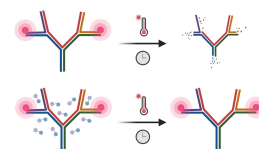


Aug 24, 2023

🌐 Sensor Synthesis and Storage Protocol

DOI

<https://dx.doi.org/10.17504/protocols.io.rm7vzxxz4gx1/v1>



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Protocol Citation: Nikita Srinivasan, heather Ann clark, Kristine Ma, James Monaghan 2023. Sensor Synthesis and Storage Protocol. [protocols.io https://dx.doi.org/10.17504/protocols.io.rm7vzxxz4gx1/v1](https://dx.doi.org/10.17504/protocols.io.rm7vzxxz4gx1/v1)

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Protocol status: Working

We use this protocol and it's working

Created: August 24, 2023

Last Modified: August 24, 2023

Protocol Integer ID: 86926

Keywords: fabrication of dna nanosensor, dna nanosensor, detailed protocol of the dna scaffold stability study, dna scaffold stability study, dna scaffold, dna, acetylcholinesterase, enzyme, sensor synthesis, phab dye

Funders Acknowledgements:

SPARC

Grant ID: 1-OT2OD024909-01

Abstract

This protocol explains the fabrication of DNA nanosensors with pHAb dye used for stability analysis, its characterization, and the detailed protocol of the DNA scaffold stability study over a period. Here, the DNA nanosensor and Acetylcholinesterase (AChE) enzyme were analyzed separately for the stability study. The enzyme binding with the DNA scaffold might affect the stability and structural integrity. One of the significant downsides of analyzing AChE with DNA is cost-effectiveness.

Guidelines

Custom amine functionalized single-strand DNA oligonucleotides were ordered from Sigma-Aldrich, and the strands (L2 and L4) were conjugated with pHAb dye (Amine reactive, Promega). The DNA scaffold was self-assembled by incubating the five distinct DNA strands (L1, L2, L3, L4, and L5) following a temperature gradient transitioning from 95°C to 4°C over a period of 6 hours. This controlled temperature change was facilitated using a thermocycler (Mastercycler® nexus X2, Eppendorf) within a Tris-EDTA buffer (1X TE buffer, 10mM Tris and 1Mm EDTA). The DNA scaffold was purified and isolated from various impurities using a size-exclusion column (Superdex 200 increase 10/300 GL column, GE Health). A stability study was performed using various protectants like sucrose and trehalose at various temperatures (room temperature and 50°C) over a period of one week. Followed by that, Gel electrophoresis was used to analyze the structure formation. Based on this analysis, it has been verified that both sucrose and trehalose serve as effective stabilizers for maintaining structural integrity during a one-week shelf study.

Materials

The custom Oligonucleotides were ordered from Sigma-Aldrich providing specifications such as dry-state condition and amine-modified sites.

Oligo Custom Sequence

L1 TCTGAAAGTACTGACGAGCTAACATGGCTGCGGCAGAATCCCTCACTATGCGAGTTGACC

L2 [Amine C6] GGTCAACTCGCATAGTGAGGGAGTCGTGAGTACTAATAGT

L3

ACTATTAGTACTCACGACTCGATTCTGCCGCAGCCATGTTTCGCCAGAATGCCAGTCAGCATTAAAGGAGAGCTCAGGGC
A

L4 TGCCCTGAGCTCTCCTTAATAGCCTACATCCTACCAGAGG [Amine C7]

