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SafeForever™

Ensuring Eternity

Case Studies- Downstream processing of SafeForever™ stored samples

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Introduction

SafeForever™ (SFE) has been utilized to store samples from humans, animals, plants, and soil. For the experimental techniques, samples such as blood, saliva, and tissue (from humans), soil samples, animal tissues, and plant components were used. The above SFE preserved samples were processed downstream to isolate DNA, RNA, and protein and to perform molecular tests such as PCR, Next Generation Sequencing (NGS), and the ELISA assay. The study's findings are summarized below.

Application	Test performed	Specimen type
Genetic analysis	DNA isolation	Human specimen-blood and saliva
	DNA isolation	Agriculture -Plant parts
	RNA isolation	Human specimen-tissue/ saliva Agriculture -Plant parts
Genotyping	Polymerase Chain Reaction (PCR)	Human specimen-blood and saliva
	SNP detection	Human specimen-blood and saliva
Next Generation Sequencing	NGS of cancer hotspot panel	Human specimen-Blood/Saliva/Liver tissue
	NGS of 16srRNA for microbiome study	Human specimen-Endoscopic biopsy/soil
Proteomics	Protein extraction and ELISA	Human specimen-Blood/Saliva
Histopathology	Hematoxylin and eosin (H&E) stain	Human specimen-Uterus tissue

Usability Note:

- DNA isolation can be performed from different sources of samples like blood, saliva, tissue, plant materials and soil after storing the sample in SafeForever™ version 1. DNA isolation from cell cultures and rumen fluid etc. will be undertaken in the second phase of the study.
- As proof-of-concept RNA isolation from solid materials like tissue and plant parts has been established using SafeForever™ version 1, further modifications in the SafeForever™ version is under process to establish its utility in RNA isolation from liquid samples like blood, saliva and serum.
- Total Protein isolation from blood and saliva is validated using SafeForever™ version 1 of the product. Further validation required to test protein isolation or its application when storing serum/plasma/urine samples.
- Metabolomics analysis profiling of the sample stored in SafeForever™ has to be validated.

Genomic studies

A. DNA extracted from animal specimens kept in SFE

Blood and saliva samples were collected from healthy volunteers and stored in SFE as well as at -200C and -800C for comparison. DNA was extracted from the previously stored samples. The concentration of DNA in SFE-stored samples was found to be similar to the standard cold storage temperatures of -200C and -800C (Figure 1).

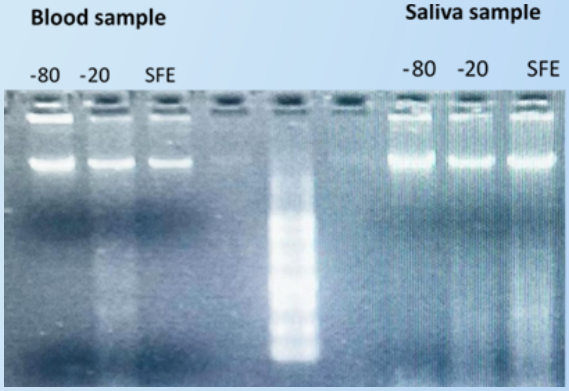


Figure 1. Electrophoresis image showing representative DNA extracts from blood and saliva samples stored at different temperature

B. DNA extracted from plant specimens kept in SFE

For the purpose of extracting DNA, plant components (stem, root, and leaf) were kept in SFE (Figure 2a). The agarose gel was used to run the extracted samples, and the results are displayed below (Figure 2b).

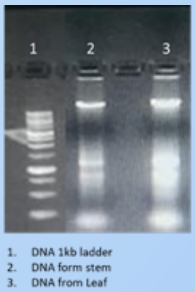
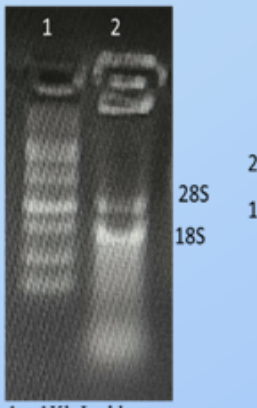


Figure 2a. Plant specimen stored in SFE
Figure 2b. DNA isolated from stem and leaf

C.RNA isolated from different sample kept in SFE

The TRIzol Protocol was used to isolate RNA from plant material and saliva samples. The information is presented in Figure 3(a).
The suitability of the isolated RNA from blood for Realtime assay was verified by real-time PCR for the housekeeping gene RNase P. The graphical representation of the amplification plot for these samples is shown in Figure 3(b). The Ct (threshold cycle) value of the amplification is also provided (5b).



