PROTOCOLS AND MATERIALS

iGEM VILNIUS-LITHUANIA 2021

CONTENTS

Terrific Broth (TB) culture media

TB is a highly enriched medium used for the cultivation of bacteria.
Dissolve tryptone, yeast extract and glycerol in water to a final volume of 900 mL and autoclave for 15 min at 121°C. Let cool down to room temperature before adding the 10X TB salts.
TB medium:

- 1.2% tryptone.
- 2.4% yeast extract.
- 0.5% glycerol.

10X TB salts:

- 0.17 M KH_2PO_4.
- 0.72 M K_2HPO_4.

DNA Digestion

Fast digestion of DNA protocol is taken from Thermo Fisher Scientific website:

Component	Volume		
	Plasmid DNA	Unpurified PCR product	Genomic DNA
Water, nuclease-free*	15 µL	17 µL	30 µL
10x FastDigest buffer or 10 x FastDigest Green buffer	2 µL	2 µL**	5 µL
DNA	2 µL (up to 1 µg)	10 µL (0.2 µg)	10 µL (5 µg)
Fast Digest enzyme	1 µL	1 µL	5 µL
Total volume	20 µL	50 µL	50 µL

1. Prepare the reaction mixture at room temperature in the order indicated in the table above.
2. Mix gently and spin down.
3. Incubate at 37°C in a heat block or water thermostat for 5 min. ***
4. Inactivate the enzyme(s) (optional). ***

Note:

* The volume of water should be corrected to keep the indicated total reaction volume. The volume of DNA can be scaled up to 10 µL or down to 0.5 µL depending on the DNA concentration.
** Only 2 µL of 10X FastDigest® buffer is required for unpurified PCR product in a 30 µL reaction volume.
*** See the Certificate of Analysis for enzyme and substrate-specific incubation time and enzyme inactivation conditions.

Fast simultaneous plasmid vector linearization and dephosphorylation

This protocol has been taken from the Thermo Fisher Scientific website.

1. Prepare the following reaction mixture containing:

Components	Volume
Plasmid DNA	1 µg
10X Thermo Scientific™ FastDigest™ Buffer	2 µL
FastDigest™ Restriction Enzyme	1 µL
FastAP™ Thermosensitive Alkaline Phosphatase	1 µL
Water, nuclease-free	to 20 µL
Total volume	20 µL

2. Mix thoroughly, spin briefly and incubate at 37 °C for 10 min.
3. Stop reactions by heating at 65 °C for 15 min or at 80 °C for 20 min (if restriction enzyme is not inactivated at 65 °C).

Note. For FastDigest™ SphI (PaeI) (#FD0604), simultaneous digestion and dephosphorylation is not recommended. Perform digestion, spin column purification and then dephosphorylation.

Oligos annealing and ligation into vector

1. Prepare reaction mixture as described below:

Water, nuclease-free	5.5 µl
Forward oligo (100 µM)	1 µl
Reverse oligo (100 µM)	1 µl
10x FastDigest Buffer	1 µl
10 mM ATP	1 µl
Polynucleotide kinase	0.5 µl

2. Incubate at 37°C for 30 min.
3. Incubate at 95°C for 5 min.
4. Stepwise decrease the temperature by 0.1°C per 1 s.
5. Dilute oligos 200 times in sterile water (1 µl of oligos in 199 µl water).
6. Prepare the ligation mixture:

Water, nuclease-free	to 20 µl
Linearised plasmid	30 ng
1:200 diluted oligos	2 µl
Ligation buffer*	2 µl
DNA ligase	1 µl

7. Incubate ligation mixture at room temperature for 15 min.

* If ligation buffer does not have a specific smell, it is recommended to add 0.5 µl of 100 µM DTT.

Chemical transformation

1. Take bacteria out of the 80 °C freezer or prepare chemically competent bacteria before the transformation.
2. Turn on the UV sterilization function in the working box for about 15 minutes to sterilize the working environment before opening the test tube with the bacteria to avoid any risk of contamination.
3. Take out petri dishes with required medium and antibiotic out of the refrigerator.
4. Put 2 µL of plasmid into 50 µL of competent cells culture (or 20 µL ligation mixture into 100 µL of competent cell culture). Also, prepare the transformation controls:
    a. contamination control - transform bacteria without DNA.
    b. negative control (check if DNA is properly digested) - transform bacteria with a linearized digested vector.
    c. positive control (check transformation efficiency) - transform bacteria with purified non-digested plasmid DNA.
5. Hold the samples on ice for 10-30 minutes.
6. Perform a heat shock for 1 min at 42 °C.
7. Incubate samples on ice for 5-10 minutes.
8. Suspend the bacteria with 1000 µL of LB (Luria Broth).
9. Incubate samples in the 37 °C* heat block for 20-60 minutes**.
10. Centrifuge cells for 3 minutes at 4400 RPM.
11. Discard the supernatant in one quick movement and resuspend bacteria in the remaining LB medium. Plate out this mixture on an LB petri plate with the correct antibiotic.
12. Incubate the plates overnight at an optimal temperature for selected bacteria.

Note:

* The temperature for recovery can vary depending on the used plasmids or bacteria strains.

** The length of the period of bacteria recovery after heat shock depends on some variables (plasmid copy number, antibiotic, etc). You can follow these recommendations - if cells are transformed with high copy number plasmid containing:

- kanamycin resistance gene, recovery should be performed for ≥ 40 min.;
- spectinomycin resistance gene, recovery should be performed for ≥ 60 min.;
- ampicillin resistance gene, recovery should be performed for ≥ 20 min;
- erythromycin resistance gene recovery should be performed for ≥ 40min.

