to raw data folder.

Step 13. Freeze DNA and store at -20°C (those to be shipped for lab analyses within 6 months) or -80°C (long term storage).

Note:

CIA solution: mixture of chloroform and isoamyl alcohol in a ratio of 24: 1.

TE buffer: 10 mM Tris HCl, pH 8.0, 1 mM EDTA.

8.4.3 Expected Yield

The expected yield using this procedure in whole blood samples is around **30-70 µg DNA/ml** blood. The yield is much more variable in tissues and depends on tissue type and the amount of the initial/starting sample.

8.4.4 Sample Quality

Purity: With this DNA extraction method, it is crucial to work very carefully to avoid contamination of the DNA solution by remnants of phenol or chloroform. The occurrence of traces of phenol or chloroform may affect the absorbance ratios that indicate the purity of the sample.

The samples obtained by this method have an **A260/A280 ratio around > 1.7, although A260/A230 ratios less than 1.5** are relatively frequently observed.

Integrity: The samples show a well-defined, prominent band at the top of the agarose gel after electrophoresis, indicating an optimal DNA integrity.

Functionality: Most samples obtained by this method can amplify large (> 17 kb) DNA fragments by PCR. For samples with which bands >17 kb are not amplified, it is possible to amplify DNA fragments of approximately 7 kb long.

SPECIFICATIONS FOR DIFFERENT SAMPLE TYPES (BY METHODS OF SALT PRECIPITATION & ORGANIC SOLVENTS)

9.1 Whole blood samples: A prior selective method for lysis of red blood cells is required. To do this, add three volumes of red blood cell lysis solution to the initial volume of whole blood to be lysed. Mix the sample by inversion and leave for 5 minutes; invert the tube gently several times during incubation. Once the sample is completely lysed, a colour change to a clearly darker shade is observed.

Next, centrifuge to precipitate the leukocytes of the sample; they remain attached to the bottom of the tube while the supernatant containing the lysed red blood cells is decanted. A small residual volume of 200-400 µl of liquid should be left to resuspend the leukocytes by vigorous vortexing for about 20 seconds. Add lysis solution to lyse the leucocytes and if cell bodies are observed in the solution, incubation at 37ºC or 65ºC is recommended to achieve complete homogenization of the sample.

An additional lysis of erythrocytes can also be done with samples of peripheral blood mononuclear cells (PBMCs) if red blood cells are observed in the sample.

9.2 Saliva samples: before processing saliva samples collected in a container with a preservative such as Oragene® preservative, it is recommended to incubate the sample at 50ºC for 1-2 hours to maximize the final yield of DNA. This incubation time can be increased if it is deemed necessary to increase the final yield of the sample.

9.3 Tissue samples: tissue samples must be homogenized so that the cells are more exposed to the action of the reagents. There is a wide variety of methods for the disruption of tissue, such as sonication, mechanical grinding, using a mortar, adding glass beads, and vigorous stirring. According to preference in different DNA extraction protocols, the cell lysis solution is added after homogenization of the sample, while in others homogenization is carried out in the lysis solution itself. Because tissue disruption involves the release of proteases and other enzymes involved in the degradation of various cell components, enzyme inhibitors are commonly used to prevent cell degradation during processing. In addition, working quickly and in the cold with such samples minimizes the risk of enzymatic degradation.

In recent years, more sophisticated methods of DNA extraction and detection of haemoglobinopathies are increasingly in use. However, in developing countries, where advanced techniques are not available or limited, simpler and convenient methods of DNA extraction from frozen blood samples and even DBSs are proven suitable and ideal.

QUALITY CONTROL/ASSESSMENT

After DNA has been extracted, quality control is necessary to ensure there is sufficient DNA for sequencing and that it is not contaminated. Quality check on a Bioanalyzer or a 0.8% agarose gel electrophoresis device is recommended. High quality genomic DNA should give a major band of 10-20 kb on the gel. Refer to SOP on DNA quantification.

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REVISION HISTORY

Revision No	Effective Date	Description of Changes Made from Preceding Revision	Approved by/ Date
		Updated materials, reagents, procedure, and quantification	

DOCUMENTATION OF SUGGESTED CHANGES TO THIS SOP

CLAUSE	SUGGESTION	BY	DATE