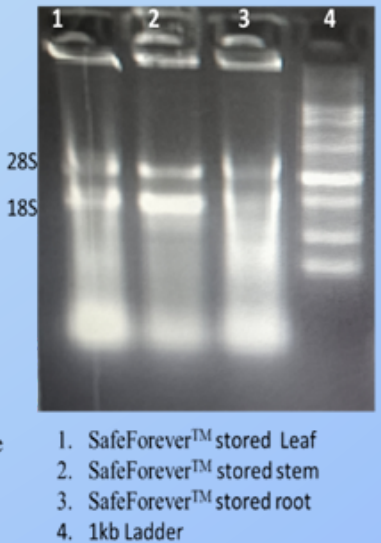


Figure 3. RNA isolation from different samples

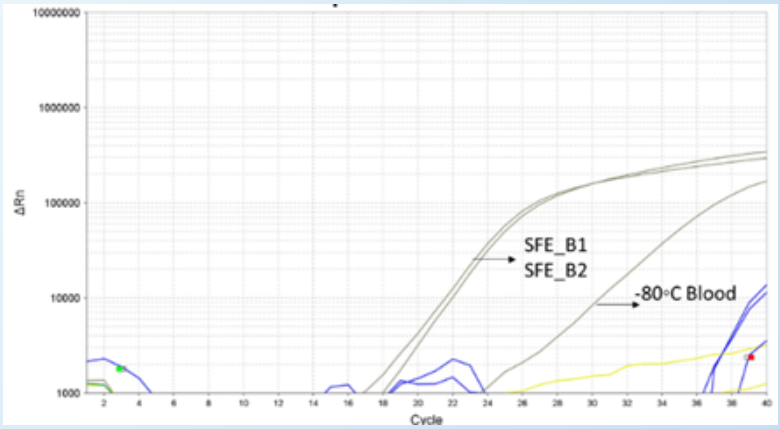


Figure 3b. Amplification plot of RNase P for Fresh and SafeForever™ stored blood

DNA from samples maintained with SFE was examined using PCR.

The DNA obtained from saliva and blood samples maintained under various conditions was subjected to PCR analysis to determine its eligibility. The CYP26 gene was amplified using particular primers from all of the above-mentioned DNA samples. The results indicate that the DNA acquired from the samples maintained in SFE gel at room temperature is of good quality, and the results obtained after amplification are shown below (Figure 4).

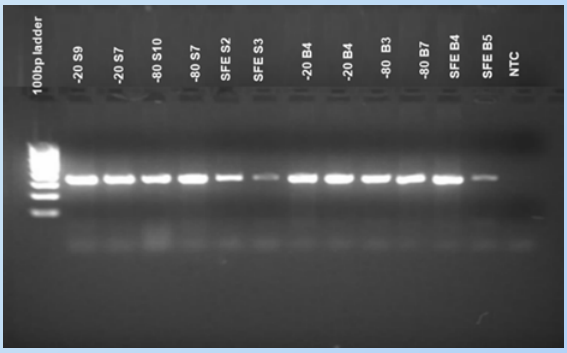


Figure 4: The agarose gel electrophoresis of the PCR products obtained with CYP2D6 primers

SNP-based detection using real-time PCR assay

TaqMan Real-Time SNP-based assay was used to test the quality of the DNA in properly naming the variations of interest utilizing real-time assay. HLA-B27 variations were examined using various isolated DNA samples. Although there was a three-cycle difference between DNA extracted from SFE stored samples and DNA isolated from -20°C and -80°C kept samples. The test findings verified that the SFE DNA is adequate for reliably assessing the SNP calling using the RT-PCR assay (Figure 5).