Protein induction

1. Cultivate transformants overnight on a plate with LB broth.
2. Transfer a single transformant colony to a 12 mL flask with LB medium with an appropriate antibiotic concentration.
3. Incubate flask in a water bath at 37 °C until OD600 = 0.5.
4. Once OD600 = 0.5 is reached, transfer the flask to 4 °C for 10 min.
5. After incubation transfer 1 mL of bacterial culture into a clean 1.5 mL tube.
    a. All collected tubes needs to be prepared in this way:
        i. Centrifuge sample at 13 000 RCF at room temperature for 1-2 min and discard the supernatant.
        ii. Add 50 µL of SDS loading dye (10%) and β-mercaptoethanol (10%) solution, resuspend the pellet.
        iii. Incubate for 10 min at 100 °C in a dry bath.
        iv. Store samples at -20 °C.
6. For induction, add 1 mM IPTG inducer into bacterial culture. Incubate flasks in a shaking water bath under selected conditions and induction duration.
7. After induction, centrifuge the remaining bacterial culture volume at 4 °C, 4000 RPM for 10-15 min, carefully decant the supernatant making sure not to lose any of the precipitate or evidently more viscous fluid.
8. Pipette away any of the remaining supernatant.
9. Remaining precipitate is then stored at -20 °C or used further in a sonication process.

Cell lysis by sonication

1. Resuspend the bacterial cell biomass with 1.5 mL of resuspension buffer (Tris-HCl pH 8.0, 50 mM).
2. Sonication was carried out using the Bandelin Ultrasonic Disintegrator HD2070.
   3. Sonication process parameters:
    - 30% power
    - 3 cycles of 30 s ON / 30 s OFF sonication.
3. After sonication, centrifuge the sample at 13 000 RCF at 4 °C for 1-2 min and resuspend with 1.5 mL of resuspension buffer.
4. Repeat the sonication process.
5. Once again centrifuge the sample at 13 000 RCF at 4 °C for 1-2 min.

6. Remove the supernatant, prepare a supernatant electrophoresis sample: 20 µL of supernatant + 20 µL of SDS loading dye (10%)/β-mercaptoethanol solution (10%).
7. Resuspend the precipitate with 200 µL of resuspension buffer.

Protein Electrophoresis (SDS Page)

Separating gel

1. Prepare the gel casts in a container. Fill the container with water and after 5 min check for leakage. After a leakage check, remove water from the gel casts and dry residual gel with a paper towel.
2. To prepare a separation-gel, mix the reagents listed in the table below in proper volumes to produce a gel of desired composition.
   NOTE! Add TEMED and APS last, as solidification of the gel will occur when these reagents are added.
3. Pipette gel mixture into gel casts up to a height approximately 1 cm below the gel's comb.
4. Fill the remaining cast with isopropanol (if not available use distilled water).
5. Allow the gel to harden for 30-60 min.
6. Pour of all the isopropanol (or water) from gel casts and dry the gel with paper towels.
   - Ammonium Persulfate (APS) - 20 mg/mL
   - Acrylamide 30% solution
   - TEMED (99.9%)
   - Separating Gel Buffer - 1.5 M Tris-HCl pH 8.8 with 0.4% SDS. Stored at 4 °C.
   - Stacking Gel Buffer - 0.5 M Tris-HCl pH 6.8 with 0.4% SDS. Stored at 4 °C

1 Table.: Percentage of Acrylamide, depending on protein size.

Acrylamide [%]	M. W. Range [kDa]
7	50-500
10	20-300
12	10-200
15	3-100

2 Table.: Separating gel composition

Acrylamide [%]	6% [mL]	8% [mL]	10% [mL]	12% [mL]	15% [mL]
Water	5.2	4.6	3.8	3.2	2.2
Acrylamide/Bis-acrylamide (30%/0.8%/w/v)	2	2.6	3.4	4	5
1.5 M Tris, pH 8.8	2.6	2.6	2.6	2.6	2.6
10% (w/v) SDS	0.1	0.1	0.1	0.1	0.1
10% (w/v) ammonium persulfate (APS)	0.1	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01	0.01

Stacking gel

1. To prepare a stacking-gel, in a tube, mix the reagents listed in table 3 below with the proper volumes to receive a gel with desired composition. *NOTE! Add TEMED and APS last, as solidification of the gel will occur when these reagents are added.*
2. Pipette gel mixture on top of a separating gel to fill the cast and remove all air bubbles.
3. Fill the remaining cast with isopropanol (if not available use distilled water).
4. Insert the comb whilst the gel is still in liquid form. The comb will form wells to load samples into when the gel turns solid.
5. Allow to harden for 45-60 min.

3 Table.: Stacking gel composition

Material	Amount [mL]
Water	2.975
0.5 M Tris-HCl, pH 6.8	1.25
10% (w/v) SDS	0.05
Acrylamide/Bis-acrylamide (30%/0.8%/w/v)	0.67
10% (w/v) ammonium persulfate (APS)	0.05
TEMED	0.005

1. Mount the gels into the tank, remove combs and top off the inner chamber of the tank with 1X running buffer. Fill a third of the outer chamber of the tank with 1X running buffer.
2. Add 2 µL of ladder and 10 µL of sample into each well.
3. Connect the power supply and run at 100 V for 60 min or until the blue line approaches the bottom of the gel.
4. Once electrophoresis is done, remove the gels from the glass plates.
5. Carefully wash all equipment used for electrophoresis with water. 10x Running buffer

Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 mL of water. The pH buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature and dilute to 1X before use.

Coomassie Blue Staining

1. Prepare the staining solution containing 0.1% Coomassie Blue in 40% ethanol, 10% acetic acid.
2. After electrophoresis, incubate 1 or 2 gels in a staining container containing 100 mL of Coomassie Blue staining solution at room temperature for 1 hour until bands are visible.
3. Decant the stain and rinse the gel once with deionized water.
4. Prepare 100 mL of destaining solution containing 10% ethanol and 7.5% acetic acid.
5. Gently shake the gel at room temperature on an orbital shaker until the desired background is achieved.

