Lab notebook

Noa Notea, Aviv Feiertag

28.03.22

Prepared six primers of the FUSED poxB\LeuRS protein with pBEST-HIS-MBP-GPP12

Genes are from MG1655 genome

29.03.22

:Pyruvate osxidase and linker segment of fused protein

Segment length: 2580 bp

Leus_F mt=67.6 Celsius

Leus_R mt=62.4 Celsius

min elongation 2.5

(50X TAE Buffer preparation (100 ml

Tris Base: 24.2g

Glacial acetic acid: 5.71 ml

EDTA(0.5M): 10 ml

Ran DNA gel with 4 wells of back-bone PCR products

completed PCR and DNA gel for all of the 3 segments for Gibson*

next step is Gibson assembly*

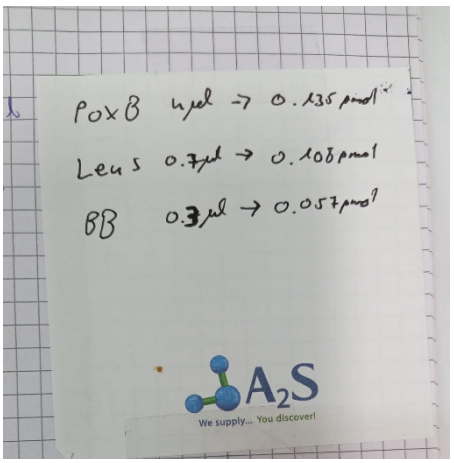
31.03.22

:DNA gel extraction

Leus = 247 ng/ul

poxB=34.5ng/ul

pBEST=301.ng/ul



03.04.22

Gibson mix = 15ul

Maximum DNA = 0.5pM

Insert=4x backbone

:GIBSON ASSEMBLY DESIGN

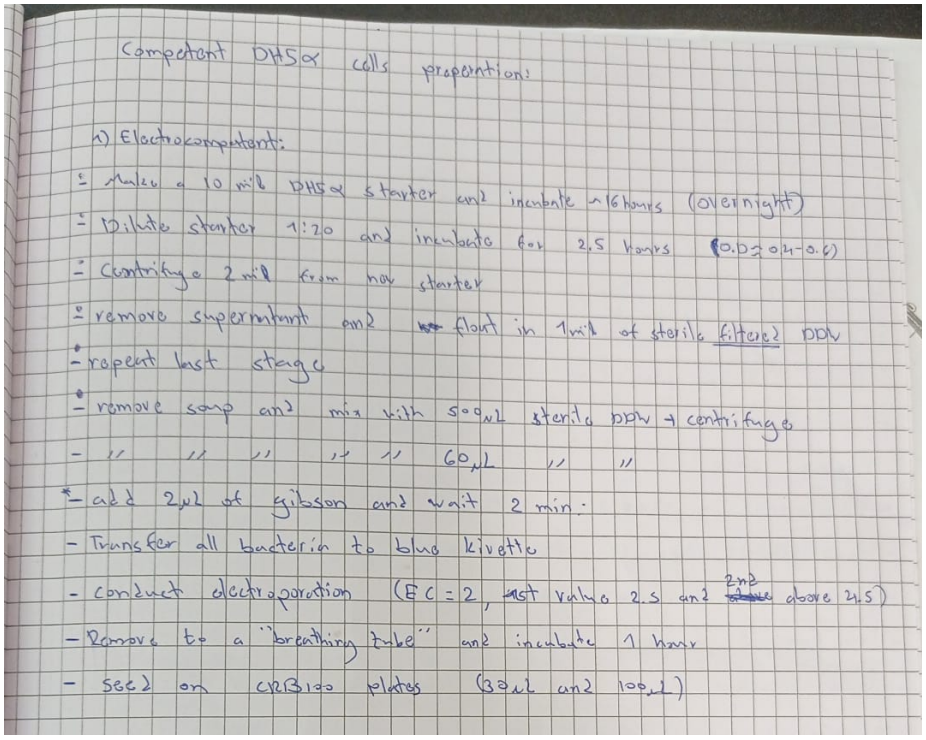
poxB length = 1644bp [34.6ng/ul] 0.033pmol