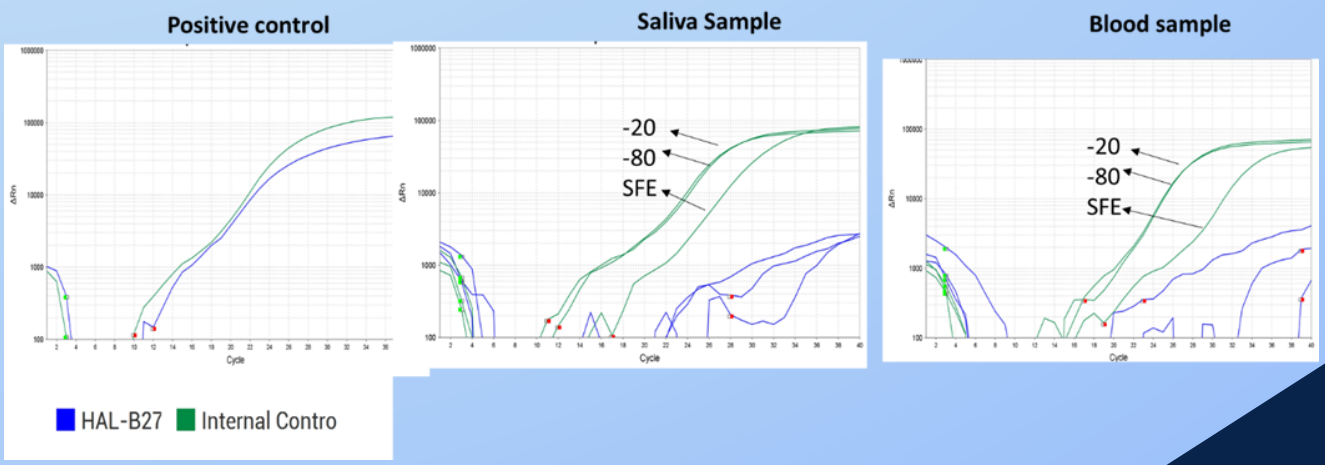


Figure 5. Real time PCR assay of HLA B27 from the DNA obtained from blood and saliva samples stored at different temperature and SFE

Next Generation Sequencing (NGS) of Cancer Panel on Ion GeneStudio S5

The DNA obtained from saliva and blood samples preserved in SFE was used in Next Generation Sequencing. A panel of cancer genes called the Ion AmpliSeqTM Cancer Hotspot Panel v2 is utilized in NGS to find mutations in 50 oncogenes and tumor suppressor genes. The 207 amplicons of the Cancer Hotspot Panel v2 are intended to cover over 2,800 COSMIC.
The graphic below depicts the coverage analysis report and the filtered variants (Figure 6a, Figure 6b). The variations in the -80°C preserved sample and the SFE DNA sample were compared. The resulting sequence variations were similar, showing accurate variant calling, which is comparable to SFE stored samples. This highlighted the stability of DNA recovered from materials maintained in SFE at standard storage settings for high throughput next-generation sequencing research.