Western blot

Solutions:

1. 30% acrylamide – bis solution:

30 g acrylamide and 0.8 g N,N'-methylenebisacrylamide are dissolved in 100 mL of deionized water. The solution is filtered and stored at 4 °C, no longer than 30 days.

2. 1.5 mol/l Tris-HCl, pH=8.8:

18.15 g Tris dissolved in 70 mL of H_2O; pH is corrected with HCl. Solution is diluted to 100 mL, filtered and stored at 4 °C, in the dark for no longer than 30 days.

3. 0.5 mol/l Tris-HCl, pH=6.8:

3 g of Tris are dissolved in 35 mL of H_2O, pH is adjusted using HCl. The solution is diluted
to 50 mL, filtered and stored at 4 °C, in the dark for no longer than 30 days.

4. 10% SDS solution:

5 g of SDS are dissolved and diluted to 50 mL. The solution is stored at room temperature

5. 10% APS (ammonium peroxide sulphate):

25 mg of APS are dissolved in 250 µL of H_2O. Solution must be used on the day of preparation.

5X "Loading" buffer solution
Each of these components:

- 350 µL of 0.5 mol/L Tris-HCl (pH=6.8),
- 150 mL of glycerol,
- 50 mg of SDS,
- 46 mg of DTT,
- 10 µL of bromophenol blue are to be dissolved in water one after the other. The solution is then frozen. Protein samples are heated up once mixed with this buffer, and before loading into the gel – cooled down.

10X PBS (phosphate buffer solution) (1 liter):

- 80.06 g of NaCl;
- 2.01 g of KCl;
- 11.4 g of Na_2HPO_4 ($Na_2HPO_4•7H_2O$ – 21.6 g; $Na2HPO_4•12H_2O$ – 29.0 g);
- 2.04 g of KH_2PO_2.

are to be dissolved in 800 mL of deionized H_2O in the order that the components are listed. Dilute to 1 L. Pipette in 100 mL portions and store at 4 °C.

Protein transfer buffer solution:
For 100 mL: 300 mg Tris, 1.12 g glycine, 10 mL methanol. Dilute with H_2O to 100 mL. (400 mL is the amount to be made).

Blocking solution:

- PBS with 2% milk powder.

Tween-20 solution:

- Dilute 5 mL of concentrated Tween-20 to 50 mL. 1 L PBST 10 mL diluted Tween-20 (Tween 10%/10x).

PBS-T:

- PBS + 0.1% Tween-20.

Chloronaphthol solution:

- 1 tablet of chloronaphthol + 10 mL of methanol. For membrane resolution use 10 mL of PBS 2 mL chloronaphthol dissolved in methanol 30 L 30% H_2O_2.

Blotting protocol:

1. Electrophoresis - everything done in this step is covered in the SDS-PAGE protocol.
2. The protein from the polyacrylamide gel is transferred onto the PVDF membrane (Polyvinylidene fluoride). The membrane is cut to be nearly the same size as the gel, same sized filter papers are also prepared.
3. After electrophoresis, the polyacrylamide gel is submerged in the protein transfer solution. The membrane is washed with methanol and also soaked in the transfer solution. The same is done to the filter papers.
4. Semi-dry fractionated protein transfer onto the membrane is performed. On the protein transfer apparatus' cathode base, a piece of soaked filter paper is placed, afterwards the membrane, gel, and lastly, another layer of filter paper. Everything is pressed down using the anode base and for 40 minutes the protein transfer is performed, the current is adjusted based on the area of the gel. (Length cm x width cm = area cm2 mA usually 40 mA) (always check if there is an already set protein transfer current based on the lab you are working in).
5. The membrane is washed with PBST and afterwards blocked with PBS + 2% milk powder solution for 60 minutes at room temperature (can be stored at 4 °C overnight).
6. The membrane is washed 4 times (for 5 min) with PBST. The antibody solution is prepared: PBST + 2% milk powder. If the hybridoma medium is used, it can be diluted to 1:1-1:20 overall solution volume is 10 mL [50 µL hybridoma medium]. The membrane is incubated with the antibodies for 1 h at room temperature.
7. Membrane is washed 6 times with PBST. The secondary antibody solution is prepared with 2% milk powder PBST. The conjugate is diluted to 1:1000 – 1:5000 (usually 1:4000), based on the manufacturer's recommendations. Incubate for 1 h at room temperature.
8. Membrane is washed 4 times with PBST, incubating 2–3 times for 5 min on a shaking platform. Afterwards, wash 2–3 times with distilled water.
9. The membrane is treated with chloronaphthol solution, and only if nothing is seen or is barely resolved, TMB – blotting substrate for the peroxidase (ready-to-use) is used (before TMB wash the membrane with water).
10. The membrane is washed with water, dried, and scanned. Due to light exposure the peroxidase substrate blurs out in 3-7 days.