L5 CCTCTGGTAGGATGTAGGCTGCTGACTGGCATTCTGGCGAA GCTCGTCAGTACTTTTCAGA

Unless specified differently, reagents were acquired from either Sigma-Aldrich or Fisher Scientific and utilized without further modifications. The GelRed® Nucleic Acid stain was purchased from Biotium. The Trirack DNA Loading dye (6x), TBE buffer, and Gene Ruler 100 bp DNA ladder were purchased from Thermo Fisher Scientific. Absorbance Spectrophotometry was carried out using a spectrophotometer (Nanodrop™ 2000c, Thermo Scientific). The concentration of the DNA scaffold was assessed based on the absorption of the DNA structure at 260nm, with an attenuation coefficient (ϵ) = 2675900 M⁻¹cm⁻¹. pHAb amine-reactive dyes were purchased from Promega (Catalog #G984A). The DNA strands were filtered using a 3k centrifugal filter (Amicon, ultra – 0.5mL) and the DNA scaffold was reconcentrated using a 100k centrifugal filter (Amicon, ultra – 0.5mL). NAP-10 columns were obtained from Cytiva – Sephadex G-25 DNA grade. HPLC (Infinity 1260, Agilent) was used to purify the individual strands and the scaffold for both columns (size exclusion and reverse phase). The column used for the reverse phase was purchased from Agilent Technologies (Eclipse Plus C18 5µm 4.6×250mm column), and the size exclusion column was purchased from GE Health (Superdex 200 increase 10/300 GL column). The Lyophilizer was acquired from Labconco, specifically the Centrivap series. The Sonicator (BRANSON 2000) was purchased from Sonics.

Before start

General warnings:

1. If any saturated peak is observed in HPLC, make sure to inject Nanopure or MilliQ water (20 µL). Always stick with 100 µL injection in HPLC.
2. Be careful while pipetting the samples during the EtOH precipitation. You could lose some nmoles.
3. Always wash the column for 30 – 40 mins, there could be buffer, or sample get stuck in the column.
4. Make sure to have n=3 replicates while performing stability study.
5. For long term storage, store the extra scaffold in -80°C.
6. While adding AchE to the cuvette, make sure to add it very quickly atleast within 2 – 3 seconds.

Conjugation of pHAb to single-stranded DNA oligonucleotides

1

As shown in Figure 1, the buffer exchange was performed for the strands L2 and L4 (20 nmole each) for the pHAb conjugation in 0.1M NaHCO_3 solution of pH= 8.1 using a 3k centrifugal filter (Amicon Ultra-0.5mL). Followed by that, strands (L2 and L4) are placed in the centrifuge at 9000xg for 45mins at -4°C . Equal volumes of DNA and Dimethyl sulphoxide (DMSO, VWR life science) (1:1) were added directly to the dried pHAb in the manufacturer-provided tube (Promega). The sample was placed on the shaker at room temperature overnight for DNA-pHAb conjugation.

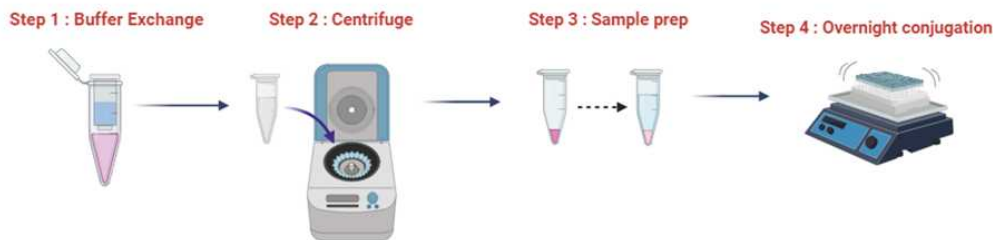


Figure 1: This scheme depicts the overall steps involved in DNA-pHAb conjugation.

2

Purification of the pHAb conjugated Oligonucleotides

3

After the overnight conjugation process, the samples will be subjected to ethanol precipitation to separate the modified single strands from the excess pHAb dye as visually represented in Figure 2. In an Eppendorf tube, it is important to combine the total volume of DNA strand (1x) with 3M sodium Acetate from sigma (0.1x), followed by the addition of 100% Ethanol(3x) and 2 μL of glycogen. The purpose of glycogen is intended to enhance the efficiency of the precipitation process. Samples are stored in

a deep freezer (-80°C) for 30 – 40 mins and later placed for centrifuging at the rate of 2000xg for 30 – 40 mins at -10°C . Finally, excess ethanol was removed, and the sample was resuspended in 900 μL of nanopure water.

