Experiments

Introduction

During strenuous exercise, insufficient oxygen delivery will eventually lead to the shift from aerobic to anaerobic respiration in muscle cells (Brooks, G. A et al., 2009). As a byproduct of glucose metabolism, lactate accumulates in the blood during exercise. Thus, lactate levels are a good indicator of exercise intensity and can be used to predict acute fatigue as well as efficacy of interval training (Huang T et al., 2025). As of today, athletes often rely on lactate analysers to monitor blood lactate levels between exercise intervals (Swart J, et al., 2004). This instrument requires a blood sample and will only inform of current lactate levels. Over the years, interest has grown for the potential development of continuous lactate monitoring (CLM) methods.

The potential of such an instrument has been tested to moderate success. A common minimally invasive approach involves a biosensor embedded in a patch containing microneedles that penetrate the skin outer layer. By sampling interstitial fluid (ISF), the device enables lactate monitoring, setting it apart from conventional blood lactate meters. Such a method can be found in the paper by Freeman et al. (2023). Of course, the main issue with this is the known delay of lactate levels accumulating in the ISF due to diffusion from the blood, but this is something than will have to be accommodated for by using i.e. machine learning to optimise pattern recognition over time.

Objective

We aim to develop a proof-of-concept minimally delayed CLM system that uses enzyme-coated microneedles to convert lactate in the ISF into a measurable signal. To achieve this, two types of experiments need to work in parallel: 1. cloning, expression, large-scale production, and purification of functional enzymes suitable for this reaction; and 2. developing a standardised electrochemical protocol to detect the enzyme-catalysed reaction and quantify the signal for different lactate concentrations. Two enzymes were selected for this purpose: lactate dehydrogenase (LDH) and lactate oxidase (LOD), as both can efficiently catalyse the conversion of lactate in the body. See Figures 2 and 4 for structure models of both enzymes.

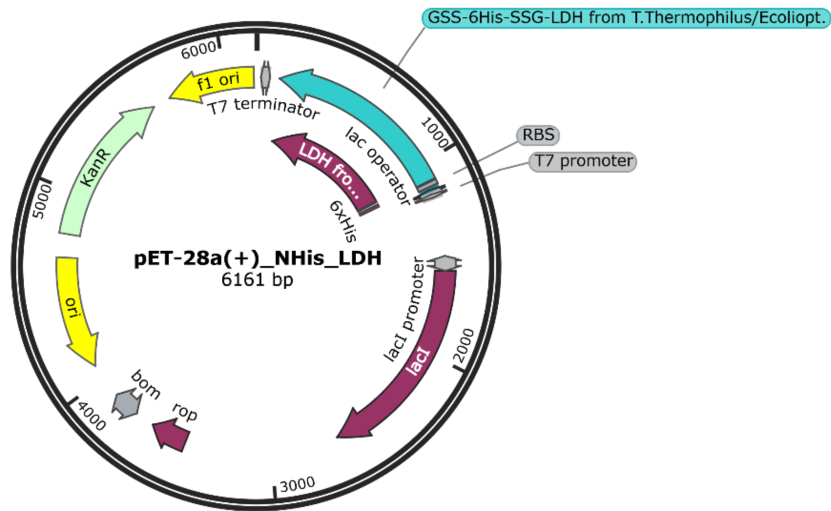

***Figure 1**: Map of the designed pET28a vector with LDH insert from* T. thermophilus*, with His-tag and optimised for E. coli expression. Made with Snapgene Viewer 8.2.*

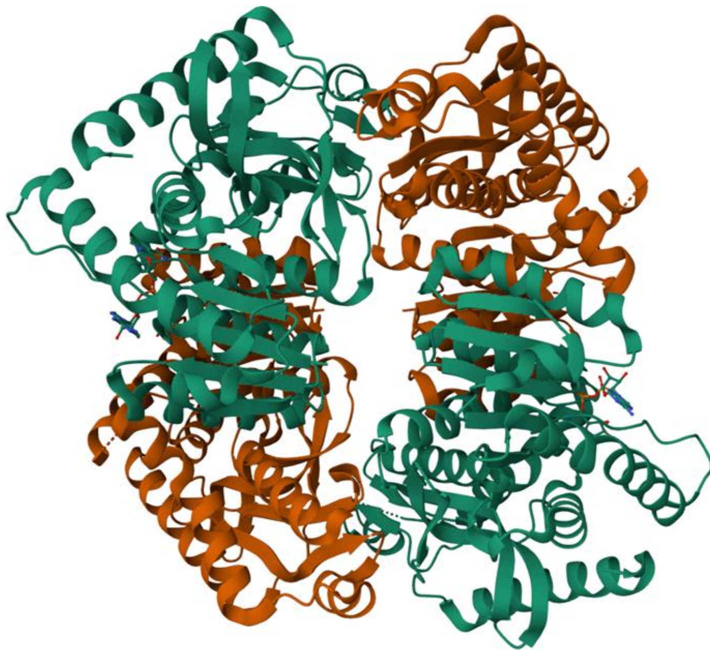

***Figure 2**: X-ray model (2E37) of entry Q5SJA1 (L-lactate dehydrogenase from* T. thermophilus*) retrieved from UniProt (The UniProt Consortium, 2025) and visualised using RCSB PDB. Water molecules were removed.*

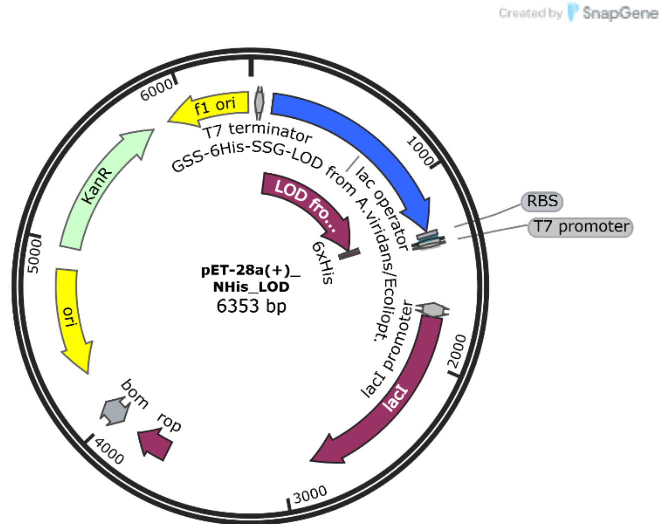

Figure 3*: Map of the designed pET28a vector with LOD insert from* A. viridans*, with His-tag and optimised for E. coli expression. Made with Snapgene Viewer 8.2.*

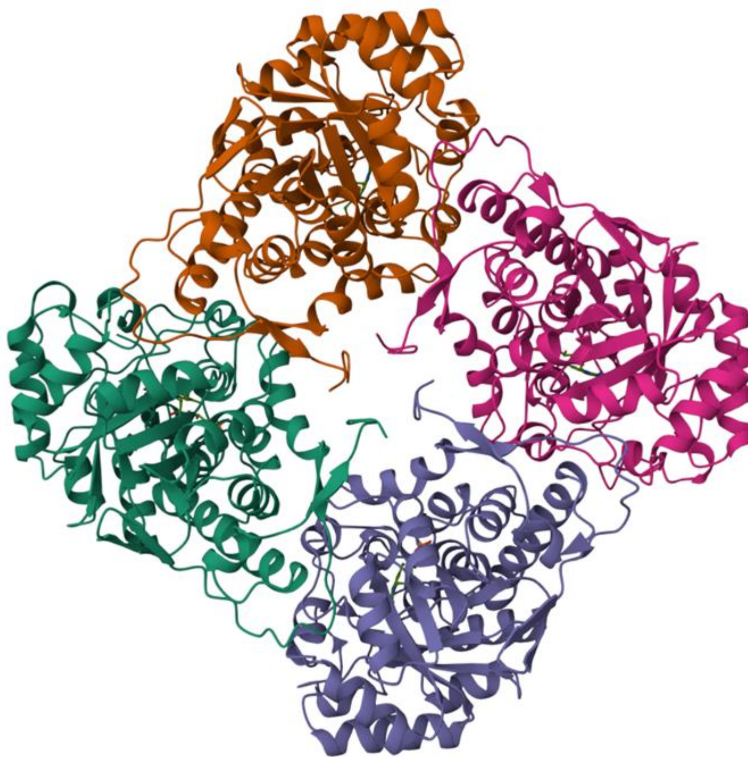

Figure 4*: SWISS-MODEL (A0AAU8UAJ4_5-374:2j6x.1.D) of entry A0AAU8UAJ4 (lactate oxidase from A. viridans) retrieved from UniProt (The UniProt Consortium, 2025) and visualised using RCSB PDB. Water molecules were removed.*

Regarding the expression of our own proteins, the LDH gene utilized for constructing our recombinant originated from *Thermus thermophilus* (*T. thermophilus*) while the LOD originated from *Aerococcus viridans* (*A. viridans)*. The reference for these constructs was

both the article by Godino et al. (2023) and the UniProt entry A0AAU8UAJ4 for LOD, and the UniProt entry Q5SJA1 for LDH. Searches were performed and eventually items were codon-optimized for *Escherichia coli* (*E. coli)* (see sequences below). For reconstruction, the plasmid used as vector for the gene inserts was pET28a (see Figure 5).