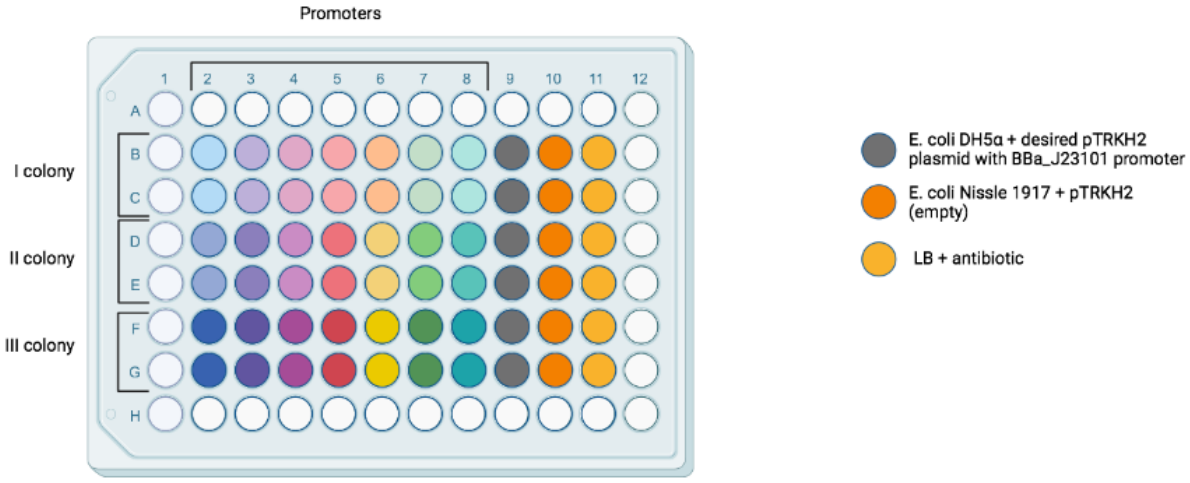
Note: in aptamer-based Western blot we use 0,1 µM aptamer PBS-T working solution.

Fluorescence measurement using a plate reader

Introduction: This protocol is for measurement of fluorescence and OD600.

Preparation of bacteria

1. Transform bacteria with plasmid containing your target genes.
2. Pick three colonies from each plate and inoculate in 5 mL of LB medium with the right antibiotic.
3. Make a 1:10 dilution of each overnight culture in LB medium with the right antibiotic.
4. Measure OD600 and write down the values of OD600.
5. Dilute the culture to 0.05 OD600 in an eppendorf tube. The sample is placed on ice.
6. The samples should be laid out according to the plate down below:



PCR product purification

This protocol for GeneJET PCR Purification Kit has been taken from Thermo Scientific website and is as follows:

1. Add a 1:1 volume of Binding Buffer to the completed PCR mixture. Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet,

add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mixture will become yellow.

2. If the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol. Mix thoroughly.
3. Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s at 13,000 RCF. Discard the flow-through.
    - Note.
        - If the total volume exceeds 800 µL, the solution can be added to the column in stages.
        - Close the bag with DNA Purification Micro Columns tightly after each use!
4. Add 700 µL of Wash Buffer to the GeneJET purification column. Centrifuge for 30-60 s at 13,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
5. Centrifuge the empty GeneJET purification column for an additional 1 min at 13,000 RCF to completely remove any residual wash buffer.
    - Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
6. Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube. Add 20 µL of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min at 13,000 RCF. Repeat elution with 20 µL of Elution Buffer.
    - Note:
        - For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.
        - If the DNA fragment is > 10 kb, prewarm Elution Buffer to 65 °C before applying to a column.
        - If the elution volume is 10 µL and the DNA amount is ≥5 µg, incubate the column for 1 min at room temperature before centrifugation.
7. Discard the GeneJET purification column and store the purified DNA at -20 °C.

Gel extraction

This protocol for GeneJET Gel Extraction and DNA Cleanup Micro Kit has been taken from Thermo Scientific website and is as follows:

1. Remove the gel from the electrophoresis system.
2. To visualize the DNA prior to extraction one cannot use UV illuminators due to risk of mutation caused by UV exposure. Instead, a Blue-light illuminator in a dark room can be used, which is sufficient for accurate gel excision.
    - Note. If the possibility of avoiding UV irradiation is out of the question, please try to keep the exposure of the gel to UV under 10 seconds to avoid damaging DNA.
3. Excise the bands with the DNA of interest keeping the excess of gel to a minimum as it decreases the yield of DNA after cleanup.
4. Follow the Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit, which is listed below as follows:
5. Excise a gel slice of up to 200 mg containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize gel surplus. Place the gel slice into a 1.5 mL tube.
6. Add 200 µL of Extraction Buffer. Mix thoroughly by pipetting.
7. Incubate the gel mixture at 50-58°C for 10 minutes or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved.
8. Add 200 µL of ethanol (96-100%) and mix by pipetting.
9. Transfer the mixture to the DNA Purification Micro Column preassembled with a collection tube. Centrifuge the column for 30-60 seconds at 14,000 RCF. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
    - Note:
        - If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 RCF.
        - Close the bag with DNA Purification Micro Columns tightly after each use!
10. Add 200 µL of Prewash Buffer (with ethanol) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
    - Note. If the DNA fragment is ≥ 10 kb, centrifuge the column for 1-2 minutes at 14,000 RCF.

11. Add 700µL of Wash Buffer (with ethanol) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
    - Note. If DNA fragment is ≥ 10 kb, centrifuge the column for 1-2 minutes at 14,000 RCF.
12. Repeat step 11.
13. Centrifuge the empty DNA Purification Micro Column for an additional 1 minute at 14,000 RCF to completely remove the residual Wash Buffer.
    - Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
14. Transfer the DNA Purification Micro Column into a clean 1.5 mL microcentrifuge tube (not included).
15. Add 10 µL of Elution Buffer to the DNA Purification Micro Column. Centrifuge for 1 minute at 14,000 RCF to elute DNA.

    - Note:
      - If a DNA fragment is ≥ 10 kb the elution volume should be increased between 15-20 µL to get optimal DNA yield. Elution volume less than 10 µL is not recommended.
      - A lower volume of Elution Buffer for DNA Micro Kit can be used (6-10 µL) to concentrate eluted DNA. Please notice that <10 µL elution volume slightly decreases DNA yield.
16. Discard the purification column and store the purified DNA at -20 °C.