Coverage Analysis: Blood Sample stored in SFE

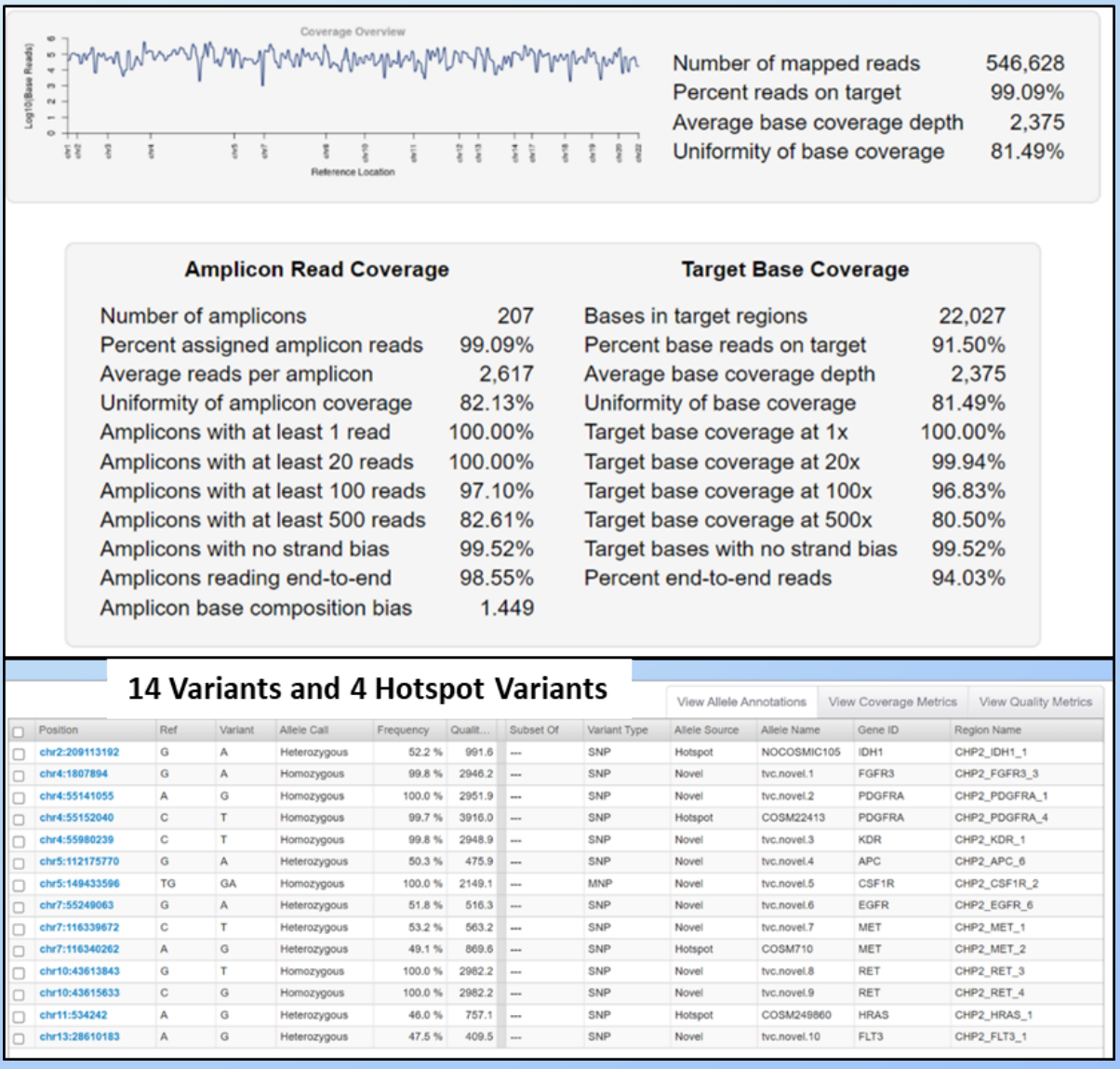


Figure 6a. Coverage analysis and the variants called from Ion Reporter analysis from the DNA obtained from blood stored in SafeForever™

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Coverage Analysis: Saliva Sample stored in SFE

Number of mapped reads	513,163
Percent reads on target	99.58%
Average base coverage depth	2,394
Uniformity of base coverage	94.41%

Amplicon Read Coverage

Number of amplicons	207
Percent assigned amplicon reads	99.58%
Average reads per amplicon	2,469
Uniformity of amplicon coverage	95.17%
Amplicons with at least 1 read	100.00%
Amplicons with at least 20 reads	100.00%
Amplicons with at least 100 reads	100.00%
Amplicons with at least 500 reads	95.17%
Amplicons with no strand bias	99.52%
Amplicons reading end-to-end	98.07%
Amplicon base composition bias	1.834

Target Base Coverage

Bases in target regions	22,027
Percent base reads on target	91.97%
Average base coverage depth	2,394
Uniformity of base coverage	94.41%
Target base coverage at 1x	100.00%
Target base coverage at 20x	100.00%
Target base coverage at 100x	100.00%
Target base coverage at 500x	94.41%
Target bases with no strand bias	99.49%
Percent end-to-end reads	95.67%

11 Variants and 1 Hotspot Variant

View Allele Annotations | View Coverage Metrics | View Quality Metrics

Position	Ref	Variant	Allele Call	Frequency	Qualit...	Subset Of	Variant Type	Allele Source	Allele Name	Gene ID	Region Name
chr4:1807894	G	A	Homozygous	100.0 %	2981.4	—	SNP	Novel	tvc.novel.1	FGFR3	CHP2_FGFR3_3
chr4:55141055	A	G	Homozygous	100.0 %	2938.2	—	SNP	Novel	tvc.novel.2	PDGFRA	CHP2_PDGFRA_1
chr4:55152040	C	T	Heterozygous	52.8 %	1007.5	—	SNP	Hotspot	COSM22413	PDGFRA	CHP2_PDGFRA_4
chr4:55980239	C	T	Heterozygous	44.5 %	337.6	—	SNP	Novel	tvc.novel.3	KDR	CHP2_KDR_1
chr5:112175770	G	A	Homozygous	100.0 %	2973.3	—	SNP	Novel	tvc.novel.4	APC	CHP2_APC_6
chr7:55249063	G	A	Heterozygous	50.2 %	480.2	—	SNP	Novel	tvc.novel.5	EGFR	CHP2_EGFR_6
chr10:43613843	G	T	Heterozygous	47.8 %	415.9	—	SNP	Novel	tvc.novel.6	RET	CHP2_RET_3
chr10:43615633	C	G	Heterozygous	45.2 %	356.1	—	SNP	Novel	tvc.novel.7	RET	CHP2_RET_4
chr13:28602292	T	C	Heterozygous	47.5 %	409.9	—	SNP	Novel	tvc.novel.8	FLT3	CHP2_FLT3_3
chr13:28610183	A	G	Homozygous	100.0 %	2982.2	—	SNP	Novel	tvc.novel.9	FLT3	CHP2_FLT3_1
chr17:7579472	G	C	Homozygous	98.0 %	2790.9	—	SNP	Novel	tvc.novel.10	TP53	CHP2_TP53_2

Figure 6b. Coverage analysis and the variants called from Ion Reporter analysis from the DNA obtained from Saliva stored in SafeForever™

Liver Tissue DNA extraction and downstream Next Generation sequencing analysis to understand the genetic variants

SFE (Sample ID IP1) and -80C (Sample ID A8) were used to store liver tissue samples. DNA was extracted from 30mg of tissue, and quality control was conducted using the Qubit assay (Table 1). The Ion AmpliSeqTM Cancer Hotspot Panel v2 was used to prepare the libraries, which were designed to amplify 207 amplicons covering about 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes. Before sequencing on the 520 chips on the Ion Gene studio S5 equipment, the libraries were measured using Qubit to ensure their quality.The data unmistakably shows good reads produced by the SFE stored liver (tissue) sample's Ampliseq Cancer panel (Figure 7). Variant Visualization in the genes that was picked up by the Ion Reporter Software (Figure 8).