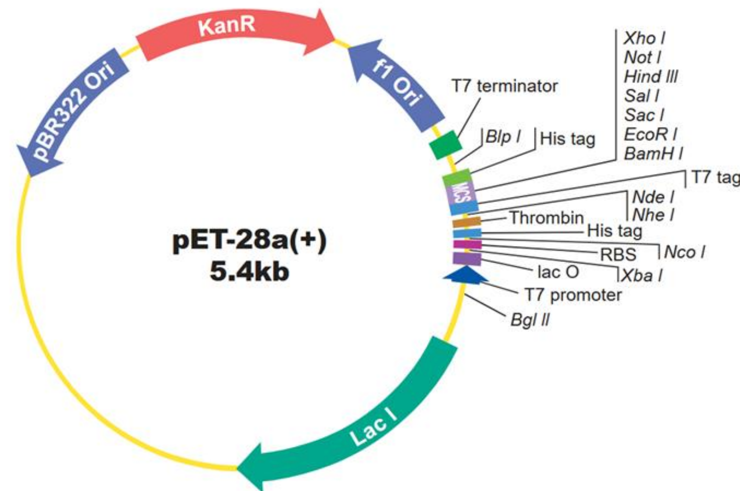

***Figure 5**: Visualisation of the vector pET28a, retrieved from GenScript (GenScript, n.d.).*

```
> GSS-6HIS-SSG-LDH from T. thermophilus E. coli optimised (969 bp)
AAAAACCCAGAGCGAAGGCCGCTTCTTTCAGGATCTCCGCGCTACGACGCAGGGCTTCACGTTCCTCCGGAGACAGGC
TCGGATAAACGGTGCCCTCGACACCACCCGCGCCCAAGATGCGCGGCAGGGACAGGCTCACCTCTAACACGCCTTCAACC
TCCGGGGTGAATGCGCTAACGGTGTACACACCCTTTTCATCGGTCAGGATCGCACGCACCAGGCGAGCGAGACCCGCACC
AATGCCGTAATACGTCGCACCTTTTCCTTCAATGATGCGGTATGCCGCACGACGAACGCCTTCATCAATGCGCGCGCGGT
CCTCCGGGGACAACGCGCGACCTCTTGCCTCCGCGAACTCGAGCAGCGGAACACCGCCAACTTGAGCGCTCGACCACACC
AGCACTTCGCTGTCGCCGTGCTCGCCCAGCACGTACGCATGAACGCTCTGTGGCGCAACGCGCAGATACTCGGCCAGGAG
CGCACGAAAACGCGCGGTATCCAGAATGGTGCCGGAACCAACAACACGACCAGGCGGCAGACCAGACAAACGGTAGGCCA
CCTGGGTCATAACATCCACCGGATTCGTTGCGACCAACAACACAGCTTCCGGTGCCGCCTCAAGAACACGTGGTACTACC
TGAGCGAACACTTGTGCGTTGCGGTCTAACAGTTGCAGACGGGTTTCACCCGGACGTTGGGCCACGCCCGCGGCCAACAC
GACTGCACGCGCGCCCTCCAGGTCACCATAGCTGCCCGCACGGACCCAAACCGGATGGGCAAACGGAGTCGCGTGCAGGA
TGTCTTCCGCGTGCGCCTGAGCCAGCTTTCTGTCCAGATCAACCAGCACAACCTCGCGGGCGACGCCCAACAACGCCAGT
GCGTAAGCCGTCGCTGAACCCACCATACCTGATCCTACTATCCCAACTTTGCCGCTGCTGTGATGATGATGATGATGGCT
GCTGCCCAT
```