Plasmid purification

This protocol for GeneJET PCR Purification Kit has been taken from Thermo Scientific website and is as follows:

1. Use 1-5 mL of *E. coli* overnight culture in LB media for purification of high-copy plasmids. For low-copy plasmids use up to 10 mL of culture.
2. Resuspend the pelleted cells in 250 µL of the Resuspension Solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be entirely resuspended by vortexing or pipetting up and down until no cell clumps remain.
    - Note. Ensure RNase A has been added to the Resuspension Solution.

3. Add 250 µL of the Lysis Solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
    - Note. Do not vortex to avoid shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.
4. Add 350 µL of the Neutralization Solution and mix immediately and thoroughly by inverting the tube 4-6 times.
    - Note. It is important to mix thoroughly and gently after addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.
5. Centrifuge for 5 min to pellet cell debris and chromosomal DNA.
6. Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
    - Note. Close the bag with GeneJET Spin Columns tightly after each use.
7. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
    - Note. Do not add bleach to the flow-through.
8. Add 500 µL of the Wash Solution to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
9. Repeat the wash procedure using 500 µL of the Wash Solution.
10. Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.
11. Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube. Add 20 µL of the Elution Buffer to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 5 min at room temperature and centrifuge for 2 min.
12. Repeat elution with 20 µL Elution Buffer and incubate for 5 min at room temperature and centrifuge for 2 min.
    - Note. An additional elution step (optional) with Elution Buffer or water will recover residual DNA from the membrane and increase the overall yield by 10-20%. For elution of plasmids or cosmids >20 kb, prewarm Elution Buffer to 70 °C before applying to silica membrane.
13. Discard the purification column and store the purified DNA at -20 °C.

Electrocompetent *E. coli* preparation

1. 1 M $CaCl_2$ stock solution preparation. 0.1 M $CaCl_2$ and 0.1 M $CaCl_2$ + 15% glycerol working solutions preparation. HMCK buffer preparation.
2. Inoculate 1 mL of LB with *E. coli*, place in shaking incubator at 37 °C and 200 rpm and incubate for 12-16 hours.
3. Subculturing overnight culture: add 1 mL of overnight culture to 99 mL of fresh LB (1:100 dilution, no antibiotics). Shake incubate at 37 °C and 200 rpm for 3-4 hours or until OD reaches 0.4
4. Ensure that all reagents ($CaCl_2$ solutions, HMCK buffer, centrifuge tubes, centrifuge) are ice-cold or at 4 °C.
5. Separate culture into multiple centrifuge tubes and place on ice for 20 minutes.
6. Discard the supernatant by tipping tubes over a discard bin and then aspirating any remaining media.
7. Resuspend each pellet with 20 mL ice-cold 0.1 M $CaCl_2$, incubate on ice for 30 minutes.
8. Centrifuge at 4 °C at 4000 rpm for 10 minutes.
9. Additionally cells were incubated for 10 min in the HMCK buffer on ice.
10. Centrifuge at 4 °C at 4000 rpm for 10 minutes.
11. Discard the supernatant and combine pellets by resuspending in 5 mL ice-cold 0.1 M $CaCl_2$ with 15% glycerol.
12. Use for downstream transformation or store in -80 °C freezer.

Chemically competent *E. coli* Nissle 1917 preparation

1. Inoculate one colony from the LB plate into 2 mL LB liquid medium. Shake at 37 °C overnight.
2. Inoculate 1 mL overnight cell culture into 100 mL LB medium (in a 500 mL flask).
3. Shake vigorously (170 RPM) at 37 °C to OD600 0.25-0.3.
4. Chill the culture on ice for 15 min. Also make sure the 0.1 M $CaCl_2$ solution and 0.1 M $CaCl_2$ plus 15% glycerol are on ice.
5. Centrifuge the cells for 10 min at 5000 g at 4 °C.

6. Discard the medium and resuspend the cell pellet in 30-40 mL cold 0.1 M $CaCl_2$. Keep the cells on ice for 30 min.
7. Centrifuge the cells as above.
8. Remove the supernatant, and resuspend the cell pellet in 6 mL 0.1 M $CaCl_2$ solution plus 15 % glycerol.
9. Pipet 0.2 mL of the cell suspension into sterile 1.5 mL microcentrifuge tubes. Use for downstream transformation or store in -80 °C freezer.

E. coli Nissle 1917 genome editing

Electrocompetent cells preparation and λ-Red induction:

1. Add 0.5 mL* of the overnight culture (grown at 30°C) to 35 mL of LB medium supplemented with kanamycin to final 50 µg/mL concentration to maintain pCas plasmid and with arabinose (10 mM final concentration) for λ-Red induction. Dilute the overnight culture at least 70-fold!
2. Place the flask in the 30°C H_2O bath and grow cells with shaking for about 2 hr until the cells reach an OD_{600} of 0.4–0.6. It is important not to over-grow the cells, since stationary phase cells are not optimal for recombineering.
3. Transfer half the culture to a 125 mL Erlenmeyer flask and place that flask to shake in a 42°C H_2O bath; keep the other flask at 30°C. Shake for 15 min at 200–220 rpm. The culture at 42°C is induced for recombination functions and the 32°C culture is the uninduced control.
4. Immediately after the 15 min induction, rapidly chill both cultures in an ice-water slurry; swirl the flasks gently. Leave on ice for 5–10 min. Label and chill two 35–50 mL centrifuge tubes for each set of induced and uninduced cells.
5. Transfer both the induced and uninduced cultures to the centrifuge tubes and centrifuge 7 min at 4600 × *g* at 4°C. Using sterile technique, aspirate or pour off supernatant.
6. Add 1 mL ice-cold sterile distilled H_2O to the cell pellet and gently suspend cells with a large pipet tip (do not vortex). Add another 30 mL of ice-cold distilled H_2O to each tube, seal, and gently invert to mix, again without vortexing. Centrifuge tubes again as in Step 10.
7. Promptly decant the 30-mL supernatant very carefully from the soft pellet in each tube and gently suspend each cell pellet in 10 mL ice-cold sterile 1 M $CaCl_2$. CRITICAL STEP Remove tubes from the centrifuge promptly at end of spin. The pellet is very soft and care should be taken not to dislodge

it or lose the cells, especially when processing multiple tubes. If necessary, leave a little supernatant in the tube. Centrifuge as in a 10 step.