leus length = 2580bp [247ng\ul] 0.1549 pmol

pBEST = 2555bp[301ng/ul] 0.19pmol

04.04.22

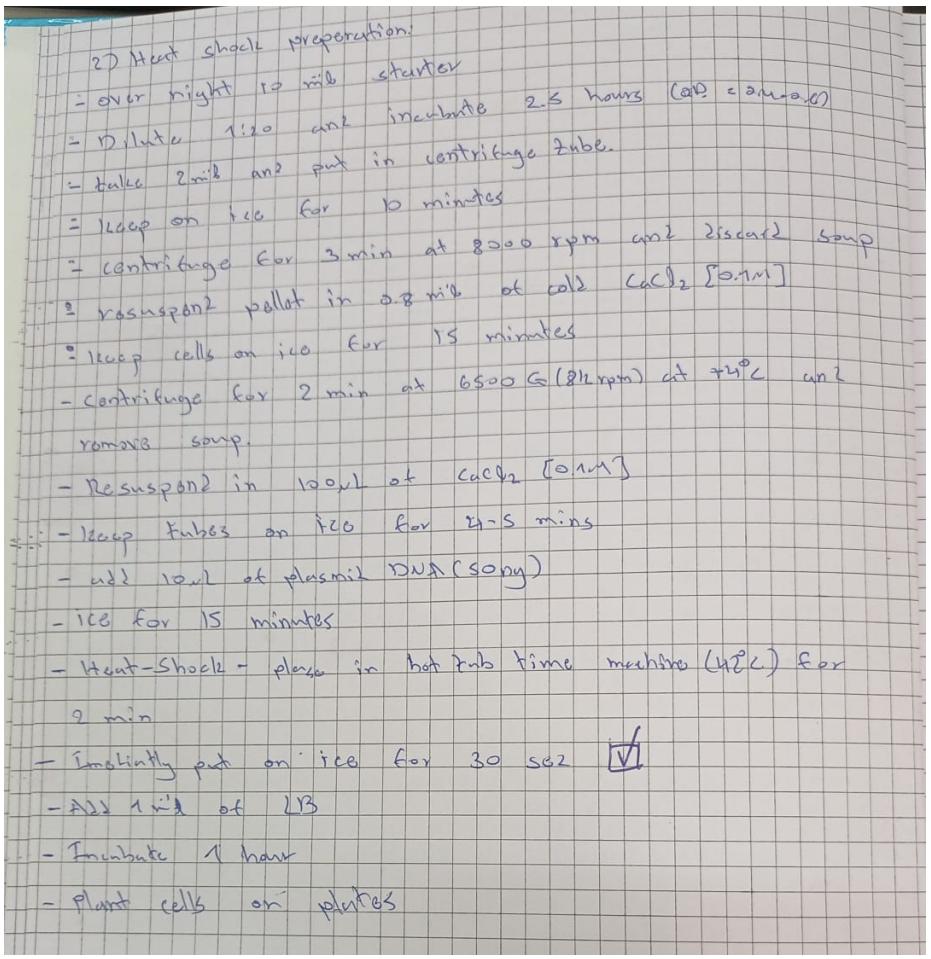
(Competent cells protocol (electroporation and heat shock



Competent DH5α cells preperation:

1) Electrocompetent:

- Make a 10 ml DH5α starter and incubate ~16 hours (overnight)
- Dilute starter 1:20 and incubate for 2.5 hours (O.D ≈ 0.4-0.6)
- Centrifuge 2 ml from new starter
- remove supernatant and ~~add~~ float in 1 ml of sterile filtered DDW
- repeat last stage
- remove soup and mix with 500 µL sterile DDW + centrifuge
- // // // // // 60 µL // //
- add 2 µL of gibson and wait 2 min.
- Transfer all bacteria to blue kivette
- conduct electroporation (EC = 2, last value 2.5 and 2nd above 2.5)
- Remove to a "breathing tube" and incubate 1 hour
- seed on CRB100 plates (30 µL and 100 µL)



2) Heat shock preperation:

- over night 10 ml starter
- Dilute 1:20 and incubate 2.5 hours (OD ≈ 0.4-0.6)
- take 2 ml and put in centrifuge tube.
- keep on ice for 10 minutes
- centrifuge for 3 min at 8000 rpm and discard soup
- resuspend pellet in 0.8 ml of cold $CaCl_2$ [0.1M]
- keep cells on ice for 15 minutes
- centrifuge for 2 min at 6500 G (8k rpm) at +4°C and remove soup.
- Resuspend in 100 µL of $CaCl_2$ [0.1M]
- keep tubes on ice for 4-5 mins
- add 10 µL of plasmid DNA (sony)
- ice for 15 minutes
- Heat-Shock - place in hot tub time machine (42°C) for 2 min
- Immediatly put on ice for 30 sec ☑
- Add 1 ml of LB
- Incubate 1 hour
- plant cells on plates

05.04.22

:Prepared Gibson reaction

15ul Gibson mix

4ul poxB dna

pBEST backbone 0.3

leus dna 0.7

Prepared both electroporation and heatshock competent cells. Transformation performed .and 4 agar plates with CRB100 were seeded resulting with 3 small colonies on plate #1

07.04.22

Miniprep to extract plasmids from 3 samples and sent to sequencing

1A=84ng\ul

2A=48ng\ul

A3 = 51ng\ul

10.04.22

Prepared working primer solutions for poxB_MG1655_pET28a_kanR and for leus_MG1655_Pet28a_kanR

.PCR for leus and pox Gibson assembly to pET28a_kanR was performed

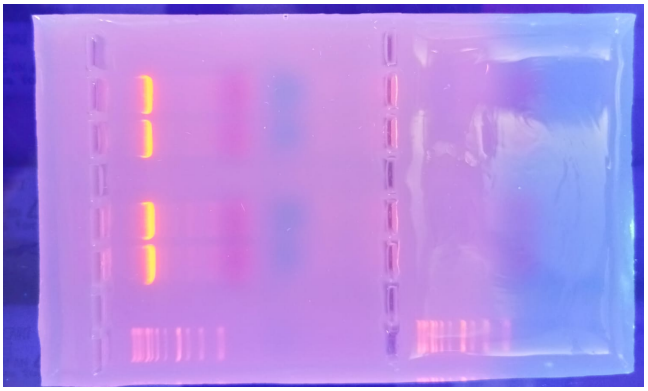
11.04.22

Miniprep to pox and leus pet28a_kanR samples for sequencing

12.04.22

.PCR for pox and leus

.Pox appeared but leus did not appear on the gel



18.04.22

Used the wrong template for poxB? Used BL21 instead of MG1655

19.04.22

Ran PCR with 12 samples. All of which were amplified

Ran gel purification

.Issues with PCR program, 2 mins 90 degrees Celsius were not enough to lysis the cells