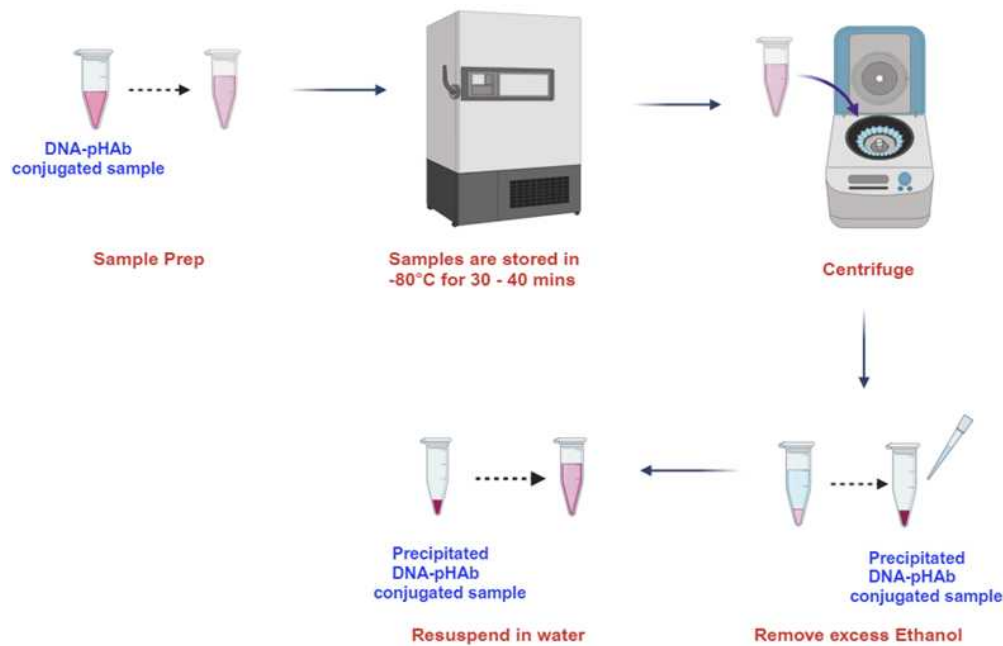


Figure 2: This workflow illustrates the comprehensive ethanol precipitation procedure.

- 4 Following the ethanol precipitation step, the NAP-10 column purification was performed using Cytiva's Sephadex G-25 DNA grade column. As depicted in Figure 3, the columns were washed thrice. Afterward, when the sample was introduced, 1.5 mL of nanopure was added. Subsequently, a 1.5 mL sample volume was collected and stored in a deep freezer (-80°C) for 30 minutes before undergoing lyophilization using the Centrivap lyophilizer from Labconco for approximately 4-5 hours. The resulting samples were then reconstituted in 100 μL of nanopure water.

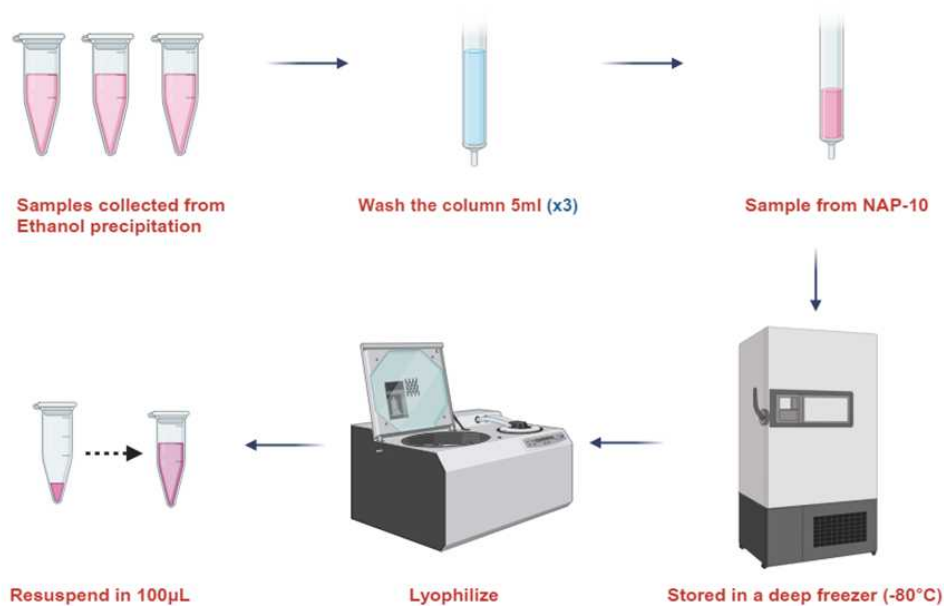


Figure 3: Workflow of NAP-10 Column Purification

4.1

HPLC Purification of the conjugated single-strand DNA

- 5 After DNA – pHAb conjugation, the strand was purified using a Reversed phase chromatography (Eclipse Plus C18 5µm 4.6×250mm column, Agilent technologies) via HPLC (Infinity 1260, Agilent). This purification method is used to remove unreacted reagents and isolate the pHAb-DNA conjugate. The mobile phase used for this method was 50 mM TEAA (triethylammonium acetate) buffer, pH 7. As shown in figure 4, the sample of L2 and L4 was collected in the time interval between 7.5 mins and 11 mins. Collected fractions were compiled and lyophilized.

