PROTOCOLS

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General Protocols

Reviving *E.coli Nissle 1917* (EcN) from Mutaflor

AIM:

Revive *E.coli Nissle 1917* from Mutaflor and make it calcium competent

MATERIALS:

- Agar plates
- Culture tubes
- 200 mL LB medium
- 25 mL 100 mM CaCl2
- 25 mL 100 mM MgCl2
- 8 mL 85 mM CaCl2 (with 15% Glycerol)
- 280 µL DMSO
- 1 Pill of Mutaflor

PROTOCOL:

Day 1:

- Prepare antibiotic-free LB-agar plates.
- Ensure the workplace is sterile (wipe down your bench with ethanol, wear gloves, etc.): break open the Mutaflor capsule and suspend it in 5 mL LB.
- Grow the culture overnight.

Day 2:

- Make 1:1, 1:10, and 1:100 dilution series of the EcN culture.
- Plate 100 µL each on antibiotic fee plates under sterile conditions.
- Grow overnight at 37°C.

Day 3:

- Pick a colony using a pipette tip and resuspend it in liquid LB overnight culture.
- Grow overnight at 37°C.

Day 4:

- Make the obtained cells calcium competent according to the protocol on page 6.

GeneJET Plasmid Miniprep Kit by ThermoFisher Scientific

AIM:

Extraction and purification of DNA from a bacterial calcium-competent:

MATERIALS:

- Centrifuge
- 4 mL overnight culture of a strain with the plasmid
- Resuspension solution
- Lysis solution
- Neutralisation solution
- Wash Solution
- Eppendorf tubes (as many as bacterial cultures)
- Thermo Scientific GeneJET spin columns (one per culture harvested)

PROTOCOL:

- Centrifuge cells for 5 min at 6800g, then discard the supernatant.
- Add 250 µL of Resuspension solution and vortex until the pellet dissolves completely.
- Transfer all liquid into an Eppendorf tube.
- Add 250 µL Lysis solution and invert the tube 4-6 times.
- Add 350µl of Neutralisation solution and invert the tube 4-6 times.
  The solution should become cloudy and more viscous.
- Centrifuge for 3 min at 12000g (10‘000 - 14‘000 rpm).
- While centrifuging, get the GeneJET spin columns and attach them to the vacuum pump (ensure all other attachment sites are correctly closed).
- Transfer the supernatant to a GeneJet spin column, start the vacuum, and stop when the liquid is sucked through. During this step, the DNA binds to the membrane in the spin column.
- Add 500 µL of wash solution to SpinColumn and vacuum through.
- Repeat the previous step one more time.
- Discard the flow-through and then centrifuge the empty column (keep it in its container) for 1 minute at 12000g. Ensure that the lid of the spin column points away from the direction of the centrifuge turns.
- Transfer the entire Spin Column into a new, clean Eppendorf tube.
- Add 50 µL Elution Buffer to elute the purified DNA.
- Incubate at room temp for 2 min.
- Centrifuge for 2 min.
- Collect flow-through, discard spin column.
- Measure the concentration using the NanoDrop.

GenBuilder Cloning

- Set up the following reaction on ice:
  - DNA Fragment: 0.1pmol
  - Linear Vector: 0.1pmol
  - GenBuilder MM: 10µl
  - Water: add to 20µl
- Gently mix the reactions by pipetting.
- Incubate the reaction in a thermocycler at 50 °C for 15 minutes. For DNA assembly reaction involving more than six fragments, the incubation time may be increased to 60 minutes
- Transform 2.5 µl of the assembly product into competent *E. coli* cells. For electroporation, dilute the reaction product 5-fold and use 1 µl for transformation
- Spread 1/10 volume of the recovered cells onto selection plates.
- Incubate the plates overnight at 37°C

Gibson Assembly

Gibson Master Mix Chemicals:

- 320 µL ISO buffer (5x)
- 699 µL MilliQ (ddH2O)
- 0.64 µL T5 exonuclease (10U/ µl)
- 20 µL Phusion polymerase (2U/ µl)
- 160 µL
- Taq ligase (40 U/µL)
  Total = 1.2 mL

Make 15 µL aliquots, snap freeze, and store at -20°C.

PROTOCOL:

- Mix backbone and fragments in a 1:2 ratio into a total volume of 5 µL. (2:3 for complicated assemblies with large or many inserts).
- Add the DNA mixture to one 15 µL Gibson Master Mix aliquot.
- Incubate the aliquot for 15-60 min at 50°C. (Usually one hour works fine but may need to be optimized for complicated assemblies with large or many inserts).

Transformation of competent cells with Gibson assembly:

- Thaw cells for 10 min on ice.
- Add 5 µL of Gibson mix to the cells and stir with a pipette tip to mix.
- Incubate cells on ice for one hour.
- Heat shock cells for 45 s at 42°C.
- Place cells on ice for 3 min.
- Add 0.5 mL LB medium.
- Incubate for one h at 37°C, 600 rpm.
- Plate 100 µL of culture.