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>GSS-6His-SSG-LOD from A. viridans E. coli optimised (1161 bp)
TTAGTACTCATAACCGTACGGGTTGTCGAACAAGTCCAAGCCCTTCAGGTCCTCTACGTTCTGGCTACCAGTCAACTGCA
TCACACGGGTGAGATCTTTTTGGAAATAATCCAGAACAGAGTACGCACCTTGCCAACCGCCTAATGCCAGACCGAACAAC
ACCGGGCGGCCCAGAGCCACCACGTCCGCACCGGAAGCCAACGCCTTCGCAACGTGCTCTCCGCGACGCACGCCAGAGTC
GAAAACGATCGGAACACGTTTGTTGACACGTTCCGCAATTGCCGGCAGGGTGTCAAAGCTACCAGGTGCTTCGTACAGCT
GGCGAGCACCATGATTGCTAACCCAGATGCCGCTCGCGCCTGCCTTAATGGCCATGTCTGCATCTTCCGGATGTTGAATA
CCTTTCACGAAAACCGGCAAGCCGCTGTGGGCAGCGATTTCTTCGATGTCACGCGGGGAGATTTTCTGTTTGGACGCGCC
GTAGATGTTGTTCAGCGACATGCCTTCGGCCGTACCACGCAGATAACGCTGAACAATTGGCATGCCGAACGGATAAACAA
ACTTATTTTTCACGTCGCGATCACGATTACCGGAAACGGTGCTGTCGGCAGTGAGAATAATCGCCGTCGCACCATCGCTC
TTCGCCTCGTCCAGGATGTCACGGTTTTGCTGATCGTCCTTCGCCATGTAAATCTGAAACCAGCGCGGACCGCCGTTCAG
ACCCTCCGAGATCTCTTCAAAGGTAGCACCGCTATACGCGCTAATACTCATAATCGTGCCGAACTCGCTCACTGCTCTCG
CGGTACCGGCTTCTTTGGTGGTGTGTGCAAGCCCGTGAGCGGCAATCGGAGCCATGATAAATGGGGCTTTGATCTTGTGG
CCCAGGATTTCCGTGCTGGTATCCGGCGCCTCGACATCTTGTGCCAGACGCGGGTACAGCAGCTTATGTTTCCACGCACG
ATCATTCGCGCGCTTGGTCCATTCATCGCCAGAAGCACCCGCAATATAATTAAAACCACCATGCGGAACAACTTTCGACG
CCTCCTCTTCCAGATCATAGGTGTTCACGACGTCGATGTACTTGATCTCGCTCGGAGCATTATATTCTATATCATTGTTA
TTGCCGCTGCTGTGATGATGATGATGATGGCTGCTGCCCAT
```

Commercially available LDH and LOD were also used during experiments in our standardised electrochemical protocol as an additional reference. To this end, our commercially bought LDH originated from making a recombinant in *E. coli* while LOD originated from *Lactococcus lactis* (*L. lactis*) subsp. c*remoris* IFO3427 (Toda & Nishiya, 1998). Sizes were determined as about 34 kDa for the commercial *E. coli* recombinant and around 33 kDa for the *T. thermophilus*, both determined by the gels run during the project. As for the LOD, the size of the *L. lactis* subs. *cremoris* is reported as about 40 kDa determined from sequencing the construct in the paper by Toda et al. (1998). Meanwhile the size of our *A. viridans* LOD construct is about 42 kDa, determined from the gels run during the project. This can also be calculated by having a look at the sequence additions (Table 1).

Table 1*: Calculations for predicted protein lengths of constructs of LDH (T. thermophilus) and LOD (A. viridans) optimised for E. coli.*

Calculation for the genes and proteins
1. LDH construct - Total amino acids: 322 AA - Mass of native LDH (309 AA) = 32.7 kDa - Extra 13 AA = His$_6$ (6 AA) + GSS + SSG (6 AA) + start codon Met (1 AA) Approximate extra mass: - His$_6$: 6 × 155 Da = 930 Da ≈ 0.93 kDa - GSS + SSG: 6 AA (Gly/Ser) → Gly = 75, Ser = 105 GSS = 75 + 105 + 105 = 285 Da, and SSG = 105 + 105 + 75 = 285 Da GSS + SSG Total = 570 Da ≈ 0.57 kDa Start codon Met = 149 Da ≈ 0.15 kDa Extra mass total = 0.93 + 0.57 + 0.15 ≈ 1.65 kDa **Total LDH protein mass = 32.7 + 1.65 ≈ 34.35 kDa**
2. LOD construct - Total amino acids: 386 AA - Mass of native LOD (373 AA): we approximate using average amino acid mass: ~110 Da per residue - 373 × 110 Da ≈ 41,030 Da ≈ 41.03 kDa - Extra 13 AA = His$_6$ (6 aa) + GSS+SSG (6 aa) + start codon Met (1 aa) - Extra mass total = same as LDH extra = 0.93 + 0.57 + 0.15 ≈ 1.65 kDa **Total LOD protein mass = 41.03 + 1.65 ≈ 42.68 kDa**

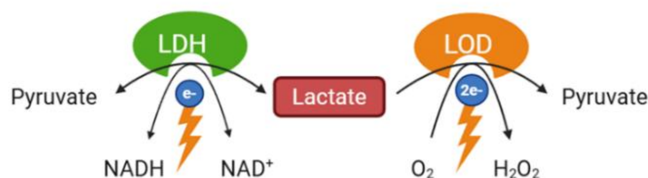

Figure 6*: The formation of pyruvate from lactate through the catalyzation of LDH and LOD respectively. Made with BioRender.*

Like how lactate and nicotinamide adenine dinucleotide (NAD^+) can be created by catalyzing pyruvate and NADH (the reduced form of NAD^+) with LDH as described by Kozinetz et. al.

(2024), the reverse reaction can also take place by sampling ISF lactate through LDH-coated microneedles. NAD^+ will act as a coenzyme, transferring the hydride ion and converting to NADH through LDH catalyzation (Lafuente et. al 2024). By attaching an electrode surface to the microneedle with potential, oxidation of NADH will take place and generate current inferring to the present lactate concentration.

Likewise, sampling through LOD-coated microneedles allows LOD to catalyze a reaction with the lactate present in the ISF to create pyruvate and hydrogen peroxide (H_2O_2), such as described by Chien et al. (2022). An electrode surface attached to the microneedles would also be required to catalytically decompose H_2O_2, producing oxygen gas (O_2), water (H_2O), and electrons ($2e^-$). By monitoring these generated electrons, the electrode surface would be able to determine the given quantity of H_2O_2 proportional to the present lactate concentration.

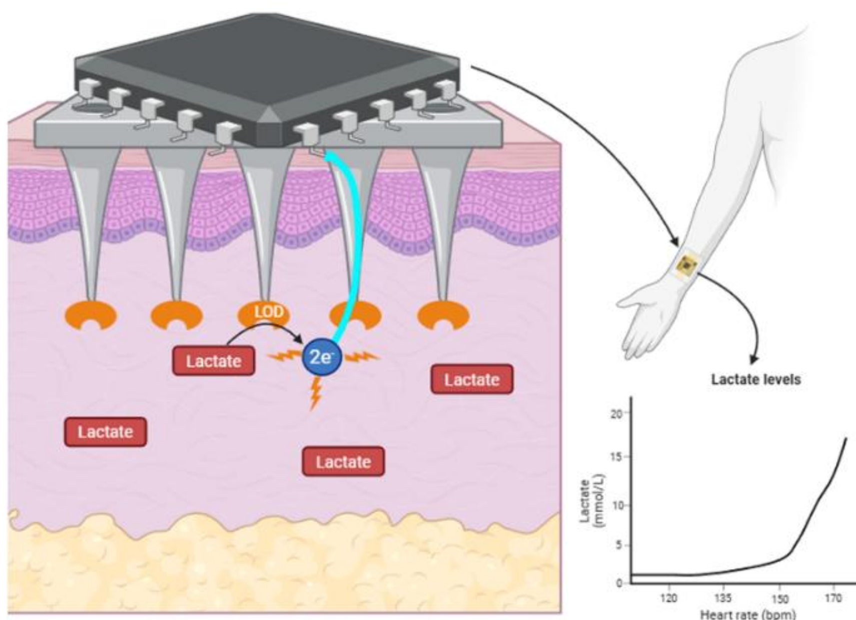

Figure 7*: Use of enzyme-coated microneedles through ISF to generate electron current for lactate detection. Made with BioRender.*

Isolation of plasmid

Materials: See protocols featured.

This protocol is to isolate a plasmid from grown bacteria, either to use as vectors or to isolate constructs already transformed into the bacteria to use for other purposes.

1. Thaw 60 µL of cells on ice.
2. To plate the cells, follow **[Plating of bacteria]** protocol.
3. The day after, check the plates for successful growth and pick colonies following the following protocol: **[Pick colonies]**.
4. After overnight (O.N.) incubation period, follow the following protocol for miniprep: **[Miniprep]**.

Solutions for bacteria growth

Luria-Bertani (LB) medium

- LB Broth (Merck, Cat. #L3522)
- dH_2O

From Merck's own page on the product with Cat. #L3522 (Merck, n.d.) it is recommended that one suspends 25 grams (g) of medium in 1 L of deionized water (dH_2O) and mix well, before sterilization by autoclaving at 121°C for 15 minutes (min). Keep the medium in a cold room. Before use, add antimicrobials to a final concentration of 50 µg/mL.

Kanamycin

- Kanamycin (ITW Reagents, Cat. #A1493,0025)
- dH_2O

Dissolve Kanamycin to a final concentration of 50 mg/mL in dH_2O (50 g of Kanamycin to 1 L dH_2O) and filter-sterilize the solution. Aliquot into 1.5 mL Eppendorf tubes and store at -20°C. If using in LB medium, add 1 mL of stock per 1 L to achieve a final concentration of 50 µg/mL.

Super Optimal broth with Catabolite repression (SOC) medium

- Tryptone (VWR, Cat. #84610)
- Sodium chloride (NaCl) (Merck, Cat. #S7653)
- NaOH (Merck, Cat. #28244.295)
- Glucose (Merck, Cat. #G7021)
- $MgCl_2$ (Merck/Sigma-Aldrich, Cat. #M8266)
- Yeast extract (Merck, Cat #70161)
- Potassium chloride (KCl) (Merck, Cat. #60130)
- dH_2O

Note: Protocol used was developed by the lab.

Prepare 1 M glucose by dissolving 18 g of glucose in 90 mL of dH_2O. After the sugar has dissolved, adjust the volume to 100 mL with dH_2O and filter the solution using a 0.22-µm filter. Then, dissolve 20 g tryptone, 5 g yeast extract and 0.5 g NaCl in 950 mL dH_2O. Add 10 mL of 250 mM KCl and adjust pH with 5 M NaOH to pH 7.0. Adjust the volume to 1 L with dH_2O and autoclave the solution. Add 20 mL of the 1 M glucose solution to the medium. Just before use, add 5 mL of sterile 2 M $MgCl_2$.

Incubate single colony

Materials:

- Falcon tubes of appropriate size
- LB medium from **[Solutions for bacteria growth]**
- Kanamycin from **[Solutions for bacteria growth]**

Follow this protocol to incubate single bacteria colonies for growth in medium.

Follow protocol **[Screening for successful transformant]**. Identify individual colonies separate from other bacterial growths. In a hood, use a sterile loop to transfer a colony to a tube containing LB medium with antimicrobials. Pick one colony per tube, stir the loop in the medium, and discard the loop in the hood waste. Label the tubes and incubate O.N. on a shaker at 37°C, 200 rpm. To allow optimal air access during growth, loosen the lid of the tube.

Plating of bacteria

Materials:

- LB plates
- LB medium from [Solutions for bacteria growth]
- Kanamycin from [Solutions for bacteria growth]

You can plate bacteria for growth using other recently grown bacteria cells, thawed cell samples, incubated LB medium or scraped-off material from bacteria in cryogenically frozen tube.

1. If growing your own cells, centrifuge at 12,300 × g for 1 min, discard supernatant under sterile conditions (such as a biosafety cabinet or near a Bunsen Burner) and resuspend in 60 µL of the remaining supernatant (avoiding bubbles).
2. In a hood, spread cells evenly on LB plates with antibacterials. You may include a plate for cells with the empty plasmid vector as control. If using a cryostock solution to streak the plates, ensure that streaking occurs quickly to prevent the bacteria stock from thawing. This is repeated freeze-thaw cycles will create ice crystals that will kill the bacteria.
3. Lid the plates and label appropriately. Incubate O.N. at 37°C in an incubator containing a water tray to prevent drying and agar cracking.

Miniprep

Materials:

- GeneJET Plasmid Spin kit (ThermoFisher, Cat. #K0502)

Miniprep is conducted to isolate your recombinant plasmids from the cloning host. Normally, one will follow the protocol belonging to the product used to isolate the plasmids. In this case, a kit was used with its own protocol. Starting out with harvesting the cloning bacteria transformed with your sequence of interest, one lyses the bacteria, renatures the plasmid DNA, followed by isolating it using specialized columns. Read on the product with Cat. #KO502 on ThermoFisher's website (Thermo Scientific, n.d.).

1. Prepare the resuspension solution by adding the ribonuclease (RNase) A solution provided to the Resuspension Solution, then mix thoroughly (stored at 4°C).