Sample Type	Sample ID	Qubit DNA conc. (ng/ul)	Library Conc (ng/ml)
Live tissue at -80◦C	LP-T2	22.7	2100
Liver tissue in SafeForever™ gel	SFE_LP-T2	22	1812

Table 1: Qubit Assay

Sample	Bases	Q20	Reads	Mean Read Length	Read Length Histogram
LP-T2	4,207,491	4,010,600	36,375	115 bp	
SFE_LP-T2	4,408,493	4,200,249	38,381	114 bp	

Figure 7. Reads obtained from sample stored in SFE (IP1) compared to -80 stored sample

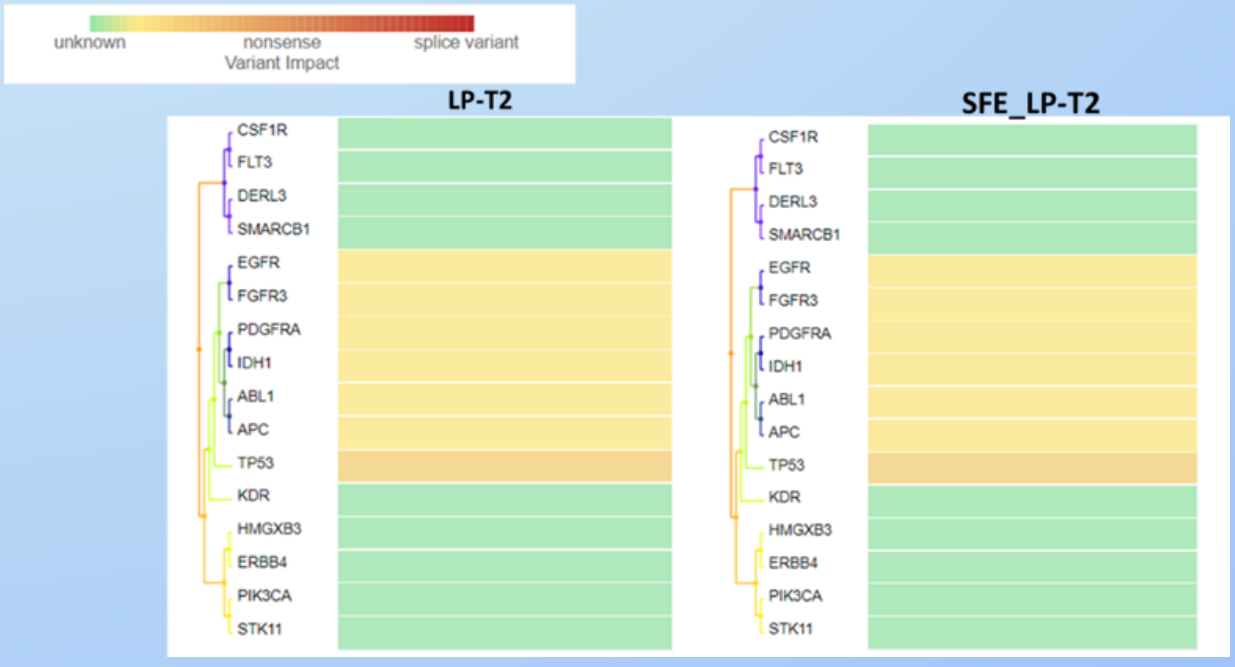


Figure 8. Variant Visualization in the genes

Next Generation Sequencing on Oxford Nanopore for 16srRNA sequencing Endoscopic biopsy stored in SFE useful for Gut microbiome study

Testimonial: https://youtu.be/CSA3Qu9fe34, (Clinician sharing the usefulness of SafeForeverTM stored biopsy sample in Gut microbiome analysis, 1min50sec-3min52sec)

In order to research metagenomics, the endoscopic biopsy samples kept in SFE were subjected to Oxford Nanopore 16srRNA sequencing. Using the primers V1–V9 and the 16S Barcoding Kits and EPI2ME 16S analysis methodology, the full-length 16S rRNA gene was sequenced using a Nanopore MinION sequencer. The table (2) below displays the DNA concentration from these samples.

Sl No.	Sample ID	Qubit DNA Conc (ng/ul)	260/280
1	Sample 1	299.506	1.824
2	Sample 2	334.851	1.823
3	Sample 3	223.883	1.827
4	Sample 4	323.801	1.827

Table 2: DNA Concentration of samples

Understanding the makeup of a microbial community in the gut of the samples that were kept in SFE was made possible by the 16S process. Screenshot of the report that assisted the doctor in making a more accurate diagnosis based on the microbiome analysis of the samples that were taken, kept, and transported in SFE medium.