25.04.22

DPN1 for pet segments followed by dna gel and dna gel extraction

26.04.22

Concentrations if plasmid dna were measured for Gibson assembly planning

24.04.22

Gibson assembly for pet28a segments performed

25.04.22

DH5a colonies grew

08.04.22

BL21 transformation

26.05.22

Choose 1 colony from each agar plate

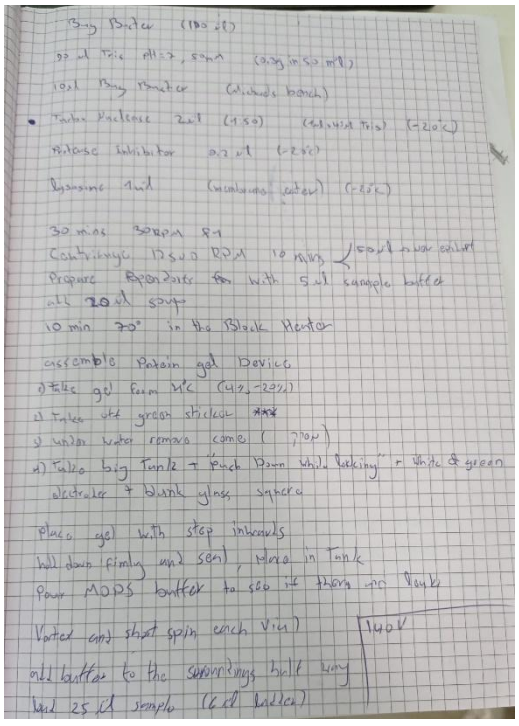
13.05.22

Three different temperatures and incubation periods tested for protein expression

15.05.22

BL21 samples did not grow

DH5a sample did grow and stored in -20 for western blot



23.05.22

Colony PCR performed to comfirm transformation

31.05.22

DH5a pBEST fused prepared and kept in -80

02.06.22

. Another colony PCR

12.06.22

Backbone for pet28a-kanR PCR

And placing DH5a and BL21 samples for 48 and 24 hours for western blot

15.06.22

Collecting samples for western blot from overnight cultures and 24h cultures

16.06.22

Samples of 48 hours collected

20.06.22

.Western blot performed with half sized protein results

28.06.22

new colonies were chosen from original fused protein plate 5

29.06.22

Miniprep for the 5 colonies and sent to sequencing

02.07.22

Colonies 1a and 2a did not show plasmid transformation

.3b and 4a showed plasmids

05.07.22

New PCR for new Gibson assembly of fused protein since there is a mutation in the original .batch

10.07.22

All plated showed colonies. Will choose 5 colonies for 48 and 24 hour starters. And sequencing

15.07.22

4a and 3d starts were placed

16.07.22

.3b did not grow, only 4a and #5 grew

08.07.22

.Western blot performed and did not work properly. Nonspecific 45kda sized protein signals

28.07.22

New IDT constructs has arrived

30.08.22

Transformation to DH5a of new IDT constructs

10.08.22

Gibson reactions for pox, leus and fused. Did not work 3

14.08.22

.New Gibson assembly reactions for leus pox and fused

21.08.22

(plates with several colonies of FUSED IDT protein (BL21 2

plates with several colonies of leus IDT with TEV and histag 2

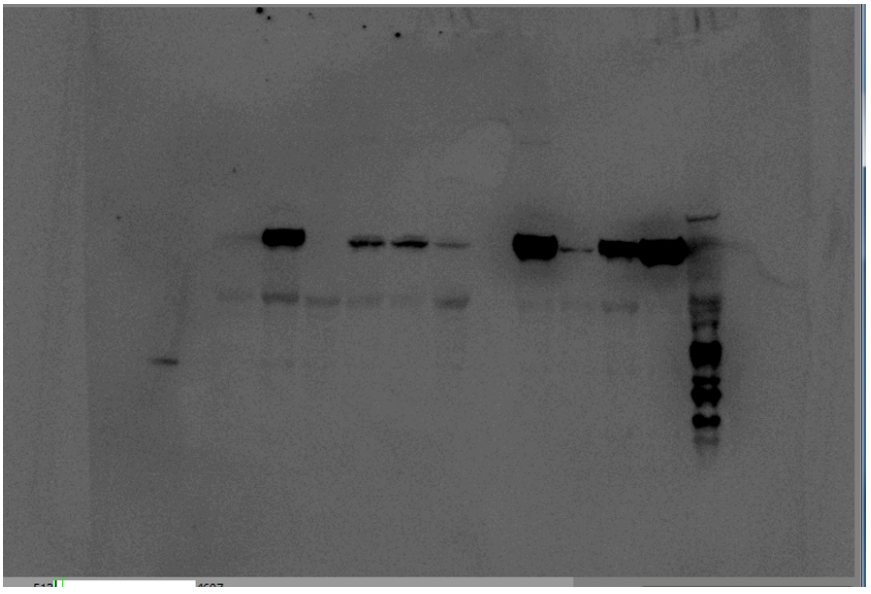
plates with several colonies of pox IDT with TEV and histag 3

24.08.22

Tac polymerase colony PCR confirms transformations

29.08.22

Western blot shows number of 45kda proteins. These are not the sizes we are expecting