2. Harvest bacteria by distributing 3 mL of the bacteria sample into two separate Eppendorf tubes and centrifuge for 2 min at room temperature (RT), 7,000 × g. Discard the supernatant safely.
3. Add 250 µL Resuspension Solution to the pellet and vortex the tube.
4. Add 250 µL Lysis Solution and invert the tube 4 – 6 times, before waiting for a few min as the cells are lysed by the alkaline environment.
5. Add 350 µL Neutralization Solution and invert the tube 4 – 6 times. This solution is meant to neutralise the acidic solution and renature plasmid DNA (pDNA) while precipitating gDNA, cellular debris and proteins out of the solution.
6. The tubes were centrifuged for 5 min at 12,300 × g, and the supernatant was transferred to the GeneJET Plasmid Spin Columns. These columns were centrifuged for 1 min at 12,300 × g and supernatant discarded once again.
7. The columns were washed 2X with 500 µL Wash Solution, centrifuging for 1 min each time, 12,300 × g, and flowthrough was discarded each time. Then the columns were centrifuged for 1 min.
7. The columns were transferred into new tubes and 40 µL Elution Buffer (dH_2O) was added to each column. The tubes were set to incubate 2 min at 56°C, then centrifuged for 2 min, and the flow-through was collected. This flow-through hopefully contains the isolated plasmid.
8. If sending the plasmids for sequencing, follow the protocol [Prepare for sequencing] following miniprep.

Prepare for sequencing

In order to verify the sequences of your constructs, you may send your samples in for sequencing to an external lab. Requirements vary between both purposes and lab, so make sure to make note of what is required of your samples.

1. Run your samples on an agarose gel to ensure a nice, clean band by **[Amplification and verification].**
2. Follow **[NanoDrop measurement]** to test concentration by A260, and test purity with A260/A280. Typically, a ratio of 1.8 - 2.0 (indicating high purity) is required for sequencing.
3. Prepare tubes of samples consisting of minimum 15 µL containing your sequence in a 50 – 100 ng/µL concentration with nuclease-free H_2O.
4. Generally, it is required to send mixes of your sequence with the Forward and Backward primers separately as well, at a concentration of 5 – 10 µM with a volume of minimum 30 µL.
5. After receiving the results, you need to align the sequences provided with your initial sequence design to determine whether the sequences are of good enough quality to be used for further experimentation. One can do this e.g. by using BLAST through SnapGene.

Amplification and verification

PCR

Materials:

- Forward and Reverse primers (purchased from ThermoFisher)
- Q5 Reaction Buffer (BioLabs, Cat. #B9027S)
- Deoxynucleotide (dNTP) Solution (BioLabs, Cat. #NO447L)
- Q5 High Fidelity DNA Polymerase (BioLabs, Cat. #MO491L)

According to Khehra et al. (2025), the polymerase chain reaction (PCR) is used to amplify a DNA template through the use of primers, a polymerase and deoxynucleotide triphosphates (dNTPs). Mixing these components together, you denature the double-stranded DNA (dsDNA) template to single-stranded DNA (sDNA) sequences using high temperatures, thus allowing access for primers specifically designed to be complementary to parts of your template (denaturation step).

The primers will bind to the template (annealing step), and the polymerase added will recognise these primers and make use of the dNTPs to make the sDNA strands into dsDNA once more, doubling the initial amount of dsDNA (elongation step). By repeating these steps

for several cycles, the amount of dsDNA is amplified. The protocol was based on the one provided by BioLabs for the QT Polymerase (Cat. #MO91L) (BioLabs, n.d.).

Note: Individual contents were mixed prior to use, and all reaction components were assembled on ice. PCR tubes were used for the PCR mixes themselves.

1. Prepare primer stocks by mixing them in nuclease-free H_2O to a final concentration of 100 µM. Store at -20°C. Before use, dilute 20 µL of primer stock with 180 µL nuclease-free H_2O.
2. Prepare a PCR mastermix in one PCR tube and distribute 25 µL per tube before adding the DNA template to the respective tube. See Table 2 for the required volume of all components per tube to be prepared. The Q5 polymerase is to be added last to the mastermix and kept in the fridge until then. **Note**: You may centrifuge the samples at 12,300 × g for 2 min before splitting the tubes.
3. The reactions were quickly transferred to a preheated thermocycler (98°C) and PCR was run with the required parameters. 25 cycles are sufficient for amplification, and one may set the lid temperature to 105°C. For Q5, the program used is as observed in Figure 8. The temperature of the annealing step is dependent on what is being analyzed and will have to be calculated, while the time for the extension step is dependent on the size of the product (1 min per kb).

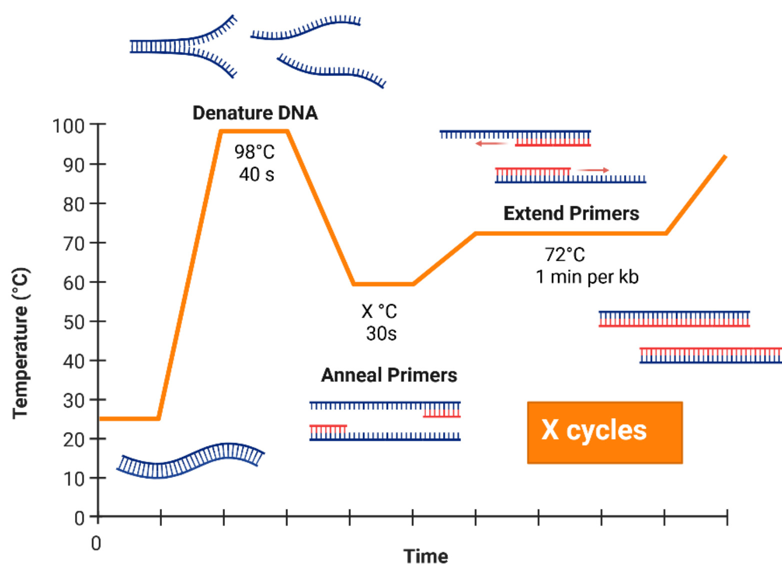

Figure 8: *Basic PCR program when using Q5 polymerase. The amount of cycles, annealing temperature and extension time will have to be predetermined by user. Made with BioRender.*

4. Mix the contents of the two tubes.

Table 2: *PCR mastermix – volume per sample to be prepared.*

Components	**Amount per sample**
5X Q5 Reaction Buffer	5 µL
10 mM dNTPs	0.5 µL
10 µM Forward Primer	1.25 µL
10 µM Reverse Primer	1.25 µL
Template DNA	0.5 µL
Q5 Polymerase	0.25 µL
Nuclease-free H_2O	*To total volume*
Total	*25.5 µL*

Gel electrophoresis

Materials:

- Agaros Standard (Saveen Werner, Cat. #A1000-500)
- 50X Tris-Acetate-EDTA (TAE) (ThermoScientific, Cat. #B49)
- GelRed Nucleid Acid Gel Stain, 10,000X (Biotium, Cat. #41003)
- Ladder (ThermoFisher, Cat. #26619)

Gel electrophoresis can be used to separate DNA by size. It makes use of an electrical field to move the negatively charged DNA molecules through agarose gel towards a positive electrode – smaller molecules will move more quickly, thus travelling further along the gel (Addgene, 2018). By running your samples alongside a known control (DNA ladder), observing the band will inform you of the approximate length of your sample (Addgene, 2018). A standard protocol is provided by Addgene (Addgene, 2018). The gels were imaged using the Gel Doc XR+ with software ImageLab 5.1.

Note: The concentration of agarose in a gel will vary depending on the properties of what you're investigating, but generally a 0.8% gel works for a wide range of sizes.

1. Prepare a 0.8% gel agarose in 1X TAE buffer by adding 0.56 g agarose powder in 70 mL of the buffer. At 50X this buffer consists of 40 mM Tris, 20 mM acetic acid and 1 mM EDTA in dH_2O, and it needs to be diluted in dH_2O to 1X. Boil the solution using e.g. a microwave (making sure it doesn't spill!) and cool it down by running cold water on the flask.

2. Once the gel is cooled down, add 7 µL of gel dye. Then, pour the solution into a gel casket with appropriate wells and leave to set for about 1h.
3. Dilute gel loading dye to 1X with H_2O, for instance 1:5 if the dye is 6X.
4. Prepare samples by either diluting with H_2O or adding the dye directly. If the former, prepare mixes as seen in Table 3. If not, add 5 µL dye directly to the samples and load 2 µL of the ladder and 7 µL of each sample to their respective wells.
5. Run the gel for 1h, at 90V. Gently disassemble the casket and remove the gel. Image the gel.

Table 3: *The mixes loaded onto the gel.*

Ladder	
Dye	2 µL
Nuclease-free H_2O	9 µL
Ladder 1 kB	1.5 µL
Total	*12.5 µL*
Sample	
Dye	2 µL
Nuclease-free H_2O	7 µL
DNA	3 µL
Total	*12 µL*

NanoDrop measurement

To measure using a NanoDrop machine, wash the measurement plate with dH_2O and a piece of paper, then blank with the appropriate buffer. Load with 2 µL droplets and ensure the droplet makes a solution bridge in the device. Use the appropriate program, such as A280 for protein concentration.

Gibson assembly

<u>DpnI Digestion of Circular Vector Contaminants</u>

Materials:

- DpnI (BioLabs, Cat. #R0176S)

To degrade unwanted circular parental vectors, add 1 µL of DpnI enzyme to your PCR-amplified vector fragment and incubate at 37 °C for 1h.

Ethanol Precipitation of PCR Products

Materials:

- Sodium acetate (Merck, Cat. #1.06267.1000)
- Ethanol (KiiltoClean, Cat. #33539)

To purify PCR-amplified DNA fragments, you may use ethanol precipitation, .

1. Add 5 µL of 3 M sodium acetate (pH 5.2) to 50 µL of PCR product.
2. Add 165 µL of 96% ethanol.
3. Vortex samples briefly and incubate at –80°C for 15 min.
4. Centrifuge the samples at 17,000 × g for 15 min at 4°C. Discard supernatant carefully and leave the tubes open briefly to evaporate residual ethanol.
6. Resuspend the samples in 40 µL dH_2O and vortex briefly.
7. Measure the concentration of each sample using the protocol **[NanoDrop measurement]** by adding a droplet of 2 µL sample after blanking the instrument with H_2O.

Assembly and transformation of constructs

Materials:

- Gibson Assembly Master Mix (BioLabs, Cat. #E2611)

Gibson assembly is a simple and reliable method for assembling multiple overlapping DNA fragments through the use of heat, an exonuclease, DNA polymerase and a DNA ligase (Gibson et al., 2009).

Note: Adjust insert and vector quantities to maintain a 1:1 molar ratio, accounting for approximate fragment sizes and equal DNA concentrations.

1. Per construct, mix the following:
    - 1 µL vector backbone, ~5,000 bp
    - 1 µL gBlock insert (or H_2O for control)
    - 2 µL Gibson Assembly Master Mix