During the process, two signals were monitored. Specifically, one UV signal at 260nm was used to measure the absorbance of DNA and a fluorescence signal was observed, with an excitation wavelength of 532nm and an emission wavelength of 560nm, associated with pHAb.

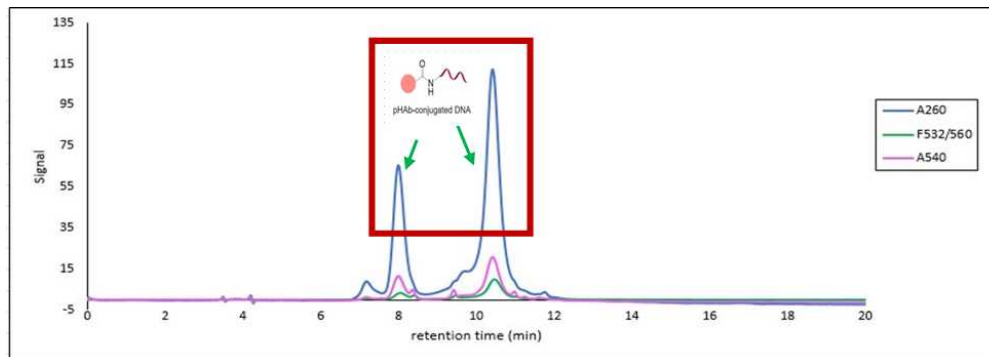


Figure 4: This HPLC chromatogram demonstrates the collected peak of the DNA+pHAb conjugate at the following retention times for L2 (7.5 mins and 11 mins).

Assembly of the DNA Scaffold

6

As shown in following figure 5, The process of self-assembling DNA scaffold involves incubating the 5 DNA oligonucleotides (Plain L1, pHAb conjugated L2, Plain L3, pHAb conjugated L4, Plain L5) together and subjecting them to a temperature gradient ranging from 95°C to 4°C over a period of 6 hours. This incubation was carried out using a thermocycler (master cycler nexus GX2).

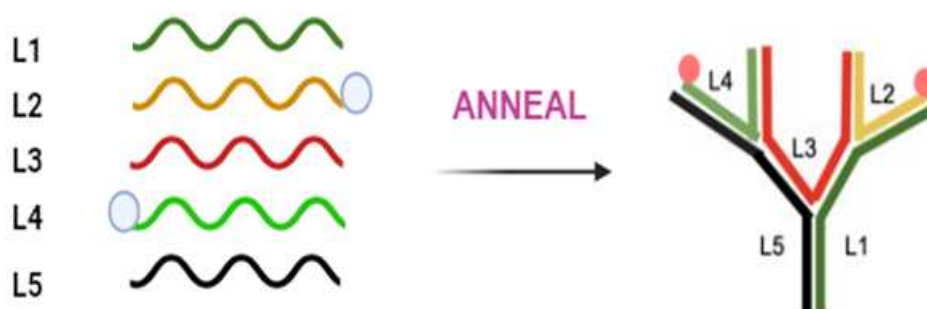


Figure 5: Scheme shows the DNA sensor assembly used for studying the storage protocols

HPLC Purification of the DNA scaffold

- 7 Following the annealing process, the DNA scaffold underwent purification using size-exclusion chromatography (Superdex 200 increase 10/300 GL column, GE health) through HPLC (Infinity 1260, Agilent). The method employed Phosphate Buffered Saline (1x) 0.0067M (PO₄), supplied by Cytiva, as the mobile phase. Much like the reversed phase (RP) column procedure, this method also involved monitoring of a single UV signal at 260nm, which provides insight into the DNA and a fluorescence signal for pHAb (Ex = 532nm, Em = 560nm).

The DNA scaffold peaks were collected from the HPLC within the time range of approximately 9.5 to 15 minutes, as indicated in Figure 6. The pHAb conjugated signals were also highlighted in red. The blue signal represents the absorbance of DNA (A₂₆₀) and the pink signal represents the fluorescence signal of pHAb (F₅₄₀).