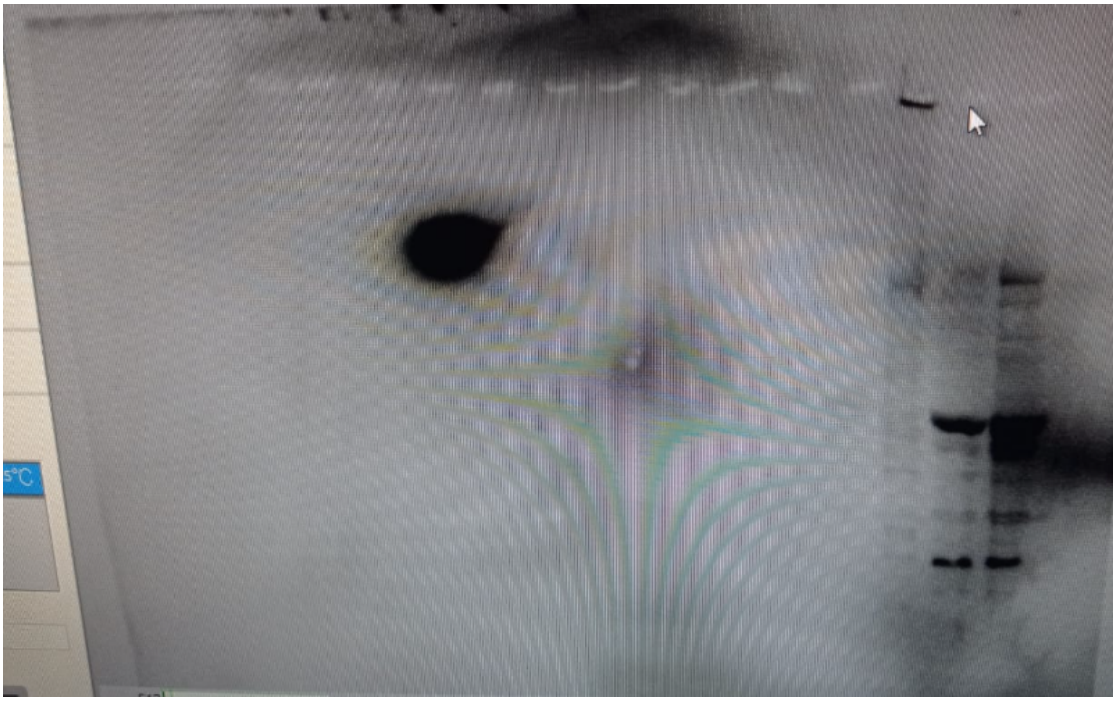
10.09.22

Second attempt of western blot with similar results

15.09.22

.Western blot shows LeuRS expression but not pox

Fused possibly stuck at "flow through" fraction



01.10.22

.rough enzyme activity assay performed. No substentional results

<u>Lab notebook</u>

Adir Eliyahu, Dorin Carmel, May Epshtein

2.1.2022

Growing competent bacteria: LB media preparation, 10mL LB starter (E.*coli* DH5a), preparation of LB agar plates with antibiotic (CRB 0.1mg/mL) and preparation for .electrophoresis

4.1.2022

Electrophoresis, inoculation of the transformed bacteria into selective LB Agar plates with .(antibiotic (CRB 0.1mg/mL

5.1.2022

.Yesterday's transformation worked! Transfer of one colony to a liquid LB media
:Preparing a glycerol stock with the bacteria
DH5alpha + PET-b(+)-His-TEV-lao1-MGBP

3.4.2022
.Creating competent E.coli BL21 bacteria
.Plasmid transformation, using heat-shock
.(Preparation of selective LB Agar plates with antibiotic (CRB 0.1mg/mL

4.4.2022
.Preparation of a starter from the colonies grown

5.4.2022
:Preparation of a glycerol stock with the bacteria
BL21-PET-b(+)-His-TEV-lao1-MGBP

6.4.2022
Preparation of Auto induction Medium (AIM), the protein can be expressed in BL21 bacteria.
Preparation of a 10mL AIM starter with CRB (0.1mg/mL) and inoculation of
.BL21-PET-b(+)-His-TEV-lao1-MGBP. Transfer to incubation, for 48h, 20 degrees

7.4.2022
We transferred 1mL of the starter to a sterile 1.5mL Eppendorf tube, centrifuge and freeze at
.-80 degrees

10.4.2022
We took the bacteria from the -80 degrees that we froze on April 7th with 10ml of LB and
.10µL of CRB. Incubated

11.4.2022
.Measurement in a spectrophotometer, using IPTG. Measured OD

13.4.2022
.Lysis using bug buster, as a preparation for western blot

25.4.2022
.Preparation of AIM
.Preparation of a starter using BL21 bacteria and lao1, in liquid LB

26.4.2022
Preparation of 100mL of AIM with CRB and 1mL of yesterdays. Incubation for 48h, 20
.degrees

28.4.2022
.His tag column + sonication

2.5.2022
.SDS-PAGE Gel plan: Lysate – FT – wash1 -wash2 – elution – ladder
.Coomassie staining and imaging of the gel with LAS4000

19.5.2022
Preparation of an activity buffer, to check the activity of the enzyme with the amino acid
.leucine

20.7.2022
Cleaning of a screen-printed gold electrode with nitric acid, then checking if the enzyme is .bound to the electrode – impedance

21.7.2022
Clean two different electrodes in two different cleaning techniques: Nitric acid and sulfuric .acid. The sulfuric acid technique was more efficient

25.7.2022
Tried to check impedance. Diluted the enzyme 1:5. Used 30μL on the electrode. Let the .electrode be soaked with the enzyme for two hours, without letting it dry. Saved in a plate

26.7.2022
Used the same electrode from yesterday. Took the enzyme, diluted 1:5 and again let the .electrode be soaked with the enzyme for two hours

28.7.2022
We cleaned more of our plasmid using sonication and his tag column, to have more enzyme. .Made agarose gel. Soaked in DPN1 for 1h. separated from gel

2.8.2022
PCR and Gibson assembly, to create a plasmid without the GBP (but with the enzyme), so we .can check its activity relative to the enzyme fused to the GBP

9.8.2022
Diluted the enzyme 1:20 with Phosphate buffer (PB) pH 7.5. Used 50μL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between -0.4 to 0.6 Voltage (V) in rate scan .of 0.05(V/S). In every run we increase the leucine concentration

23.8.2022
We take an electrode with bind enzyme. Add 50μL pb pH 7.5 and 0.25% SDS on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between -0.4 to 0.6 Voltage (V) in rate scan of 0.05(V/S). In every run we increase the leucine concentration. The SDS .damaged the sample

29.8.2022
Diluted the enzyme 1:20 with Phosphate buffer (PB) pH 7.5 and 0.05% SDS. Used 50μL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between -0.4 to 0.6 .Voltage (V) in rate scan of 0.05(V/S). In every run we increase the leucine concentration