2. Spin the solutions briefly at 12,300 × g and incubate at 50°C for 1 hour.
3. Transform your cloning cells using the protocol [Transformation] and the Gibson assemblies you just prepared.
4. Follow the protocol [Plating of bacteria] from point 1.

Transformation

Materials:

- SOC medium from **[Solutions for bacteria growth]**

To produce recombinant proteins, one requires a cloning host which will accept the assembled DNA sequence and produce plasmid copies. These will then be isolated, verified and used to transform expression hosts, and these will then produce the proteins coded by the sequence (Addgene, 2017). Transformation requires a methology which increases the permeability of the host's cell membrane, allowing for the DNA sequence to enter the host. One such method is heat-shock, the use of high temperatures for a short period.

1. If transforming cloning strain cells, mix Gibson assemblies prepared from the protocol **[Gibson assembly]** with 60 µL of cloning strain cells. If transforming expression-competent cells, add 0.5 µL of isolated plasmids from Miniprep, protocol **[Miniprep]**, to 60 – 200 µL of such cells.
2. Incubate the mixtures on ice for 30 min.
3. Heat-shock the cells for heat-shocked at 42°C for 45 seconds (s) and immediately returned to ice.
4. 1 mL SOC medium was added to each tube.
5. Tubes were incubated at 37°C for 1 hour in a PCMT incubator.
6. For plating, follow the [Plating of bacteria] protocol from point 1. **Note**: You may pre-warm the agar plates in a 37°C incubator prior to plating.

Freezing bacteria solutions

Materials:

- Glycerol (VWR, Cat. #G7893)

Bacteria samples can be frozen down long term in cryotubes. **Note**: When retrieving to streak plates, do <u>not</u> let the samples thaw. This can potentially rupture the bacteria cells. Scrape off the required material and return the sample to the cryofreezer as quickly as possible.

50% glycerol in dH_2O was made sterile in the hood by using a filter, and samples of 30% glycerol mixed with the original bacteria samples were made in Falcon tubes (0.6 mL of 50% glycerol to 0.4 mL bacteria solution). The mixes were put in cryotubes and frozen in our part of the cryofreezer.

Solutions for protein expression

Phenylmethylsulfonyl fluoride (PMSF) stock

- PMSF (VWR, Cat. #MG145)
- Isopropanol (VWR, Cat. #437145X)

In hood, measure PMSF directly into tubes on weight. Measure 100 mg PMSF and dissolve in 5 mL isopropanol to a final concentration of 20 mg/mL. Mix on rotary until dissolved, then place in a -20°C freezer.

Lysozyme stock

- Lysozyme (Roche Diagnostics, Cat. #81067121)
- dH_2O

In hood, measure lysozyme directly into tubes on weight. Mix 150 mg in 3 mL dH_2O to achieve a concentration of 50 mg/mL and mix on rotary until dissolved, then place in a -20°C freezer.

Isopropyl β-d-1-thiogalactopyranoside (IPTG) stock

- IPTG (Promega, Cat. #V395A)
- dH_2O

In hood, measure IPTG directly into tubes on a weight. Mix 0.94 mg in 4 mL dH_2O to a final concentration of 1 M. Mix on rotary until dissolved and place in a -20°C freezer. Aliquot the solution and place in a -20°C freezer. **Note**: Don't expose the solution to room temperature (RT) for too long.

Solutions for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

10X SDS-PAGE Running buffer (1 L)

- Tris (Merck, Cat. #93362)
- Glycine (Merck, Cat. #101196X)
- SDS (Merck, Cat. #L33771)
- dH_2O

Dissolve 30 g Tris, 144 g glycine and 10 g SDS in 90 mL dH_2O. Once completely dissolved, dilute to 100 mL and filter the buffer. Store at RT and dilute to 1X with dH_2O before use.

4X SDS-PAGE loading buffer (10 mL)

- Tris (Merck, Cat. #93362)
- Dithiothreitol (DTT) (ThermoScientific, Cat. #R0861)
- SDS (Merck, Cat. #L33771)
- Bromophenol blue (Merck, Cat. #B5525)
- Glycerol (VWR, Cat. #G7893)
- dH_2O

Make a 1 M Tris-HCl stock by adding 6.06 g Tris to 40 mL dH_2O and adjust pH to 6.8 with 37% HCl. Add dH_2O to 50 mL and filter the stock. Move to the hood. Mix 2 mL of that stock with 4 mL glycerol. Add 0.8 g SDS and 0.62 g DTT to the solution and dilute to 10 mL. Add 4 mg bromophenol blue to the solution.

Coomassie staining solution (1 L)

- Ethanol (KiiltoClean, Cat. #33539)
- Coomassie blue R-250 (Merck, Cat. #1.12553)
- Acetic acid (Merck/Sigma-Aldrich, Cat. #33209)
- dH_2O

Mix 1.25 g of Coomassie blue R-250 in 500 mL ethanol, add 400 mL of dH_2O. Add the acid last – 100 mL of acetic acid - and stir 1 – 2h, then filter through a Whatman filter paper. Store at RT. Wash gel 2 – 3 times, 5 min each with large volumes of deionized water. Remove all water and add about 25 mL staining solution to cover the gel. Gently shake in staining solution for 30 – 60 min.

Coomassie destaining solution (1 L)

- Ethanol (KiiltoClean, Cat. #33539)
- Acetic acid (Merck/Sigma-Aldrich, Cat. #33209)
- dH_2O

Mix 500 mL of ethanol and 400 mL of dH_2O. Add 100 mL acetic acid. Wash gel 2 – 3 times, 5 min each with large volumes of deionized water. Destain for as long as necessary.

Deoxyribonuclease (DNase) I

- DNase I (Roche/Merck, Cat. #4536282001)
- dH_2O

To make a 10 mg/mL stock, dissolve 100 mg in 10 mL of dH_2O and freeze 1 mL aliquots at – 20°C.

SDS-PAGE gel

Materials:

- Equilibration buffer from **[Solutions for protein purification]**
- DNase (ThermoFisher, Cat. #90083)
- Magnesium dichloride ($MgCl_2$) (Merck/Sigma-Aldrich, Cat. #M8266)
- Lysozyme from **[Solutions for protein expression]**
- PMSF from **[Solutions for protein expression]**
- 4X SDS loading buffer from **[Solutions for SDS-PAGE]**
- PageRuler Plus Prestained Protein Ladder (ThermoFisher, Cat. #26619)
- Novex^{TM} 4 – 20% Tris-Glycine Plus WedgeWell^{TM} Gel, 1.0 mm × 15 wells (ThermoFisher, Cat. #XP0420C)
- Comassie staining solution from **[Solutions for SDS-PAGE]**
- Comassie destaining solution from **[Solutions for SDS-PAGE]**
- White gel imaging plate

In order to verify both the content and purity of protein samples as well as to observe the effect of different conditions on protein expression, one may run an SDS-PAGE gel. For more information on the origin of this protocol as well as the belonging materials in **[Solutions for SDS-PAGE gel]**, refer to Kielkopf et al. (2021). The gels were imaged using the Gel Doc XR+ with software ImageLab 5.1.

The amount of sample to be loaded varies with both protein sample and the gel cassette used. For the gel cassette used in this project, amounts between 1 and 10 µL are appropriate. In general, it is recommended to load several different amounts, although samples such as lysate and purification flowthrough (which one expects to be unpure and contain much protein), a smaller volume is recommended.

Note: If preparing bacteria samples following the protocol **[Testing protein expression small-scale]**, start from step 1 to prepare for SDS-PAGE. If preparing for SDS-PAGE following **[Protein purification]**, start from step 7.

1. Thaw the bacteria pellet tubes.
2. Mix 4.75 mL of Equlibration buffer with 100 µL of 1 mg/mL DNAse, 100 µL of 1 M $MgCl_2$, 10 µL of 50 mg/mL lysozyme and 40 µL of PMSF 20 mg/mL.
3. Resuspend the pellets in a volume of this buffer equal to the volume taken out into the tubes initially before making pellets of the solutions. **Note**: If observing repeated instances of weak bands, you may decrease the volume of resuspension. To standardise the solutions, the same factor of dilution needs to be applied for all.
4. Gently vortex the tubes and incubate at 30°C for 30 min.

5. Freeze the tubes at -80°C for 15 – 20 min and then thaw at 37°C for 15 min. This was repeated 3X. **Note**: Ensure the samples are fully frozen and then thawed each cycle to properly lyse the samples.
6. Spin the tubes down at 12,300 × g for 20 min.
7. Transfer supernatant from each tube to a new tube and dilute 4X of SDS-PAGE loading buffer **([Solutions for SDS-PAGE]**) to 1X. Store the rest of the supernatants in new tubes at -20°C. If using samples from protein purification, instead mix 30 µL of the samples with 10 µL of the 4X SDS-PAGE loading buffer.
8. Boil the SDS-PAGE samples at 90 – 100°C in a heater block for 5 min and cool down for 5 min in a fridge. **Optional**: Spin the tubes for 2 min at 12,300 × g.
9. The SDS-PAGE gel casette was prepared with 1X SDS-PAGE running buffer.
10. Add 2 µL protein ladder in one well, and 1 – 10 µL of each sample. Store the rest of the samples at -20°C.
11. Run the gel for 1h at 30 mA. **Note**: Ensure the gel doesn't run too long.
12. Unlock the gel apparatus, discard buffer and then wash the gel as well as the apparatus with tap water.
13. Add the gel to a container and add 25 mL of Coomassie staining solution. Leave on shaker for 15 min before collecting the solution if so desired.
14. Add 25 mL Coomassie destaining solution and leave on shaker for 1h, or twice for 15 min. Discard the solution. **Note**: This solution *can* be filtered in the coal column.
15. Either leave gel in destaining solution O.N. if destained less than 1h (these risks having to repeat staining) or leave O.N. in dH_2O if having destained for 1h. **Note**: One can also repeat destaining until satisfied and image on the same day. This process depends on the loaded solutions.
16. Either the day of or after O.N. shaking, image the gel by using the appropriate machine and program, e.g. Image Lab by Bio-Rad. In general, prepare a protocol with colour Coomassie and turn off the highlighting of saturated pixels for better pixel quality. Furthermore, to visualise the gel, use a white imaging plate below the gel.