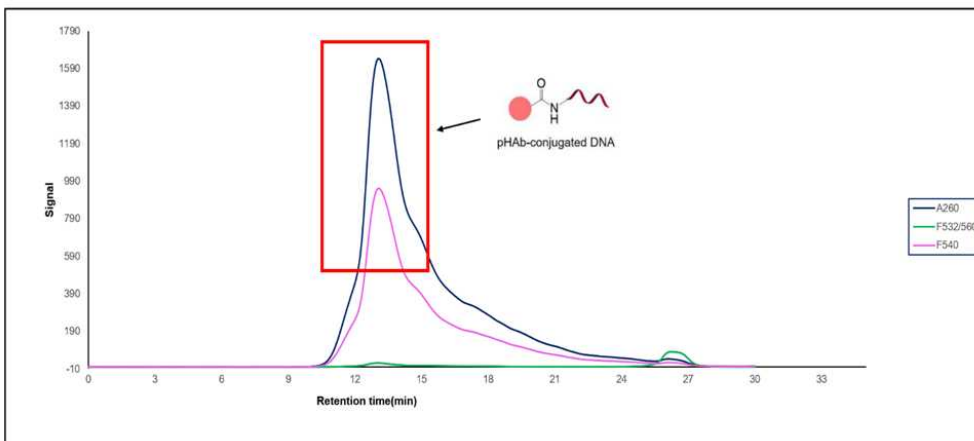


Figure 6: Depicts the peak of the DNA scaffold collected.

Lyoprotectants for Storage Study

- 8 With reference to (1) a modified DNA nano sensor structure of the DNA scaffold was designed which has five different DNA strands where two DNA strands (L2 and L4) are conjugated with pHAb dye (Amine reactive, Promega) by the vendor and the other strands (L1, L3, L5) are considered plain. The modified DNA with pHAb oligonucleotides follows the similar protocol as mentioned above.
- 9 Followed by the scaffold purification, the modified DNA was concentrated using centrifugal 100k filter by TE buffer. Samples were placed in centrifuge at 2000xg for 10mins at 4°C.
- 10 Once the purified DNA scaffolds were concentrated, the UV signal of A260 was measured using thermoscientific nanodrop/2000c. Based on the calculation, yield of the nanomole was further calculated.
- 11 Based on the calculation set the desired volume (20 μ L) for each timepoints (t=4) for the stability study. Two major lyoprotectant such as sucrose and trehalose were used to determine the shelf study of the Scaffold. The scaffold sample prepared from the stock was around 5 μ M for each desired time point.
- 12 Aliquoted samples are then stored at -80°C for 4-5 hours. Lyophilize the scaffold till it attains the powdery state

- 13 Stability study is performed to determine the maximum incubation period by the thermal decomposition of the Scaffold under various condition like with protectants (sucrose/trehalose) and no protectant to compare the stability.
- 14 Ideally, 5% of sucrose and trehalose were used for the various time points from t = 0 to a week. Make sure to prepare n=3 replicants. Here a total of 72 samples were prepared under 4 different formulations at 2 different temperatures (50°C, room temperature) for each protectant (sucrose, trehalose, no protectant).

LYO PROTECTAN T FORMULATI ON	NO PROTECTAN T	SUCROSE 5%	TREHALOSE 5%			
TIME/ CONDITION	RT	50°C	RT	50°C	RT	50° C
1 W						
96 H						
48 H						
24H						

- 15 Based on the different time points and at different temperatures, sample is placed on the room temperature and in sonicator for 50°C (water bath).
- 16 Following the study, the output was analyzed in the 4-12% Native PAGE gel (10 wells) with the below mentioned figure 7 time point order.

1	2	3	4	5	6	7	8	9	10
Ladder	-80°C	1 W	96 H	48H	24H	1W	96H	48H	24H
		50°C				Room Temp			

Figure 7: Table shows the time point analyzed in each lane of the Native PAGE gel

As depicted in Figure 8, the degradation of samples stored at room temperature and at 50°C

was observed over the course of the week. The degradation analysis was graphed using FiJi, revealing that the half-life at 50°C exhibited a notable consistency throughout the degradation process.