5.9.2022
Diluted the enzyme **1:10** with Phosphate buffer (PB) pH 7.5 Used 50μL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between -0.4 to 0.6 Voltage (V) in rate scan .of 0.05(V/S). In every run we increase the leucine concentration

7.9.2022

Diluted the enzyme 1:10 with tris buffer in different pH range between 6 -8.5, Used 50µL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between -0.4 to 0.6 .Voltage (V) in rate scan of 0.05(V/S). In every run we increase the leucine concentration

11.9.2022
Diluted the enzyme 1:10 with **tris buffer pH 8**, Used 50µL on the electrode and added 6µL off **Methylene blue** (Mb 0.1mM). Run of Cyclic voltammetry (CV) electrode potential is between **-0.5 to 0.8** Voltage (V) in rate scan of 0.05(V/S). In every run we increase the leucine .concentration

12.9.2022
We took an electrode with the bound enzyme from yesterday. Added 50µL tris pH 8 (without adding Mb) on the electrode. Run of Cyclic voltammetry (CV) electrode potential between -0.5 to 0.8 Voltage (V) in **rate scan of 0.01**(V/S). In every run we increased the concentration .of leucine. We saw that in every raise of leucine concentration the peak is also raised

15.9.2022
Diluted the enzyme **1:5** with tris buffer pH 8, used 50µL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between **-0.5 to 0.5** Voltage (V) in rate scan of 0.01(V/S). In every run we increased the leucine concentration. We saw that in every raise of .leucine concentration the peak is also raised. The correlation wad better than last test

21.9.2022
We wanted to check if the current raise just by adding leucine (without enzyme at all). Run of Cyclic voltammetry (CV), electrode potential is between **-0.5 to 1.0** Voltage (V) in rate scan of 0.01(V/S). In every run we increased the leucine concentration. We saw that in every raise of leucine concentration the peak is also raised, we think that happened because the potential .was too high

28.9.2022
We want to check again if the current raise just by adding leucine (without enzyme at all). But this time we did run of Cyclic voltammetry (CV) electrode potential is between **-0.5 to 0.5** Voltage (V) in rate scan of 0.01(V/S). In every run we increase the leucine concentration. We .saw that is **no change in the current** with every raise of leucine concentration

2.10.2022
We think that the tris(Hcl) buffer effect on the results so we want to change again to PB but this time we used in pb pH 8. We diluted the enzyme **1:5** with pb buffer pH 8, used 50µL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between **-0.5 to 0.5** Voltage (V) in rate scan of 0.01(V/S). In every run we increase the leucine concentration. We .saw that in every raise of leucine concentration the peak is also raised

3.10.2022
We want to check again if the current raise just by adding leucine (without enzyme at all) because the changed to pb pH 8. We did run of Cyclic voltammetry (CV) electrode potential is between -0.5 to 0.5 Voltage (V) in rate scan of 0.01(V/S). In every run we increase the leucine concentration. We saw that is **no change in the current** with every raise of leucine .concentration

6.10.2022

We wanted to check **interfering molecules**. We cleaned a new electrode. Diluted the enzyme 1:5 with pb buffer pH 8, used 50µL on the electrode. Run of Cyclic voltammetry (CV) electrode potential is between -0.5 to 0.5 Voltage (V) in rate scan of 0.01(V/S). In every run we increased the leucine concentration. When we put 230µM of leucine we add an 100µM of Serine, and the made more two runs with just increased leucine. We saw that in every raise of leucine concentration the peak is also raised. **Serine doesn't interfere to the .enzyme**

We made one more test but this time we made a mix of amino acid (Lysine, Glutamic acid, Serine, Glycine and every one of them was in the same concentration blood level). We made the same condition CV, but this time when we add the mix AA the peak doesn't raised. We .think that may happened because the enzyme was in Saturated

8/2/22- dissolving ferrocene dicarboxylic acid

trying to dissolve ferrocene dicarboxylic acid in only PBS pH=7.4. This works only when the solution is in 100nM and is not concentrated enough.

8/2/22- dissolving ferrocene dicarboxylic acid second try

trying to dissolve ferrocene dicarboxylic acid in only PBS that we start with high pH of 14 and then get it back to 6 pH work with a concentrated of 1mM but not more. and it's good enough.

10/2/22- learning to clean the screen-printed gold electrode

To do so you need to put 50 uL of sulfuric acid in 0.5M on the screen-printed gold electrode and use a protocol of cyclic voltammetry that starts from -0.1V till 1.2V in a jump of 0.005V and scan speed of 0.1 sec and 10 cycles. Then you need to wash the electrode with water and then with ethanol ABS(absolute) and then air the leftovers with air u need to make sure the electrode is very dry before you enter it into the potentiostat.