8. Suspend cell pellets into 1 mL ice-cold sterile 1 M $CaCl_2$. Transfer the suspended cells to pre-chilled microcentrifuge tubes. Centrifuge for 30 sec at 10,000 × g in refrigerated microcentrifuge at 4°C. Aspirate supernatant, being extremely careful with the pellet.
9. Suspend the cell pellet in 200 µl ice-cold sterile 1 M $CaCl_2$ and keep on ice until used. CRITICAL STEP For highest efficiency - use freshly processed cells.

Electroporation:

1. Mixe 50 µl of cells 100 ng of pTargetT series DNA and 400 ng of donor DNA.
2. Perform electroporation in a 2-mm Gene Pulser cuvette (Bio-Rad) at 2.5 kV, and immediately after electroporated suspend cells in 1 mL of ice-cold LB medium. Include the following controls: a. Induced cells without DNA. If colonies are present on this control plate, either the selection is not working properly or the cells have a high reversion frequency for the property selected. b. Uninduced cells plus DNA. This is a control to estimate the number of background colonies due to some contaminating factor in the DNA such as intact plasmid template from a PCR reaction. CRITICAL STEP Never leave the DNA-cell mixes on ice for more than ~10 minutes.
3. Conduct cells recovered at 30°C for 1 h.
4. Centrifugate cells at 5000 rpm for 2.5 min and carefully remove supernatant.
5. Spread cell pellets on LB agar containing kanamycin (50 mg/liter) and spectinomycin (75 mg/liter) and incubated overnight at 30°C.
6. Identify transformants by colony PCR and DNA sequencing.

Plasmid curing:

1. For the curing of pTarget series, the edited colony harboring both pCas and pTarget series should be inoculated into 2 mL of LB medium containing kanamycin (50 mg/liter) and IPTG (isopropyl-β-d-thiogalactopyranoside; 0.5 mM) for 8 to 16 h.
2. Dilute cultures and spread onto LB plates containing kanamycin (50 mg/liter).
3. To determine cured of pTarget plasmid containing colonies, their sensitivity to spectinomycin (75 mg/liter) should be shown. The colonies cured of pTarget series can be used in a second round of genome editing.

4. For pCas plasmid curing, grow the colonies overnight at 37°C non selectively.

Lactobacillus paracasei genome editing

This protocol was obtained from: Song, X., Huang, H., Xiong, Z., Ai, L., & Yang, S. (2017). CRISPR-Cas9^{D10A} Nickase-Assisted Genome Editing in Lactobacillus casei. *Applied and environmental microbiology*, *83*(22), e01259-17. https://doi.org/10.1128/AEM.01259-17.

Electrocompetent *Lactobacillus* casei BL23 *(Lacticasei bacillus paracasei* BL23) cell preparation:
Items required:

- 40 mL MRS media for overnight growth.
- 1000 mL MRS media.
- 120 mL 20 % glycine solution.
- 1 M $MgCl_2$ stock solution.
- Cold 1000mL 1 mM $MgCl_2$ solution.
- Cold 1500 mL Suc-gly solution: 0.5 M sucrose, 10 % glycerol.
- Cold 2-4 mL PEG-gly solution: 26 % PEG 4000, 10 % glycerol.

Sterilize everything in an autoclave.

First day
Inoculate one colony of Lacticasei bacillus paracasei BL23 into 40 mL MRS media and incubate in 37°C without shaking overnight.

Second day:

1. In 1000 mL MRS with 1 % glycine (remove such volume of MRS that after addition of 20 % glycine solution overall volume is still 1000 mL) such quantity of cell are added that overall optical density is 0.1.
2. Incubate for 3-4 hours at 37°C till optical density reaches 0.6-0.8.
3. After OD 0.6-0.8 (early exponential growth phase) flask is placed on ice for 10-15 min and centrifuged at 5000 RPM for 10 min at 4°C.
4. Supernatant is thrown off and cells are resuspended in 2 x 500 mL of cold 1mM $MgCl_2$ solution.
5. Centrifuge at 5000 RPM for 10 min at 4°C.
6. Supernatant is thrown off and cells are resuspended in 2 x 250 mL suc-gly solution.
7. Centrifuge at 5000 RPM for 10 min at 4°C.

8. Repeat 6-7 steps 2 times.
9. Cells are resuspended in 4 mL (2-4 mL) PEG-gly solution.
10. Distribute cell mix into 100 µl aliquots and freeze in -80℃.

Preparation of cells for electroporation:

1. Inoculate 2 mL of overnight culture in 50 mL MRSG (MRS with 1 % glycine) and incubate at 37°C without shaking until the optical density at 600 nm (OD_{600}) reaches 0.6 to 0.8.
2. Chill cells on ice for 10 min.
3. Harvest by centrifugation at 4000 RPM and 4°C for 10 min.
4. Remove supernatant and wash cells with 50 mL ice-cold 10 % glycerol.
5. Repeat the centrifugation and washing step.
6. Centrifugate at 4000 RPM and 4°C for 10 min and discard the supernatant.
7. Resuspend cells in 0.5 mL of ice-cold 10% glycerol.
8. Store 0.1 mL of the competent cells at -80°C.