DNA Scaffold degradation analysis

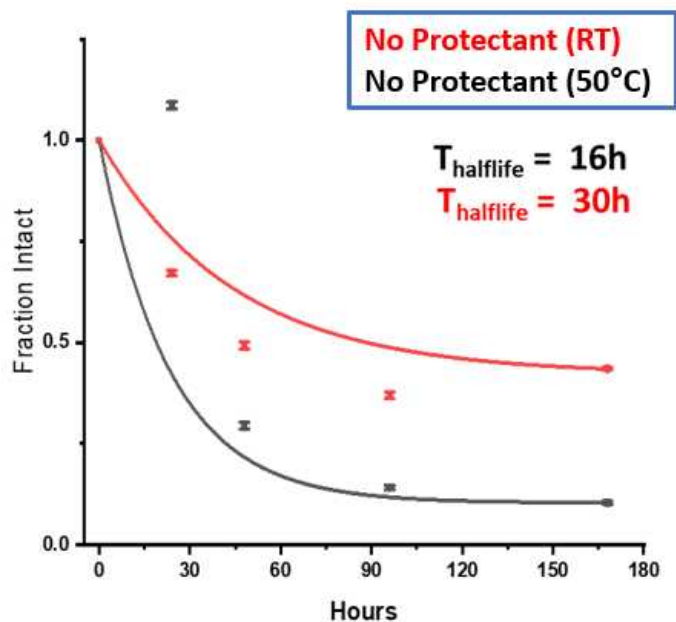


Figure 8: DNA scaffold degradation analysis from gel electrophoresis over a course of 1 week without any protective agents.

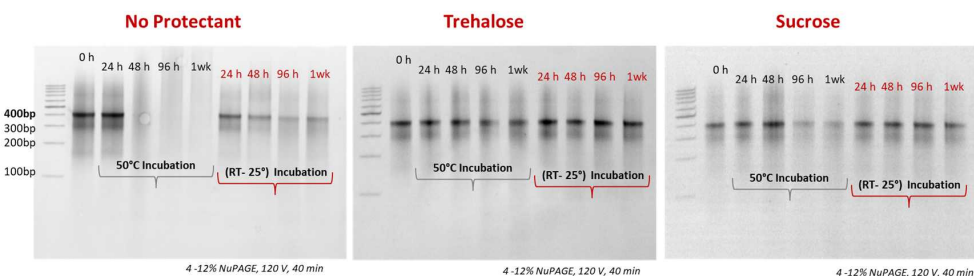


Figure 9: Representative gel image of the DNA scaffold for the no protectant group, Trehalose, and Sucrose

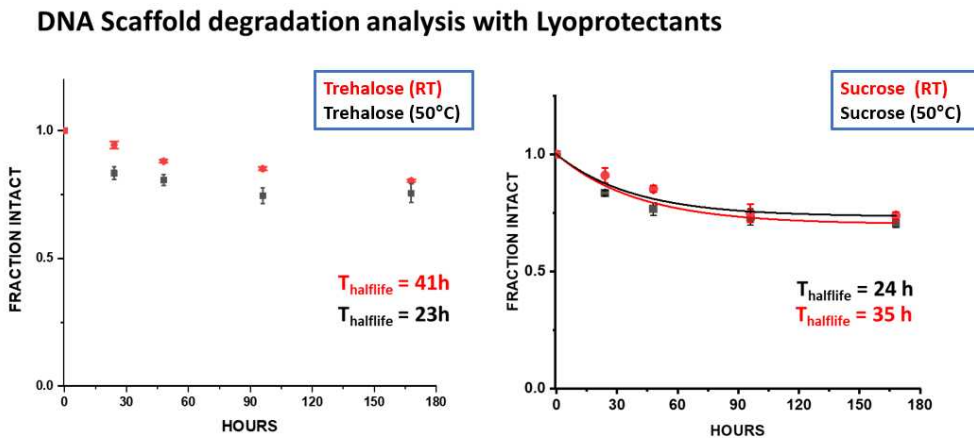


Figure 10: DNA scaffold degradation analysis from gel electrophoresis over a course of 1 week with protective agents, trehalose and sucrose.

The gel analysis of both lyoprotectants exhibits similar characteristics. From the figure 10, it is evident that both lyoprotectants, namely sucrose and trehalose exhibited a high degree of effectiveness.