10/2/22- learning to measure on the screen-printed gold electrode

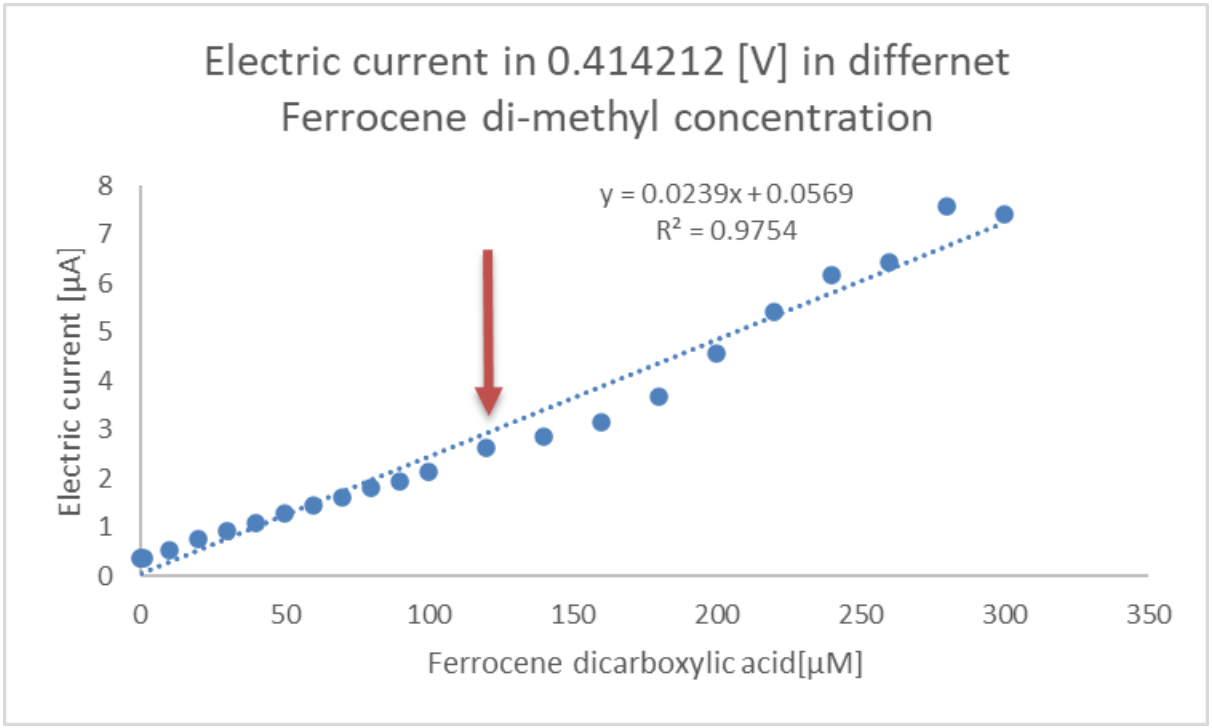
To measure cyclic voltammetry you start with cleaning the electrode and then put 50uL of your experiment tube. And use a protocol of cyclic voltammetry that starts from -0.2V till 0.6V in a jump of 0.005 and scan speed of 0.1 sec and 7 cycles which is the safe interval of water.

10/2/22- learning the voltammogram of ferrocene dicarboxylic acid

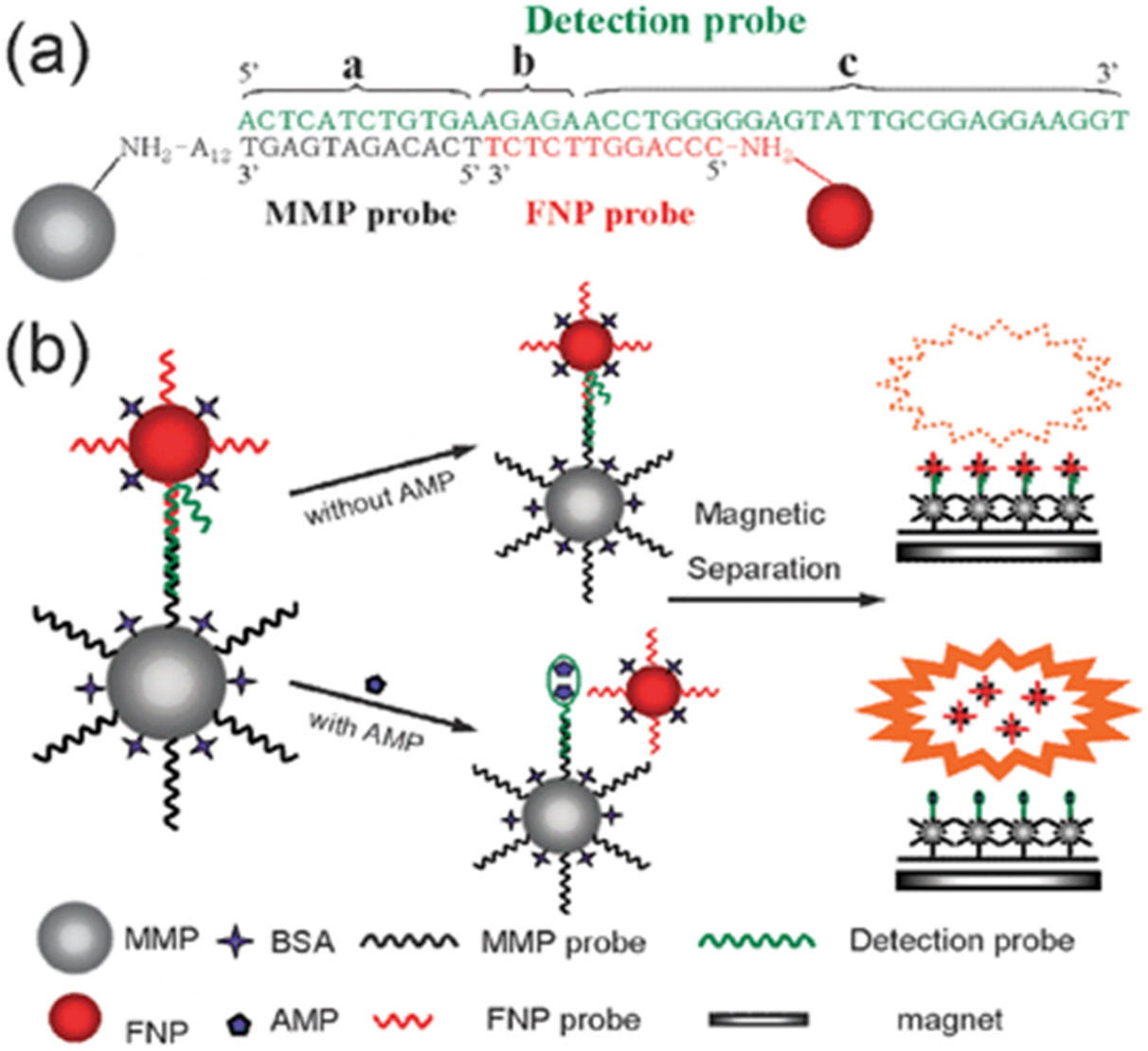
Using the protocol of measure on the screen-printed gold electrode we observed a pick on the oxidation side 410mV and on the redaction side 330mV.