Sample report of the gut microbiome

Candidate Gut Microbiome Profile

The largest group of microorganisms is called Phyla. These provide a solid foundation for assessing the general state of the stomach/gut. The graphic below compares the candidate phyla to those with a typical, balanced gut (Figure 9).

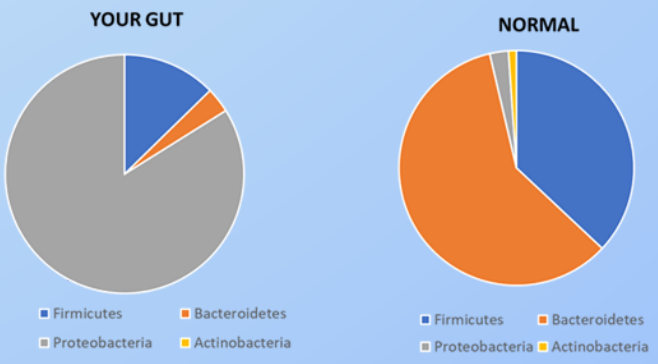


Figure 9: Gut Microbiome Profile

Interpretation: K. pneumoniae has become one of the pathogens that are frequently isolated from patients with GIT disorders. The gut microbiota component F. nucleatum, known for its pro-inflammatory properties and association with mucosa, may have a role in the etiology of inflammatory bowel disease (IBD). Below are listed the bacterial species that are most prevalent in the microbiome of the affected candidate (Figure 10).

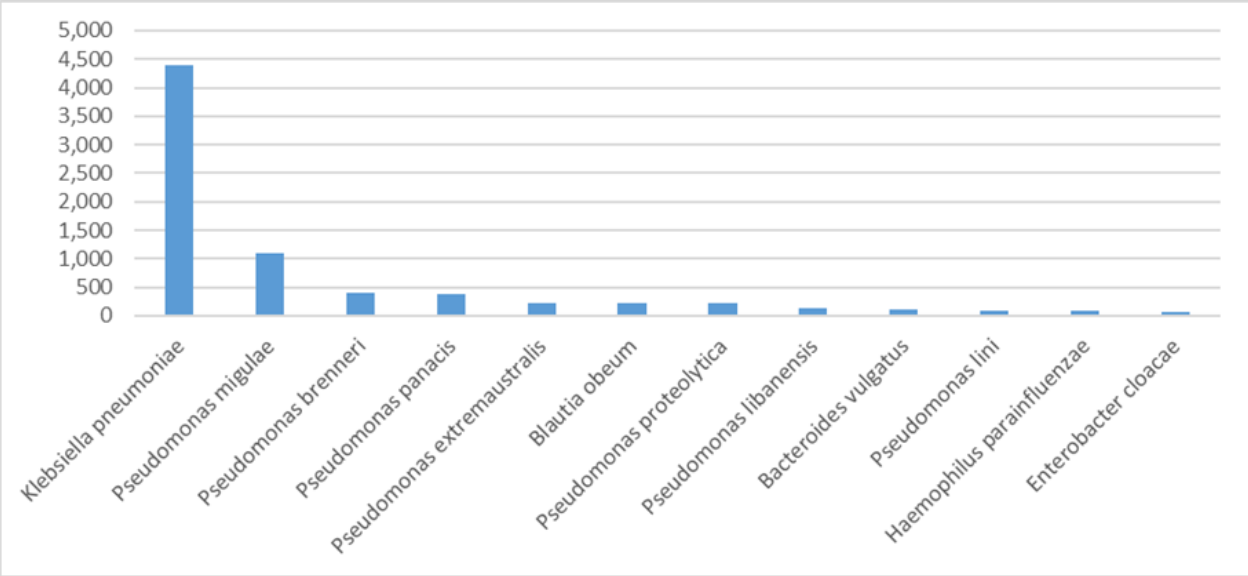
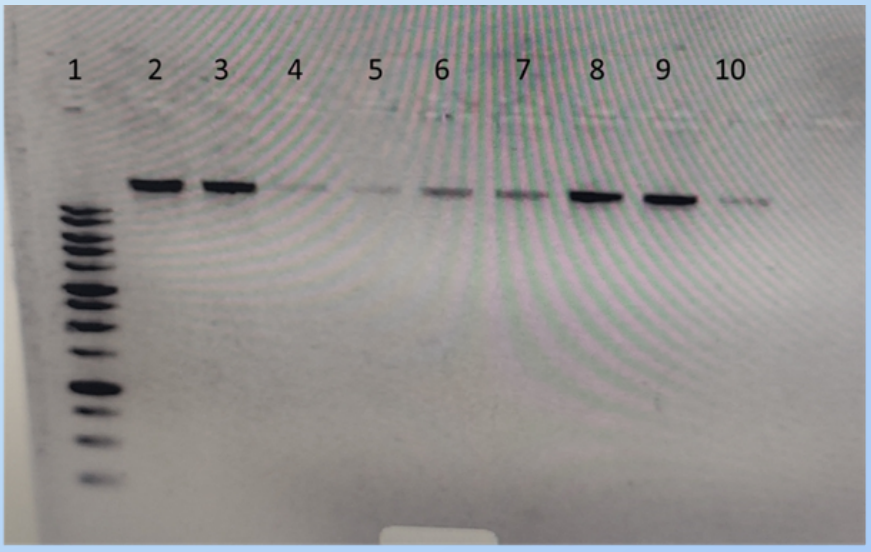