Acetylcholinesterase (AChE) Stability Study

17

For AChE purification, size-exclusion chromatography was utilized given that the commercially accessible AChE purchased from Sigma was not completely pure. Following purification, the AChE fractions were concentrated using 50kDa Amicon centrifugal filter. The final concentration ranged from 0.5μM to 2 μM, and stock solutions were prepared accordingly. To evaluate the activity of the AChE enzyme, a freshly prepared Ellman's assay solution containing 3mL 100mM NaHPO₄ (pH = 7.8), along with 40μL of Acetylthiocholine chloride (Millipore – Sigma) and 100μL of 5,5'-dithio-bis-(2-nitrobenzoic acid) DTNB (Ellman's Reagent, Thermo Scientific) was used. The entire experimental procedure was carried out using Nanodrop™ 2000c (Thermo Scientific) spectrophotometer in a UV cuvette of Z=8.5mm (ultra-micro, BRAND). This cuvette was loaded with 200 μL of Ellman's Assay solution and 400 μL of 1x PBS (filtered, Sigma Aldrich), into which 40 μL of AChE enzyme solution was swiftly introduced within a 1 to 2 second timeframe. The enzyme activity was observed at the absorbance of Ellman's Assay (412nm) and at the AChE absorbance (280nm). The

measured enzymatic activity was around 1000U – 4000U. The enzyme samples were divided into aliquots based on three distinct conditions, encompassing both with and without lyoprotectants as outlined in the table provided and lyophilized for 3 – 4 hours. Depend on the time like 24H and 48H, the enzyme activity was observed at 50°C.

Time/ Condition	Trehalose	Sucrose	No Protectant
t = 0	1175.7	1175.7	1175.7
t = 24H	1255.1	774.0	488.8
t = 48H	1238.4	516.0	464.4

Table 1: Preliminary data on AChE activity after storing them in dry state in 50C.

From Table 1, it has been analyzed that trehalose has enzyme active above 1000 U, whereas sucrose slight retains the enzyme activity (>500U). No protectant showed lowest activity (<500U).

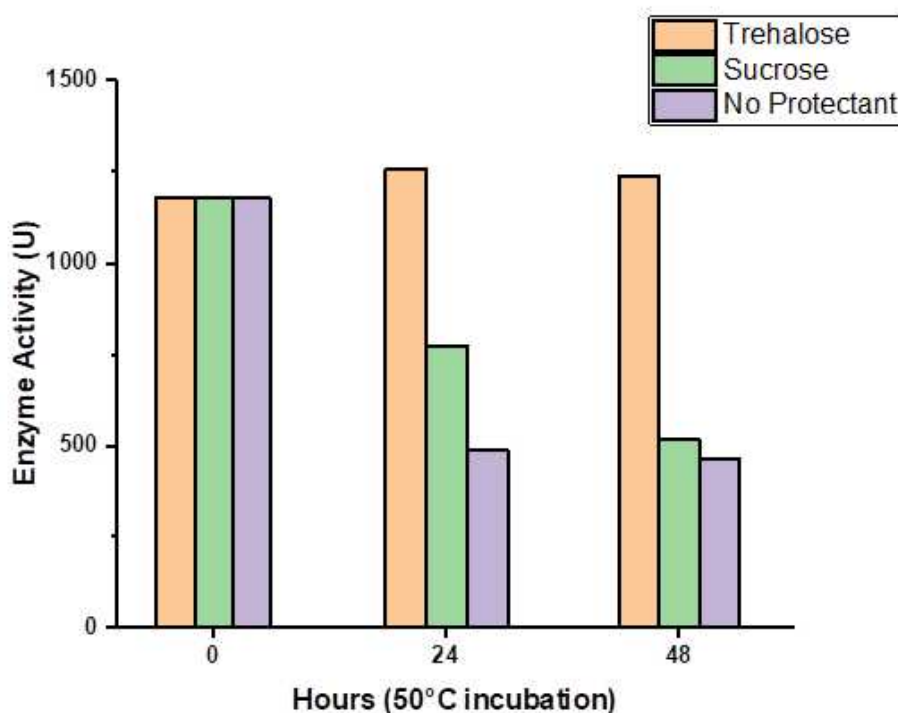


Figure 11 : Bar graph of the Enzyme activity of AChE at 0 h, 24 h , and 28h .