18/2/22- making calibration curve of ferrocene dicarboxylic acid

We made a calibration curve of ferrocene dicarboxylic acid in the range of 0-300uM.



21/2/22- making preliminary scheme of work



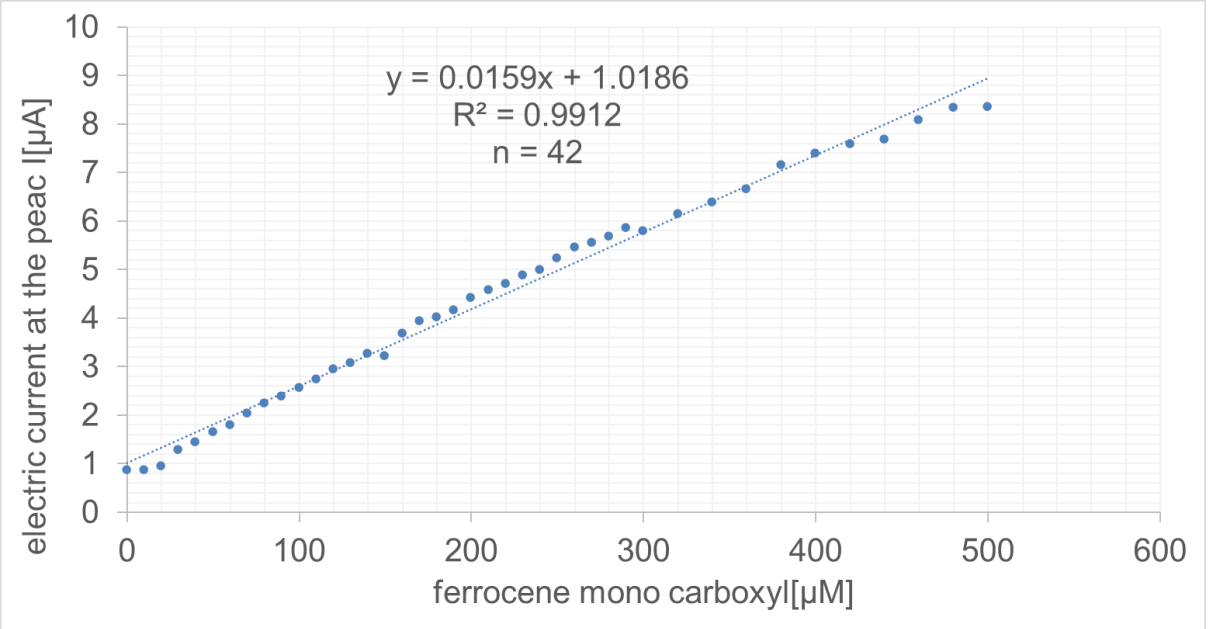
2/3/22- EDC NHS coupling between ferrocene monocarboxylic acid to Fer prob

	stock concentration	desire concentration	v from stock
Sulfo NHS	50 mM	2.5 mM	5 uL
EDC	10 mM	1 mM	10 uL
buffer MES PH=6	100 mM	10 mM	10 uL
NH2-DNA	1 mM	100 uM	10 uL
Fe-COO-	1 mM	100 uM	10 uL
DDW	-	-	55 uL

Getting together 100 uL of experiment tube that should contain Feroccen-ferProb.
mix all together and let it mix for 24 h and then store it at 4C degrees.

29/3/22,31/3/22- making calibration curve of ferrocene monocarboxylic acid

we decide to work with ferrocene monocarboxylic acid to make it one side coupling. so we make another calibration curve in a bigger range from 0-500uM.



31/3/22- EDC NHS coupling between magnetic beads to mag-prob

	stock concentration	desire concentration	v from stock
magnetic bead COO-	5gr/liter		1.8 ml that we take only the bead by magnetic sepration
mag prob NH2	1mM	100 uM	30 uL
EDC	10 mM	1 mM	30 uL
Solfu NHS	50 mM	2.5 mM	15 uL
DDW			225 uL

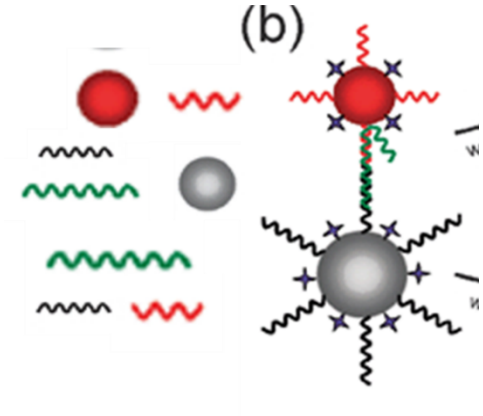
mix all together and generate 300 uL of magnetic bead-mag prob.

4/5/22- making everything together

making SSC1(25 mM sodium citrate, 150 uM NaCl), SSC2(25 mM sodium citrate, 300 uM NaCl) buffer.

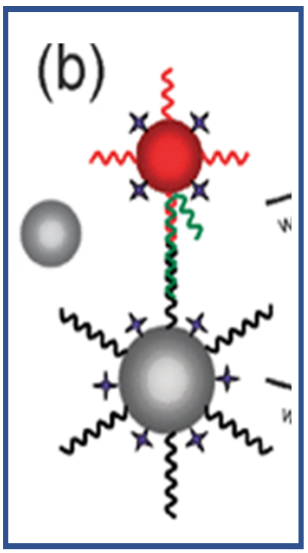
	stock concentration	desire concentration	v from stock
magnetic bead-mag prob	100 uM	40 uM	40 uL
AMP aptamer	100 uM	40 uM	40 uL
Feroccen-ferProb.	100 uM	40 uM	40 uL
SSC2			16 uL

all together have been mixed and be in room temperate for 24 h and then store in the fridge.
to make this solution.