Figure 10: Bacterial abundant Species in specimen

Soil sample stored in SFE useful for metagenomics study

DNA was extracted from soil samples (Figure 11), kept in SFE, and 16srRNA sequencing was carried out (Table 3). Due to the presence of conserved and highly variable sections, the gene is perfect for sequence-based identification of these organisms, especially in mixed samples (Figure 12).



Lane 1 ⟶ 1kb Ladder
Lane 2 -10 ⟶ DNA isolated from Soil sample stored in SafeForever™

Figure 11: DNA isolated from soil microbes

Sample type	Storage	DNA conc. (ng/ul)	260/280	16srRNA read count
SFE1_Soil	SafeForever™	2.12	1.79	1,35,596

Table 3: 16srRNA Sequencing read count

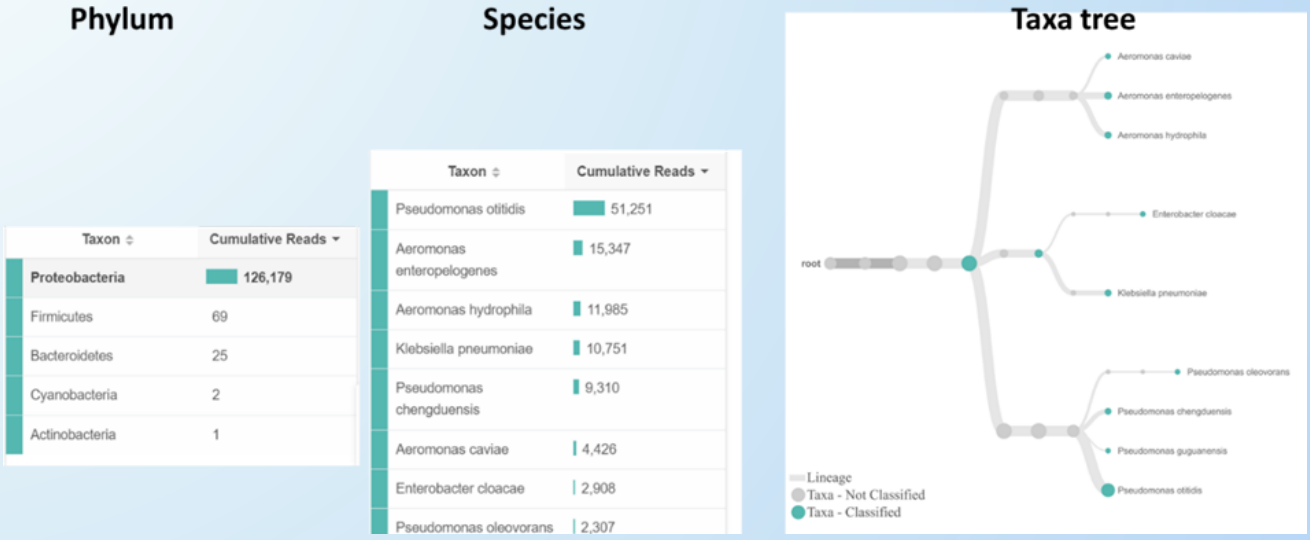
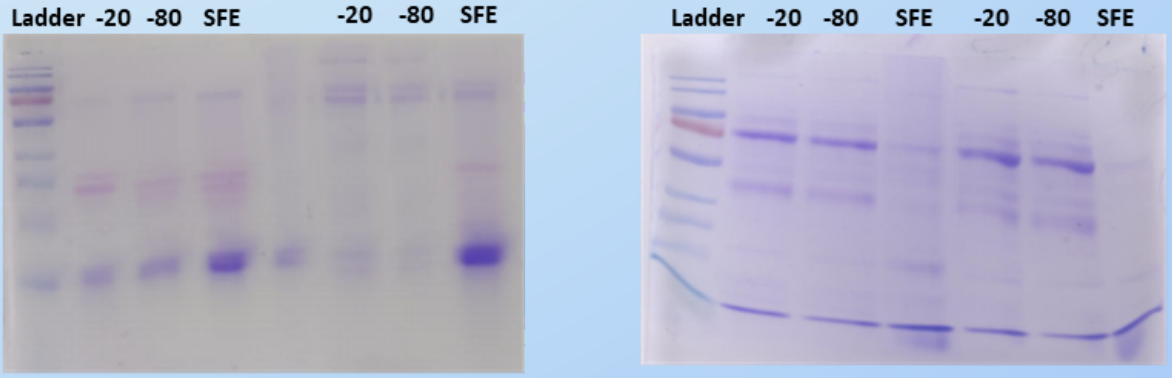


Figure 12: Phyla,species and the Taxa tree obtained form EPI2ME analysis of SFE stored soil sample

Protein extraction and ELISA

Protein was extracted from samples stored in -20°C, -80°C and SFE, using TRIzol protein extraction protocol. The results exhibited that storing saliva and blood samples in SFE preserved protein integrity (Figure 13).