Solutions for protein purification

<u>Equilibration buffer (1 L)</u>

- Tris (Merck, Cat. #93362)
- NaCl (Merck, Cat. #S7653)
- Glycerol (VWR, Cat. #G7893)
- dH_2O

Mix 6.06 g Tris in dH_2O and adjust pH to 8 with 37% HCl. Add 29.22 g NaCl, 150 mL of 15% glycerol and dilute with dH_2O to 1 L. Filter this solution.

Imidazole stock solution (250 mL)

- Imidazole (Merck, Cat. #104716)
- dH_2O

Add 34 g imidazole to 200 mL dH_2O and mix while stirring. Adjust pH to 7.5 with 37% HCl. Dilute with dH_2O to 250 mL and then filter the solution.

Testing protein expression small-scale

Materials:

- LB medium from **[Solutions for bacteria growth]**
- Kanamycin from **[Solutions for bacteria growth]**
- IPTG from **[Solutions for protein expression]**

Before committing to large-scale production of your protein(s), it is important to do a small-scale test run to test different conditions and observe what is optimal for the transformed expression-competent bacteria. This is to maximise yield of protein and to ensure that the proteins will not e.g. degrade if left to incubate for too long. The production and purification of LDH and LOD specifically was informed by the article by Godino et al. (2024).

Note: To run an SDS-PAGE gel, ensure you note down all OD600 values as well as volumes frozen down of the samples to ensure standardization of the gel volumes.

1. For each prepared bacteria solution, transfer 0.2 mL to 9.8 mL of medium with 1:1000 antibiotics. If assuming the protein will be difficult to detect, you may transfer 0.5 mL of the bacteria solution to 25 mL medium instead. Leave on shaker incubation at 200 rpm, 37°C for 1.5h.
2. With a spectrophotometer, take the OD600 of 1 mL solution after blanking with correct medium. **Optional**: You may return the 1 mL to the bacteria solution if no dilution occurred.
3. If OD600 is about 0.6, take out 1 mL of each tube to keep as non-induced control, and add IPTG to each tube to a final concentration of 0.4 mM. If making a larger solution, take out 5 mL instead.
4. Spin the 1 mL controls at 12,300 × g, 4°C and discard supernatant before freezing the pellets in a -20°C freezer. If spinning down 5 mL instead, use 4,255 × g for 15 min.
5. Take 5 mL out of each induced solution and set in a separate shaker incubator at a different temperature (e.g. 20°C), while returning the original tubes to a 37°C incubator. Both should shake at 200 rpm, and should be left to incubate for 4h.
6. Take OD600 of all tubes after incubation. At this point, one would need to dilute the sample to be measured absorbance of due to the high OD600 (e.g. 0.4 mL sample to 0.6 mL LB medium). Input this value to the spectrophotometer.

7. Using the formula $c_1v_1 = c_2v_2$, calculate the right number of cells to freeze down to standardise for SDS-PAGE. For instance, if initial OD600 was 0.6 and 1 mL was removed for control, and OD600 was 4.0 after 4h incubation, then you need to remove 0.6 × 1 mL = 4.0x => x = 0.15 mL.
8. Spin down the solution you removed at 12,300 × g for 2 min and discard the supernatant, before freezing the pellet at -20°C.
9. Leave the tubes O.N. at 200 rpm at their respective temperatures.
10. Take OD600 of all tubes after incubation, using the appropriate dilution such as 200 µL sample to 800 µL LB medium.
11. Once again, use $c_1v_1 = c_2v_2$ to calculate the appropriate volume to be frozen down of each solution to ensure standardisation.
12. Spin down the solution you removed at 12,300 × g for 2 min and discard the supernatant, before freezing the pellet at -20°C. Discard the tubes.
13. Follow the protocol **[SDS-PAGE]** from step 1 to verify results and predict the optimal conditions for large-scale expression.

Large-scale protein production

Materials:

- LB medium from **[Solutions for bacteria growth]**
- Kanamycin from **[Solutions for bacteria growth]**
- Antifoam A Concentration (Sigma, Cat. #A5633)
- IPTG from **[Solutions for protein expression]**
- PBS from **[Solutions for electrochemistry]**
- NAD^+ (Alfa Aesar Cat. #J62337)

After deciding on optimal conditions for expressing the recombinant proteins of interest, you may move on to large-scale growth (5 L solution of bacteria) of the transformed expression-competent bacteria. The production and purification of LDH and LOD specifically was informed by the article by Godino et al. (2024).

1. Follow protocol **[Incubate single colony]**, incubating a colony of the expression-able bacteria transformed with the protein of interest in 25 mL of medium supplemented with antimicrobials using a 100 mL flask O.N.
2. The next day, transfer 20 mL of the bacteria solution to 1 L of the same medium used in step 1. Use a 5 L flask and add one drop of antifoam concentrate. Leave the flask on 200 rpm, 37°C for about 1.5h.
3. Check OD600 on a spectrophotometer, blanking with the medium used to grow the bacteria. If OD600 is not 0.5 – 0.6, leave to grow in intervals of 30 min, checking OD600 until the bacteria reaches this absorbance.
4. Add 400 µL IPTG to the flask and leave to incubate at 200 rpm, 37°C for 16h.

5. Transfer the solution to a centrifugation tube and centrifuge using a JLE 8.1 rotor at 4°C for 10 min, 10,000 × g (or 15 min, 7,000 × g). Discard supernatant and leave the flask with bleach or ethanol for 45 min minimum. Discard the solution in a waste bucket, wash the flask with water and discard the water in a waste bucket, then leave to autoclave.
6. Wash the bacteria pellet with 30 mL PBS and scrape with the pipette to dissolve the bacteria in PBS, before transferring the solution to a 50 mL Falcon tube. Centrifuge at 20 min for 4°C before discarding supernatant and freezing the pellet at -20°C. Leave centrifugation tubes in bleach or ethanol and wash similarly to the flask in step 5.

Protein purification

Materials:

- PMSF from **[Solutions for protein expression]**
- DNase from **[Solutions for SDS-PAGE gel]**
- Equilibration buffer from **[Solutions for protein purification]**
- Imidazole from **[Solutions for protein purification]**
- EconoFit Nuvia IMAC 5 mL column (Bio-Rad, Cat. #12009286)

In IMAC, or Immobilised Metal Affinity Chromatography, the Buffer A (the Equilibration buffer) contains no imidazole and is used first to equilibrate the column. This buffer also contains NaCl to reduce nonspecific binding. Then, Buffer B (the Equilibration buffer with 0.5 M imidazole) is added to elute the target protein. The imidazole competes with the His-tag for the metal ions in the column, causing the protein to be released. This process happens by applying an increasing concentration of imidazole, either stepwise or using a gradient, to prevent elution of other proteins at the same time. Read more in the article by Sułkowski (1985) as well as in Yip et al. (1994). The production and purification of LDH and LOD specifically was informed by the article by Godino et al. (2024). The software used for this protocol was UNICORN (Version 1.0), and the machine was the Äkta start IMAC.

1. Thaw the bacteria pellet prepared using the protocol [Large-scale protein production] as well as 20 mg/mL PMSF prepared using the protocol [Solutions for protein expression].
2. Prepare 40 mL of Equilibration buffer using the protocol [Solutions for protein purification] was added to the pellet until dissolved, then 500 µL of 20 mg/mL PMSF was added. The PMSF is to inhibit proteases as to prevent protein degradation. **Note**: The volume of Equilibration buffer could be adjusted to accommodate for the maximum volume of e.g. the French press if using this particular method for lysis.
3. Lyse the bacteria solution, e.g. using a French press. In this case, lube the French press components and assemble it, then load the solution and run lysis for several rounds keeping the machine at the appropriate pressure as indicated on the machine.

Continue until lysis is complete (6 – 8 rounds). **Note**: It might be advantageous to add 200 µL of 10 mg/mL DNase I to reduce viscosity.

4. Split the solution to two centrifugation tubes and balance the weight using a weight scale and dH_2O. Spin the tubes with a 25.50 rotor at 20,000 × g for 20 min.
5. Collect the supernatant in one 50 mL Falcon tube by filtering through a 0.45 µm filter. Loosen the bacteria pellets using dH_2O and discard in waste buckets, then leave the tubes in bleach for 45 min minimum before leaving to autoclave.
6. Prior to purification, collect 50 µL of lysate and freeze at -20°C.
7. Purify the protein sample using e.g. an IMAC machine. If purifying a His-tagged protein, follow this protocol. Use a 5 mL IMAC column.
8. Use the Equilibration buffer as Buffer A. Prepare Buffer B by mixing 400 mL Equilibration buffer with 100 mL of 2 M imidazole solution. Filter this solution.
9. Run an appropriate purification protocol with a gradient of Buffer B, collecting the elution in different 15 mL tubes. See example of a full program script in Figure 9, appropriate for running in UNICORN start 1.0. In this case, steps of 0%, 10%, 20% and 100% of Buffer B is listed. **Note**: The program is dependent on the protein. It is recommended to start with bigger steps and then adapt the program based on the results.
10. From the absorbance peaks observed, mark tubes of interest which might contain your protein of interest.
11. Place the samples in a cold room (4°C).
12. When appropriate, run an SDS-PAGE gel to verify which tube(s) contain your protein of interest by following the protocol [SDS-PAGE gel] starting from step 7.