Electroporation:

1. Prior to electroporation, mix the competent cells with 1 µg plasmid DNA and transfer the mixture into a 4°C precooled 2-mm cuvette (do not use 1-mm cuvette).
2. Perform electroporation at 2 kV, 200 Ω, and 25 µF using a Bio-Rad GenePulser Xcell.
3. Immediately, add 900 µl prewarmed MMRS (MRS with 500 mM sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$) broth to the cuvette. and grow transformed cells to recover for 2 to 3 h without shaking at 37°C.
4. Plate transformants on MRS containing erythromycin (MRS-Em) and incubate for 2 or 3 days for CFU determination.

Identification of edited genes:

1. After electroporation, incubate plated transformants for 72 h to 96 h at 37°C.
2. Conduct PCR assay with a pair of primers flanking outside homologous arms on the chromosome or specific to inserted fragment. As a control use wild-type strain cells.

Purification of mutants and plasmid curing:

1. Streak recombinants containing editing plasmids on MRS without erythromycin about 2 to 3 times.

Emulsion PCR

There are two ways of creating emulsion for PCR. One is with Span 80, Tween 20 and Triton X-100. The other is with Abil EM 90 and Triton X-100.

Materials:

- Abil EM 90;
- Mineral oil for molecular biology;
- Triton X-100*;
- Span 80;
- Tween 20;
- PCR Master Mix.

Note: Triton X-100, 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol, is on the REACH authorisation list. Alternative materials should be used for further research. (Lucy. (2020, April 14). *TSG consulting*. TSG Consulting. Retrieved October 16, 2021, from https://www.tsgconsulting.com/advisory/commercial-use-triton-x-100-at-risk/.)

First option

Procedure:

1. Prepare sterile 2 mL microcentrifuge tube and place the same width magnetic stirring bar;
2. Turn magnetic stirrer on maximum RPM and place the tube with ice around or in any other cooling device on it;
3. Mix mineral oil with 4.5% Span 80, 0.4% Tween 20, 0.05% Triton X-100 in overall volume of 200 µL;
4. Slowly add freshly prepared ice-cold 100 µL of PCR MasterMix by 10 µL aliquots in 2 minutes;
5. Leave at maximum RPM for 10 more minutes;
6. Distribute overall volume by 50 µL into PCR tubes;
7. Perform PCR.

Second option

Procedure the same as first emulsion preparation but with 4% Abil EM 90 and 0.05% Triton X-100 in overall volume of 200 µL.

Protein binding on magnetic beads

Items required:

- HisPur™ Ni-NTA Magnetic Beads.
- Equilibration Buffer: Phosphate-buffered saline (PBS; 100 mM sodium phosphate, 600mM sodium chloride), 0.05% Tween™-20 Detergent, 10 mM imidazole; pH 8.0.
- Wash Buffer: PBS, 0.05% Tween-20 Detergent, 25 mM imidazole; pH 8.0.
- Elution Buffer: PBS, 250 mM imidazole; pH 8.0.

Protocol is adapted from Thermo Fisher HisPur™ Ni-NTA Magnetic Beads usage instructions:

1. Place 40 µL (0.5 mg) of HisPur Ni-NTA Magnetic Beads into a 1.5 mL microcentrifuge tube.
2. Add 160 µL of Equilibration Buffer to the beads and vortex for 10 seconds to mix.
3. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant.
4. Add 400 µL of Equilibration Buffer to the tube. Vortex the beads for 10 seconds and collect the beads with a magnetic stand. Remove and discard the supernatant.
5. Prepare sample by diluting the protein with an equal volume of Equilibration Buffer.
6. Add 400 µL of prepared protein extract to the tube, vortex for 10 seconds and then mix on an end-over-end rotator for 30 minutes.
7. Collect the beads by placing the tube on a magnetic stand. If desired, save the supernatant (flow-through) for downstream analysis.
8. Add 400 µL of Wash Buffer to the tube and mix well. Collect the beads with a magnetic stand, then remove and discard the supernatant.
9. Repeat wash step once.
10. Add 25 µL of Elution Buffer to the tube and vortex for 15 seconds. If needed, centrifuge the tube for 1 minute at 700 *g* to ensure all of the beads are submerged in the Elution Buffer. Incubate the beads for 15 minutes on a rotating platform. Alternatively, vortex the tube for 15 seconds every 5 minutes.
11. Collect the beads on a magnetic stand. Carefully remove and save the supernatant containing the His-tagged protein.
12. Repeat the elution step once using 25 µL of Elution Buffer. Incubate the beads for 10 minutes. Combine the two eluates, if desired.

SELEX

Materials:

- SELEX binding buffer;
  - 1 x PBS pH = 7.4;
  - 0.2 mM Dextran sulfate.
- SELEX wash buffer;
  - 1 x PBS pH = 7.4.
- Phusion High-Fidelity PCR Master Mix with HF Buffer;
- HisPur™ Ni-NTA Magnetic Beads;
- Herring sperm DNA.

For first round:

1. Bind protein on 40 µg magnetic beads as previously stated;
2. Heat for 10 min at 95°C 600 µl of binding buffer with 75 µg Herring sperm DNA and 2 nmol initial ssDNA aptamer pool;
3. Immediately add initial pool on ice to favor secondary structure ssDNA aptamer formation;
4. Place everything on magnetic beads with protein;
5. Incubate in rotor for 1.5 hours;
6. Place the tube in a magnetic rack and wait for all magnetic beads to accumulate near the walls;
7. Remove binding buffer and save it for later;
8. Wash with 500 µl of washing buffer by pipetting up and down;
9. Place the tube in a magnetic rack and wait for all magnetic beads to accumulate near the walls and remove the washing buffer. Save it as well;
10. Suspend everything in 100 µl water.