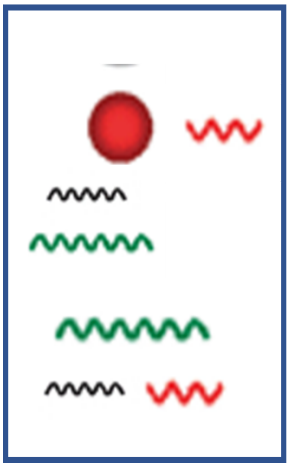


11/5/22- checking the system

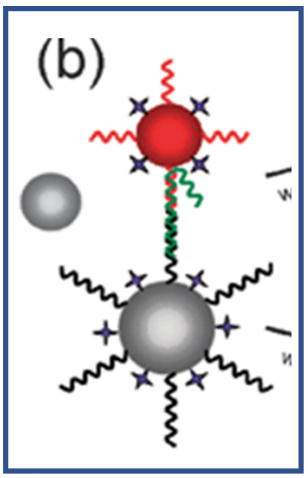
first we separate the solution with a magnet to leave two tube one that magnate and one that hasn't magnate tube 1 is called 0SSC1 that contain free Feroce, free ferProb, Free AMP aptamer, free mag-Prob. and the second tube called exp.



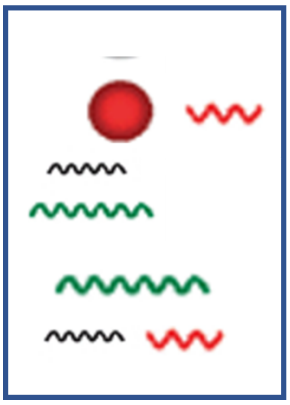
Exp



0SSC1



Exp



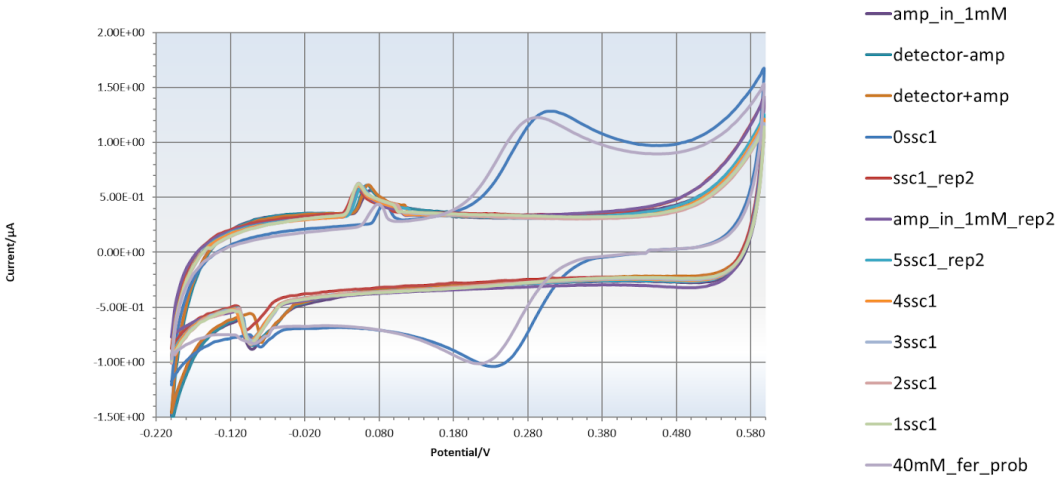
0SSC1

this Exp tube has been washed 5 times with 1ml of SSC1and separate by a magnet to remove any remains of free Feroce, etc… and this wash collects as tube 1SSC1, 2SSC1, 3SSC1, 4SSC1, 5SSC1.

the Exp tube has been suspended with 100uL of SSC1. and then split into two tubes 50 uL each. to one of them we add 1mM of AMP and name it detector AMP+, and to another we add nothing and name it detector AMP-. we magnetic separate it and get the suspension and save everything in 4C degrees. the AMP+ tube should have a Free DNA link to ferrocene hance have an electric signal and the AMP- should not have.

<u>11/5/22- testing the system</u>

checking the cyclic voltammograms got this result



the electric current of ferrocene was found only in 0SSC1 indicating that all the ferrocene was a free one that is not linked to the DNA(ie EDC NHS coupling went wrong) or the hybridization of the AMP aptamer wasn't work. So we try to do everything again by optimizing the EDC NHS coupling of the DNA to ferrocene.

18/7/22- trying to use a PCR cleaning kit to separate the free ferrocene from the ferrocene link to prob

to do so we tried to separate a 30-length DNA from a ferrocene using this kit but failed to work.

16/8/22- trying to use Sephadex column cleaning to separate the free ferrocene from the ferrocene link prob

to do so we try to separate a 30-length DNA from a ferrocene using this Sephadex G25 superfine column and succeeded make it work.

16/8/22 -1/10/22 using a lot of variation of EDC NHS coupling until one worked

Into 1000 µL solution of 3:1 PBS/DMSO (pH=7.4), we added 1.15 mg of ferrocene monocarboxylic acid and transferred 200 µL from it to another tube. To the 200 µL, we added 10 µL of 100mM Fer-DNA probe containing primary amine group, 1.917 mg of EDC, and 2.87 mg of NHS.

The final concentrations of ferrocene and DNA probe 1 were 4.76 mM each, 47.61 mM EDC, and 118.74 mM NHS. In the final step, the reaction mixture was incubated for two hours at 37 ^{0}C.