SFE A **SFE B**

Figure 13: SDS-PAGE image of the proteins obtained from Saliva Samples(A) and Blood (B) stored at different temperatures and SFE

ELISA assay

The proteins extracted from the samples maintained at -20°C, -80°C, and SFE were assessed using a human amylase alpha 1, salivary (AMY1) ELISA kit. The kit examines the levels of Human AMY1 in serum, plasma, and other bodily fluids. The findings showed that compared to samples maintained at -20°C and -80°C, the protein isolated from samples stored in SFE is equally appropriate for ELISA research (Figure 14).

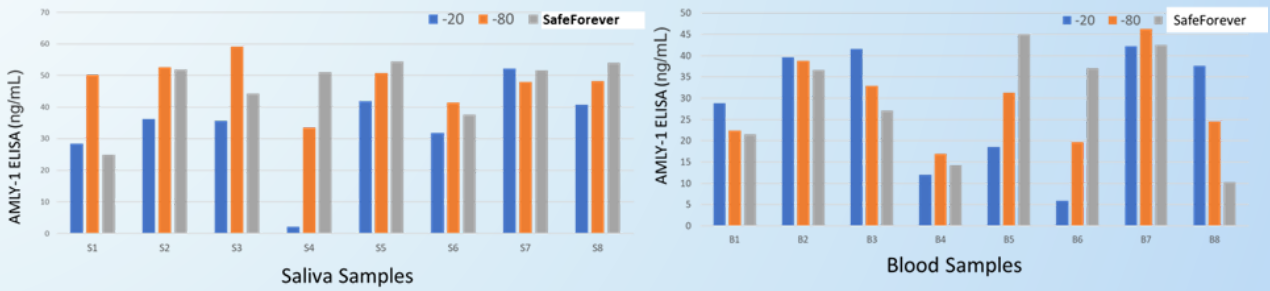


Figure 14. Human Amylase Alpha 1, AMY1 ELISA results analyzed from the protein obtained from (a) Blood and (B) Saliva samples stored at different temperatures and SFE

Histology data analysis -

Analysis of the tumor material from the SFE preserved with hematoxylin and eosin (H&E) stain (Figure 15). The report summary is tabulated below.

Case details	
Tissue	Uterus tissue
Cold ischemia	30 min
Details	Fixed in vacuum sealed and transported at 4 degree (12 hours) Following this transferred to new fixative SFE (24 hours)
Fixation Time & processing	24 hours Transferred to formalin and routine process
H&E analysis	
Tissue characteristics	Fixation adequate
Tissue architecture	Maintained
Tissue staining	Nuclear details maintained.

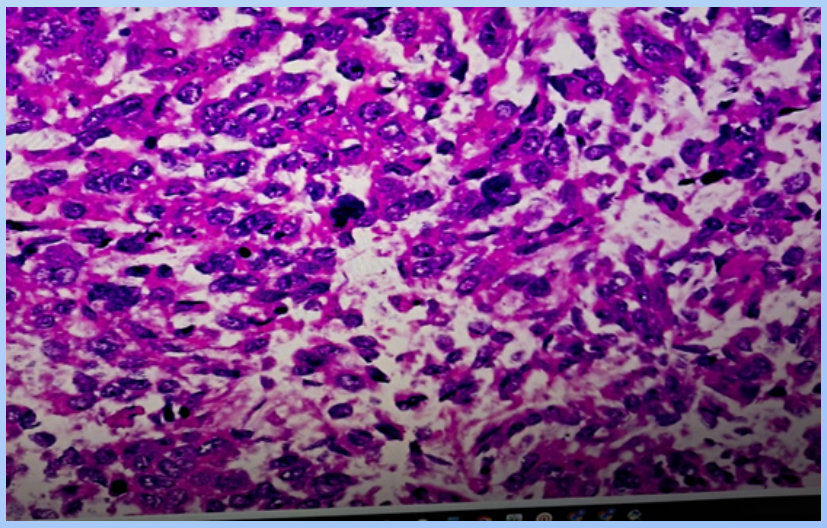


Figure 15. H&E image of the tissue after 24hrs preservation in SFE

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