Prepare preparative PCR for first round:

1. To find suitable PCR cycle count prepare 7 samples for PCR - 6 for PCR optimization and 1 as positive control.
2. Visualize in 3% TBE agarose gel.

For 2-4 rounds:

1. Bind protein on 20 µg magnetic beads as previously stated.
2. Heat for 10 min at 95°C 600 µl of binding buffer with 0.5 µg Herring sperm DNA and 2 nmol initial ssDNA aptamer pool.

Round 5 was chosen as negative control against magnetic beads:

1. Prepare magnetic beads as stated in protein binding on magnetic beads protocol steps 1-4.

2. Dilute 800-1000 ng of dsDNA in water with 20 µg magnetic beads.

Rounds 6-8 and 10* repeat everything as stated in table X.

* For rounds 7-10 include 20 µg Herring sperm DNA.

4 Table. SELEX example protocol.

Round	Input	Reaction Parameters				Output	
	Input DNA (µg)	Beads + Target structure (µg) (1:1)	Binding Incubation Time (min)	Washing Steps	Number of PCR Cycles	Output - Yield (µg)	Input/Output ratio (I/O)
1	29	80	90	1x 500 µl	12	1.816	6.3%
2	0.92	40	60		17	1.335	145.1%
3	0.72		60	2x 500 µl	24	0.964	134.8%
4	0.82		60		20	1.096	133.2%
5	0.83		60		14	5.772	695.4%
6	0.78		45		16	4.840	622.9%
7	1.50		45	2x 1000 µl	13	3.540	236.0%
8	1.12		30		18	3.236	290.2%
9	1.43		30		16	3.640	254.2%
10	2.37		15		16	6.762	285.2%

Serial dilution spotting

1. Overnight cultures of bacteria are inoculated in M9 liquid media supplemented with 120 µg/mL erythromycin and bile salts in 37 °C.
2. They were diluted to the 0.5 OD600 and then 10x serial dilutions were performed. This procedure was repeated while using M9 media with and without bile salts.

3. Serial dilution spotting was performed on M9 agar plates. The volume of one spot was 4 µl.
4. Sets of plates containing one plate with bile salts and one plate without bile salts were cultivated in different temperatures (room (~ 22°C), 30°C, and 37°C).

HPLC-MS analysis

1. Grow bacteria cultures with needed protein construct plasmids for 48 hours to ensure that enzymatic activity is fully reached;
2. Centrifuge at 6000 RPM for 5 min;
3. Collect supernatant and filter through 0.22 µm pore size filter to discard any remaining cells;
4. Distribute samples in eppendorf tubes by 1 ml aliquots;
5. Fil the tubes with chloroform up to 1.5 ml, mix for 2 min and centrifuge at 12 000 RPM for 10 min to break all bubbles and extrude proteins from LB medium;
6. Remove 200 µl of upper LB medium without disturbing middle protein layer;
7. Add 100 µl of acetonitrile, mix everything for 2 min and centrifuge at 12 000 RPM for 10 min;
8. Place samples for HPLC-MS analysis.

PDA synthesis

Materials:

- 10,12-Tricosadiynoic acid (TCDA);
- 10,12-pentacosadiynoic acid (PCDA) - M = 374.60 g/mol;
- N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl);
- Ethanolamine;
- N-hydroxysuccinimide (NHS);
- Dimethyl-2-(dimethylphosphino)ethylphosphine (DMPE) - M = 150.14 g/mol;
- Anti-HSA aptamer modified with C6-NH_2;
- PVDF membrane 0.45 µM pore size.

Procedure:

- 0.25 g (0.72 mmol) of TCDA, 0.26 g (1.35 mmol) of EDC-HCl and 0.12 g (1.07 mmol) of NHS were dissolved in 4 mL of methylene chloride.
- Stirred on low RPM at room temperature for 3 hours.
- Reaction mixture was evaporated under reduced pressure at 37ºC till white-pink solid was obtained.
- TCDA-NHS (M = 461 g/mol) was extracted with ethyl acetate and water.
- Water was evaporated under reduced pressure to yield 0.46 g white solid.
- TCDA-NHS was fully dissolved in DMSO to make a 0.1 M solution.
- Dissolve amine modified aptamer in water-DMSO to make a 200 µM solution.
- To conjugate the amine aptamer with TCDA-NHS, 900 nmol TCDA-NHS (0.41 mg) was mixed with the aptamer (10 µl of 200 µM - 2.2 nmol) in additional 30 µL DMSO and reacted for 4 h at 37 °C.
- The unreacted succinimidyl active esters were inactivated by the addition of 10 µL ethanolamine.
- Dissolve 15 mg of DMPE and 37.5 mg of PCDA separately in 1 mL chloroform to make 0.1 M solutions.
    - Dilute 10x to make 0.01 M solutions.
- To make PDA of total lipid concentration 3 mM (including 5% aptamer) reagents were mixed as follows: 7% aptamer-conjugated TCDA (9 µl of previously made TCDA-Aptamer from overall volume of 50 µl), 53% PCDA (130 µl 0.01 M in chloroform) and 40% DMPE (100 µl 0.01 M in chloroform). Diluted to 800 µl in chloroform.
    - 100 µl DMPE;
    - 130 µl PCDA;
    - 9 µl TCDA-Aptamer;
    - 561 µl pure chloroform.
- To evenly coat PVDF membrane strips of PVDF were dipped in solution two times and between each dip fully dried.
- Photopolymerization was carried out by UV irradiation (254 nm) for 1 min (to maximize efficiency of photopolymerization put UV lamp as close to membrane as possible).