```
UNICORN start 1.0
Variable list
1  Phase    Block    Variable    Value    Unit
2  Method Settings    Main    ColumnVolume    5.027    {ml}
3        Column    HisTrap FF, 5 ml    {}
4  Method Settings    METHOD SETTINGS    HighPressureValue    0.3    {MPa}
5  Method Settings,...    METHOD SETTINGS,...    FlowRate    5.0    {ml/min}
6  Prime and Equilibration    Equilibration Buffer B PumpWash    Buffer B Wash Volume    5    {ml}
7     PumpWash B Volume    5.00    {ml}
8  Prime and Equilibration    Equilibration Buffer A PumpWash    Buffer A Wash Volume    5    {ml}
9     PumpWash A Volume    5.00    {ml}
10 Prime and Equilibration    Equilibration B Concentration    Equilibration B Concentration    0.0    {%B}
11 Prime and Equilibration    Equilibrate    Equilibration Volume    5.00    {CV}
12 Sample Application    SampleApplication SystemFlow    SystemFlowRate    1.0    {ml/min}
13 Sample Application,...    Sample Application Start fractionation,...    Last tube filled action    Pause    {}
14 Sample Application    Sample Application Start fractionation    Fractionation volume (Sample Appl)    14    {ml}
15 Sample Application    Sample Application End Sample Inject from Pump    Sample Volume    43.00    {ml}
16 Sample Application    Sample Application Inject from pump complete    Chase Volume    0.50    {ml}
17 Wash out unbound    WashOutUnbound B Concentration    WashOutUnbound B Concentration    0.0    {%B}
18 Wash out unbound    Start Fractionation (WashOutUnbound)    Fractionation Volume    14.0    {ml}
19 Wash out unbound    WashOutUnbound Wash Volume    Wash Column With    10.00    {CV}
20 Wash out unbound    WashOutUnbound B Concentration_1    WashOutUnbound B Concentration_1    10.0    {%B}
21 Wash out unbound    Start Fractionation (WashOutUnbound)_1    Fractionation Volume_1    14.0    {ml}
22 Wash out unbound    WashOutUnbound Wash Volume_1    Wash Column With_1    10.00    {CV}
23 Wash out unbound    WashOutUnbound B Concentration_2    WashOutUnbound B Concentration_2    20.0    {%B}
24 Wash out unbound    Start Fractionation (WashOutUnbound)_2    Fractionation Volume_2    14.0    {ml}
25 Wash out unbound    WashOutUnbound Wash Volume_2    Wash Column With_2    10.00    {CV}
26 Elution and Fractionation    Elution Prime B concentration_(10)    Elution Prime B Concentration_(10)    100.0    {%B}
27 Elution and Fractionation    Elution Isocratic B Concentration    Elution Isocratic B concentration    100.0    {%B}
28 Elution and Fractionation    Start frac (Elution)    Elution Fixed Fractionation Volume    5.0    {ml}
29 Elution and Fractionation    Elution Isocratic volume    Elution Isocratic Volume    10.00    {CV}
30 Prime and Equilibration    Equilibration B Concentration_1    Equilibration B Concentration_1    0.0    {%B}
31 Prime and Equilibration    Equilibrate_1    Equilibration Volume_1    5.00    {CV}
```

Figure 9*: Script of IMAC purification for step-wise increase in Buffer B concentration from 0%, to 10%, 20%, then 100%. Note that volume of the sample used and the number of CVs needs to be modified.*

Solutions for LDH functional assay

Sodium phosphate monobasic (NaH_2PO_4)

- NaH_2PO_4 (Merck, Cat. #106346)
- 4 M sodium hydroxide (NaOH) (Merck, Cat. #28244.295)
- dH_2O

Mix 138 g NaH_2PO in 0.75 L of dH_2O and adjust pH using 4 M NaOH, then dilute with dH_2O to 1 L. This makes 1 M of solution. Filter before storing at RT. **Note**: To avoid potential crystallisation if the solution is to be stored, it is recommended that one prepares a 0.5 M stock instead.

Sodium pyruvate

- 100 mM sodium pyruvate (Sigma #P5280)
- dH_2O

Prepare 55 mg sodium pyruvate in 5 mL of dH_2O.

NADH

- NADH (Sigma, Cat. #N8129)
- NaOH (Merck, Cat. #28244.295)
- dH_2O

Dilute NaOH to 10 mM using dH_2O by e.g. adding 50 µL of 4 M NaOH in 20 mL dH_2O. Prepare 33.3 mg of NADH in 10 mL of the 10 mM NaOH (5 mM NADH). This solution should be prepared fresh, or frozen down in aliquots and discarded after use. To ensure the NADH is functional, you may make use of **[NanoDrop measurement]**, blanking with 10 mM NaOH and testing absorbance at 340 nm, where the NADH should have a strong peak.

LDH functional assay

Materials:

- LDH (Sigma-Aldrich, Cat. #59747, recombinant from *E. coli*)
- Self-produced LDH sample (source: *T. thermophilus*)
- NaH_2PO_4 from **[Solutions for LDH functional assay]**
- Sodium pyruvate from **[Solutions for LDH functional assay]**
- NADH from **[Solutions for LDH functional assay]**

Based on the assay described by Igwe, et al. (2024), it was decided that LDH activity can be measured using a kinetic experiment with a microplate reader. The cofactor NADH has a strong absorbance peak at 340 nm, so by observing decrease in A340 one can infer that the NADH is consumed to convert pyruvate to lactate, indicating enzymatic activity. This activity

should be dependent on the concentration of LDH. As enzymatic activity can vary with pH, it is recommended to test the assay with different pH values. Measurements occurred using the BioTek Epoch 2 and the software Gen5 3.17. Graphs were made using GraphPad Prism 10.

Note: Prepare 0.5 M NaH_2PO_4 solutions with different pH values separately.

1. Prepare 100 mM NaH_2PO_4 by diluting 10 mL of the 1 M stock solution with 40 mL of dH_2O.
2. Prepare LDH with a known concentration in 1 mL of the 100 mM NaH_2PO_4 buffers. Note down the concentration. **Note**: Prepare tubes with different pH values separately.
3. Set up a kinetics protocol in a microplate reader as such:
   - Preheat plate to 30°C.
   - Measure 340 nm
   - Measure for 5 min with reads every 30 s
   - Measure in wells A1 – H1 and A2 – H2 (see Table 4)
4. Prepare a lidless, non-sterile, transparent 96-well plate. See setup in Table 4, where
   - Buffer A is 100 mM NaH_2PO_4 with the appropriate pH
   - LDH may vary in concentration, and is suspended in Buffer A
   - Reaction Buffer is prepared fresh before each assay by mixing 1,821 µL of Buffer A with 172 µL of 5 mM NADH (0.425 mM) and 9 µL of 100 mM sodium pyruvate (0.45 mM).
   - Adding 66.6 µL of each well A1 – H1 to A2 – H2 needs to be done with a multiwell pipette right prior to starting the assay read to ensure the reaction is observed.

Table 4*: Setup of LDH assay.*

	1	2
A	100 µL Buffer A + 100 µL LDH	134.4 µL of Reaction Buffer + 66.6 µL of A1
B	100 µL Buffer A + 100 µL of A1	134.4 µL of Reaction Buffer + 66.6 µL of B1
C	100 µL Buffer A + 100 µL of B1	134.4 µL of Reaction Buffer + 66.6 µL of C1
D	100 µL Buffer A + 100 µL of C1	134.4 µL of Reaction Buffer + 66.6 µL of D1
E	100 µL Buffer A + 100 µL of D1	134.4 µL of Reaction Buffer + 66.6 µL of E1
F	100 µL Buffer A + 100 µL of E1	134.4 µL of Reaction Buffer + 66.6 µL of F1
G	100 µL Buffer A + 100 µL of F1, **discard 1**00 µL	134.4 µL of Reaction Buffer + 66.6 µL of G1
H	100 µL of Buffer A	134.4 µL of Reaction Buffer + 66.6 µL of H1

Reaction stability of LDH in various temperatures

- LDH (Sigma-Aldrich, Cat. #59747, recombinant from *E. coli*)
- Self-produced LDH sample (source: *T. thermophilus*)
- NaH_2PO_4 from **[Solutions for LDH functional assay]**
- Sodium pyruvate from **[Solutions for LDH functional assay]**
- NADH from **[Solutions for LDH functional assay]**

The purpose of this experiment is to test at which temperatures LDH are still functional to execute the reaction. See the protocol **[LDH functional assay]** for details. Measurements occurred using the BioTek Epoch 2 and the software Gen5 3.17. Graphs were made using GraphPad Prism 10.

1. Prepare a dilution of LDH samples in the appropriate 100 mM NaH_2PO_4 buffer, ensuring concentrations are the same if using different protein samples.
2. Prepare a fresh sample of Reaction Buffer by mixing 910.5 µL of the 100 mM NaH_2PO_4 buffer with correct pH with 4.5 µL 100 mM sodium pyruvate and 85 µL 5 mM NADH to achieve 0.425 mM NADH and 0.45 mM sodium pyruvate.
3. Mix 134.4 µL of the reaction buffer and 66.6 µL of the respective diluted LDH sample in a 1.5 mL Eppendorf tube and add these tubes as well as control tubes of 134.4 µL of the reaction buffer with 66.6 µL of the 100 mM NaH_2PO_4 buffer (one for each pH value tested). **Note**: Make sure to add the enzyme solution right prior to adding the tubes to the heating block, as the reaction will start instantly.
4. Leave the tubes to incubate at the temperature of interest for 15 min.
5. Transfer the solutions to wells in a 96-well plate.
6. Do an endpoint measurement on a microplate reader with the plate, testing for absorbance 340 nm.

Growth curves for bacteria

Materials

- LB medium from [Solutions for bacteria growth]
- Kanamycin from [Solutions for bacteria growth]

You may do growth curve experiments for the bacteria you grow (for instance the expression bacteria transformed with your produced plasmids) by standardising the samples and leaving them for a longer time period to read OD600 in a microplate reader. Measurements occurred using the BioTek Epoch 2 and the software Gen5 3.17. Graphs were made using GraphPad Prism 10.

1. Follow protocol **[Picking single colony]** to prepare bacteria samples.
2. Test OD600 of the bacteria samples by diluting the bacteria 100 µL to 900 µL of the medium used to grow the bacteria after blanking the spectrophotometer with the medium alone.
3. Dilute the samples in 1.5 mL Eppendorf tubes with the medium to achieve an OD600 of 0.01.
4. Transfer 200 µL of a desired number of replicates to wells of a lidless, non-sterile transparent 96-well plate.
5. Put a plastic film on the plate.
6. Run a kinetics protocol in a microplate reader, with a stabile 37°C temperature, constant orbital shaking, and the desired time period (such as 18h). Let the protocol take reads at A600 for intervals (e.g. every 30 min).

Dialysis

Materials:

- NaH_2PO_4 from **[Solutions for LDH functional assay]**
- Membra-Cel dialysis membrane (Viskase, size MC30)

After completed protein purification, one manner of exchanging the buffer of the purified sample is to perform dialysis. **Note**: The correct buffer is dependent on the enzyme sample as well as the assay(s) planned.

1. Following protein purification **([Protein purification]**) and SDS-PAGE (**[SDS-PAGE]),** pick up the purified sample with your protein of interest.
2. Follow **[NanoDrop measurement]** with the sample to get values of absorbance prior to buffer exchange.
3. Prepare a 1 – 3 L container of your desired buffer.
4. Cut a dialysis membrane in two and wash under tap water. Arrange the two to create a tube and secure the bottom with a clamp.
5. Pipette 1 mL of your sample into the makeshift container of the membranes and secure the top with another clamp as to ensure both ends are closed.
6. Put a floater foam on top of the makeshift bag.
7. Leave the bag in the container at low speed of stirring for minimum 3h.
8. Prepare another 1 – 5 L container with your desired buffer and move the dialysis sample to the new container. Discard the waste. Leave the sample O.N. **Note**: One may repeat this step if it is needed to dilute the original buffer more thoroughly.

Solutions for electrochemistry

Potassium hexacyanoferrate(II)/(III)

- Potassium hexacyanoferrate(II) trihydrate (Merck, Cat. #455989)
- Potassium hexacyanoferrate(III) (Merck, Cat. #455946)
- dH_2O

Dissolve 36.8 mg potassium hexacyanoferrate(II) and 16.5 mg mM potassium hexacyanoferrate(III) in dH_2O. Use a non-transparent tube as the solution is light-sensitive. Prepare fresh. **Note**: Do **NOT** let the solution come into contact with acids!

Phosphate buffered saline (PBS)

- Phosphate-buffered saline (PBS) (Sigma, Cat. #P4417)
- Hydrochloric acid (HCl) (Merck, Cat. #30721)
- dH_2O

Dissolve one tablet of PBS in 200 mL and adjust the solution using 2 M HCl. Filter the solution.

Sulfuric acid (H_2SO_4)

- 0.5 M H_2SO_4 (Merck #1007310510)
- dH_2O

Dilute 1.1 mL of 18.2 M H_2SO_4 to a total of 40 mL with dH_2O for a 0.5 M stock solution.

Hydrogen peroxide (H_2O_2)

- H_2O_2 (Sigma-Aldrich #216763)
- dH_2O

Add 102 µL to a total of 10 mL dH_2O on ice for a 100 mM solution and filter.

Potassium hydroxide (KOH)

- KOH (Merck #1050330500)
- dH_2O

Mix 28 mg KOH in 10 mL dH_2O for a 50 mM solution. **Note**: If the pellets are too large, you may make a stock solution of higher molarity, or dissolve 0.28 g in 100 mL dH_2O.

KOH + H_2O_2

- KOH (Merck, Cat. #1050330500)

- H_2O_2 (Sigma-Aldrich, Cat. #216763)
- dH_2O

Dissolve 28 mg KOH in 1.67 mL dH_2O and add 8.33 mL of 30% H_2O_2. This amounts to 50 mM KOH and 25% of H_2O_2. **Note**: If the pellets are too large, you may make a stock solution of higher molarity, or dissolve 0.28 g in 16.7 mL, then take out 1.67 mL of that.

Ascorbic acid

- Ascorbic acid (Merck, Cat. #A92902)
- dH_2O

To make 100 mM ascorbic acid, dissolve 0.1761 g of ascorbic acid in 10 mL of dH_2O. Filter the solution. Make another diluted 1 mM solution by adding 1 mL to 9 mL of PBS.

Horseradish peroxidase (HRP)

- HRP (Sigma, Cat. #P8125)
- dH_2O

Dissolve 2 mg of the enzyme in 100 mL dH_2O. Filter the solution.

Lactic acid

- Lactic acid (VWR, Cat. #101384Q)
- dH_2O

Dilute 833 µL of 12 M lactic acid in dH_2O to a total volume of 100 mL, or 333 µL to a total volume of 40 mL if needing less solution. This solution should be prepared fresh. **Note**: At pH levels above 4, lactic acid deprotonates to lactate, so to regulate which form the chemical takes, keep in mind the pH of the solution (National Centre for Biotechnology Information, 2025).

NAD^+

- 100 mM NAD^+ (Alfa Aesar, Cat. #J62337)
- PBS **from [Solutions for electrochemistry]**

To prepare 20 mM NAD^+, add 133 mg to 10 mL of dH_2O. This solution should be prepared fresh, or frozen down in aliquots and discarded after use.

Electrochemistry

Materials:

- 2 mm screen-printed gold electrodes (PalmSens)

- Enzymes of interest and substrates for reaction
- Nafion (Aldrich, Cat. #274704)
- Potassium hexacyanoferrate(II)/(III) from **[Solutions for electrochemistry]**
- H_2SO_4 from **[Solutions for electrochemistry]**

Electrochemistry as a method is used to detect changes in impedence as a result of chemical reactions, specifically redox reactions. Such reactions can be catalysed by enzymes, thus electrochemistry can in theory be a tool to use for detection of enzymatic activity. In this project, the Sensit Smart Potentiostat from PalmSens was used for measurements with the PSTrace 5.1 software.

1. Wash the electrodes by adding 80 µL of 0.5 M H_2SO_4 and performing cyclic voltammetry with 3 scans from 0.0 to 1.4 V vs Ag/AgCl with 0.1 mV/s, E step 5 mV. Make sure to wash 3X with dH_2O or PBS afterwards.
2. You may test the instrument and the electrodes by making a 10 mL mix of 10 mM potassium hexacyanoferrate(II) trihydrate and 5 mM potassium hexacyanoferrate(III). Running cyclic voltammetry for 3 scans, -0.3 V to 0.6 V ranging from 50 – 100 – 150 – 200 mV/s, E step 5 mV. If working correctly, you should observe a plot characteristic of this redox reaction. **Note**: To avoid toxic fumes, ensure you wash the H_2SO_4 off properly before testing this mix!
3. Prepare 100 µL of your enzyme of interest in appropriate concentrations, and add 1 µL of Nafion to each solution.
4. Incubate electrodes with 3 µL of the solutions at 37°C for 20 – 30 min, making sure to only add solution to the working electrode. Incubate one electrode with only buffer and 1% Nafion as control.
5. Prepare reaction buffers of different concentrations.
6. Test at appropriate voltage to your enzyme of interest as cited by literature, with 3 – 4 scans at appropriate mV/s and E step. Start with your control electrode and blank buffer, remove buffer between each step and add reaction buffers at increasing concentrations, running cyclic voltammetry for each concentration.
7. If you observe any oxidation peaks, make note of the voltages. If desired you may run chronoamperometry at this voltage, repeating the workflow with parameters t interval 0.5 s, t run 60 s, and E dc based on where you got the oxidation peak.

Solutions for LOD functional assay

<u>Bovine serum albumin (BSA)</u>

- 10 mg/mL BSA (Europa Bioproducts, Cat. #EQBAH62)
- dH_2O

Mix 60 mg BSA in 6 mL of dH_2O gently to avoid denaturation or foaming. **Note**: Ideally one could also prepare a larger volume and let the BSA dissolve in the solution without interference.

Sodium phosphate monobasic (NaH_2PO_4)

- NaH_2PO_4 (Merck, Cat. #106346)
- dH_2O

Mix 34.5 g NaH_2PO in 0.4 L of dH_2O and adjust pH using 4 M NaOH, then dilute with dH_2O to 0.5 L. Filter the solution.

Lactic acid

- Lactic acid (VWR, Cat. #101384Q)
- dH_2O

Dilute 83.3 µL of 12 M lactic acid in dH_2O to a total volume of 10 mL.

Ethyenediaminetraacetic acid disodium salt dihydrate (EDTA)

- EDTA (Merck, Cat. #E4884)
- NaOH (Merck, Cat. #28244.295)
- dH_2O

Dissolve 186.1 g of EDTA in 0.75 L dH_2O, adjust pH to 8.0 with 2 M NaOH and then add dH_2O to 1 L. Filter the solution and store in a cold room.

2,6-dichlorophenol-indophenol (DCIP) salt hydrate

- DCIP (Merck, Cat. #D1878)
- dH_2O

Prepare a 1 mM solution of DCIP by dissolving 14.5 mg in 50 mL of dH_2O, letting it shake continuously on an orbital shaker. **Note**: Prepare the solution fresh, **protected from light** to avoid oxidation, and store at 4°C until use.

LOD functional assay

Materials:

- LOD (Toyobo, Cat. #LCO-301, from *Lactococcus lactis* subsp. c*remoris* IFO3427 (Toda & Nishiya, 1998))
- Self-produced LOD sample (source: *A. viridans*)

- NaH_2PO_4 from **[Solutions for LOD functional assay]**
- Lactic acid from **[Solutions for LOD functional assay]**
- DCIP from **[Solutions for LOD functional assay]**
- BSA from **[Solutions for LOD functional assay]**
- EDTA from **[Solutions for LOD functional assay]**

The purpose of this experiment is to measure LOD activity using a kinetic microplate assay, which is relevant to our project goal of characterizing enzyme kinetics under varying pH conditions. Based on the assay described by Xu, et al., (1996), LOD activity can be measured using a kinetic experiment with a microplate reader. The dye DCIP has an absorbance peak at 600 nm in its oxidized form, so a decrease in A_{600} along with the solution becoming colourless indicates that the dye is being reduced. This activity should correlate with the concentration of LOD. Since enzymatic activity is influenced by pH, the assay was performed under different pH conditions. Measurements occurred using the BioTek Epoch 2 and the software Gen5 3.17. Graphs were made using GraphPad Prism 10.

Note: Prepare 0.5 M $Na_2H_2PO_4$ solutions with different pH values separately. Furthermore, ensure that the pH is not too acidic as lactic acid is present as lactate below pH 4 (National Centre for Biotechnology Information, 2025). This can be done for instance by using low concentrations of lactic acid. If too acidic, the DCIP dye will also change colour without enzymatic functionality, interfering with the final results.

5. Make 100 mM dilutions of the 0.5 M NaH_2PO_4 solutions by mixing 8 mL of the stock with 32 mL dH_2O.
6. Prepare LOD with a known concentration in 1 mL of the 100 mM NaH_2PO_4 buffers. Note down the concentration. **Note**: Prepare tubes with different pH values separately.
7. Set up a kinetics protocol in a microplate reader as such:
    - Preheat plate to 37°C
    - Measure 600 nm
    - Constant linear shaking (low speed)
    - Measure for 10 – 15 min with reads every 1 min
    - Measure in wells A1 – H1 and A2 – H2 (see Table 5)
8. Prepare a lidless, non-sterile, transparent 96-well plate. See setup in Table 5, where
    - Buffer A is 10 mM NaH_2PO_4 with the appropriate pH
    - LDH may vary in concentration, and is suspended in Buffer A
    - Reaction Buffer is prepared fresh before each assay by mixing 200 µL of 100 mM NaH_2PO_4 (10 mM in final reaction volume), 20 - 200 µL of 100 mM lactic acid (1 - 10 mM in final reaction volume), 80 µL of 1 mM DCIP (40 µM in final reaction volume), 144 µL of 10 mg/mL BSA (0.72 mg/mL in final reaction

volume) and 20 µL of 0.5 M EDTA (5 mM in final reaction volume) and filling up the volume to 1,500 µL of dH_2O.

- Adding 50 µL of each well A1 – H1 to A2 – H2 needs to be done with a multiwell pipette right prior to starting the assay read to ensure the reaction is observed.
- DCIP standards are made in the reaction buffer consisting of different concentrations of DCIP (see Table 5) in a Buffer B of 10 mM NaH_2PO_4, 0.72 mg/mL BSA, and 5 mM EDTA in dH_2O. These standards can be prepared in larger volumes or directly in the wells.

Table 5*: Setup of LOD assay.*

	1	2	3
A	100 µL Buffer A + 100 µL LOD	150 µL of Reaction Buffer + 50 µL of A1	80 µM DCIP in Buffer B
B	100 µL Buffer A + 100 µL of A1	150 µL of Reaction Buffer + 50 µL of B1	40 µM DCIP in Buffer B
C	100 µL Buffer A + 100 µL of B1	150 µL of Reaction Buffer + 50 µL of C1	20 µM DCIP in Buffer B
D	100 µL Buffer A + 100 µL of C1	150 µL of Reaction Buffer + 50 µL of D1	10 µM DCIP in Buffer B
E	100 µL Buffer A + 100 µL of D1	150 µL of Reaction Buffer + 50 µL of E1	5 µM DCIP in Buffer B
F	100 µL Buffer A + 100 µL of E1	150 µL of Reaction Buffer + 50 µL of F1	2 µM DCIP in Buffer B
G	100 µL Buffer A + 100 µL of F1, **discard 100 uL**	150 µL of Reaction Buffer + 50 µL of G1	1 µM DCIP in Buffer B
H	100 µL of Buffer A	150 µL of Reaction Buffer + 50 µL of H1	Buffer B

Reaction stability of LOD in various temperatures

Materials:

- LOD (Toyobo, Cat. #LCO-301, from *Lactococcus lactis* subsp. c*remoris* IFO3427 (Toda & Nishiya, 1998))
- Self-produced LOD sample (source: *A. viridans*)
- NaH_2PO_4 from **[Solutions for LOD functional assay]**
- Lactic acid from **[Solutions for LOD functional assay]**
- BSA from **[Solutions for LOD functional assay]**
- EDTA from **[Solutions for LOD functional assay]**
- DCIP from **[Solutions for LOD functional assay]**

The purpose of this experiment is to test at which temperatures LOD is still functional. See the protocol **[LOD functional assay]** for details. Measurements occurred using the BioTek Epoch 2 and the software Gen5 3.17. Graphs were made using GraphPad Prism 10.

Note: DCIP is light sensitive and will oxidise prematurely if exposed to light. Make sure to cover the stock solution, for instance with aluminium foil, and add DCIP after preparing the rest of the buffer (excluding the enzyme, which needs to be added right before incubation). Furthermore, note that the lactic acid might disregulate the pH levels of the tube – do not exceed 1 mM of the lactic acid. Furthermore, do not test pH levels below 4, as lactic acid at these low levels will not exist as lactate in the solution.

1. Prepare a dilution of LOD samples in the appropriate 10 mM NaH_2PO_4 buffer, ensuring concentrations are the same if using different protein samples.
2. For each different pH to be tested, make an Eppendorf tube of 10 mM NaH_2PO_4, 1 mM lactic acid, 0.72 mg/mL BSA, 5 mM EDTA, and 80 µM DCIP by mixing 20 µL of 100 mM (with correct pH), 14.4 µL of 10 mg/mL BSA, 2 µL of 0.5 M EDTA, 16 µL of 1 mM DCIP, and 8 µL of the enzyme, using dH2O to get the total volume to 200 µL. Additionally, make one blank per pH, excluding the enzyme. **Note**: Make sure to add the enzyme solution right prior to adding the tubes to the heating block, as the reaction will start instantly.
3. Leave the tubes to incubate at the temperature of interest for 15 min.
4. Transfer the solutions to wells in a 96-well plate.
5. Do an endpoint measurement on a microplate reader with the plate, testing for absorbance 600 nm.

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