Generating Electrocompetent Cells

AIM:

Make *E. coli Nissle 1917* cells electrocompetent to perform a double transformation

PROTOCOL:

- Prechill all tubes and pipets at 4 °C or –80 °C as appropriate.
- Inoculate 5 ml LB medium and grow overnight at 37 °C with rotation.
- Add the 5 ml overnight culture to 450 ml LB medium and incubate at 37 °C with vigorous shaking until the OD 600 nm is between 0.5 and 1.0. Fast cool the centrifuge with the correct rotor to 4 °C
- Pour the culture into two 225 ml centrifuge tubes.
- Place the tubes on ice for 15 min. This step can vary in incubation time between 15 min and 1 h. Longer incubation times may lead to higher competency. For the following steps it is important to keep cells cold and remove all the supernatant in each step to remove residual ions.
- Centrifuge for 10 min at 2000 g at 4 °C
- Remove supernatant and gently resuspend pellets with 200mL cold sterile water. Initially add 10-20 mL of water and resuspend by pipetting. Then add the rest of the water.
- Centrifuge for 15 min at 2000 g at 4 °C
- Remove supernatant and gently resuspend pellets with 200 mL cold sterile water. Initially add 10-20 mL of water and resuspend by pipetting. Then add the rest of the water.
- Hold on ice for 30 min
- Centrifuge for 15 min at 2000 g at 4 °C
- Remove supernatant and gently resuspend pellets with 25mL cold 10% glycerol. This can be optionally transferred to a 50 mL conical tube.
- Hold on ice for 30 min
- Centrifuge for 15 min at 1500 g at 4 °C
- Remove the supernatant and add 500 µl of 10% glycerol
- Resuspend the cells in a final volume of approximately 1 ml
- Aliquot 50 µL per tube (tubes on ice)
- Shock freeze cell suspensions in a dry ice and ethanol bath. One website recommended against using liquid nitrogen but did not justify this recommendation.
- Store at -80°C

Chemical Transformation of Calcium Competent Cells for Plasmid Amplification

AIM:

Resuspension of plasmids and transformation of competent calcium bacteria for plasmid amplification.

MATERIALS:

- Lyophilised plasmids
- ddH2O (MiliQ)
- 1 Aliquot Calcium Competent cells
- Liquid LB, no antibiotics
- LB-Antibiotic (LB-AB) plates
- Shaker
- Vortex

PROTOCOL:

Day 1:

<u>Resuspension of lyophilized plasmids</u>

- Add ddH2O to plasmids according to the table on the delivery note.
- Vortex for 1 min.
- Incubate on the shaker for 5-10 min.
- Vortex for 1 min.
- Spin down.

<u>Heat shock transformation of the bacterial strain:</u>

- 10-100 ng (0.5 µL) plasmids added to 50 µL competent cells.
- Incubate for 30 min on ice (5 min for miniprepped plasmids).
- 45-50 s heat shock at 42°C.
- Incubate for 5 min on ice.
- Add 350 µL LB.
- Incubate for one hour at 37°C, 200-300 rpm for at least 30 min (15 min for miniprepped plasmids).
- Spin down tubes for 5 min at 3000g (do not spin down miniprepped plasmids).
- Discard supernatant.
- Resuspend the pellet in the remaining liquid with the pipette.
- Plate 50 µL of remaining liquid on LB-AB plates (or how much is left).
- Let the plates grow overnight at 37°C.

Day 2:

- Set up fresh overnight culture in liquid LB with the antibiotic present in your plasmid of the 4 different colonies (4ml LB with 4ul antibiotic).

Day 3:

- Purify the DNA via miniprep
- Measure the concentration of the plasmids with the nanodrop.

Transformation by Electroporation

AIM:

Double transformation of *E. coli Nissle 1917* with plasmids containing nanobody constructs and the secretion system

PROTOCOL:

- Thaw an aliquot (100 µl) of electrocompetent EcN cells on ice and place the ligation reaction and electroporation cuvettes with a 0.2-cm gap on ice as well.
- Mix the Plasmids with the competent cells by pipetting gently up and down.
- Pulse the cells with a Gene Pulser Xcell electroporation system using 2,400 V, 25 µF and 750Ω.
- Immediately transfer the electroporated cells to 1 ml LB medium.
- Incubate the culture for 1h while shaking at 37 °C and 650 r.p.m. for recovery.
- Prepare a small preculture (5ml LB and 5ul Amp / 2.5ul Chlor)
- Add 100ul of electroporated EcN in preculture
- Plate (100ul) additionally electroporated EcN on an agar plate
- Incubate cultures over night at 37°C while shaking.

PCR

AIM:

To amplify an insert.

MATERIALS:

- 31 µL ddH2O
- 10 µL 5x Hi-Fi Phusion buffer
- 2.5 µL 10 µM forward primer
- 2.5 µL 10 µM reverse primer
- 1 µL DMSO
- 1 µL 50 mM MgCl2
- 1 µL 10 mM dNTP
- 0.5 µL template
- 0.5 µL Phusion (Hotstart) polymerase
- 0.5 µL of DpnI

PROTOCOL:

- Preparation of primers: Make a stock solution of all primers at 100 µM for storage at –20 °C and dilute them 10-fold (working concentration = 10 µM).
- Add all solutions from the materials section together (add Phusion (Hotstart) polymerase as the last part.
- Run the Thermocycler according to these settings (adjust the annealing part (3) according to your annealing temperature):

Step	Temperature	Time
1	98°C	3 min
2	98°C	30 sec
3	50°C	30 sec
4	72°C	0.5 min/kb
5	repeats 2-4	30 x
6	72°C	10 min
7	10°C	forever

- NOTE: If the template is a plasmid that carries the same antibiotic resistance gene as the destination vector, add 0.5 µl of DpnI and incubate for >1h at 37°C.
- After PCR, continue with Gel Electrophoresis.

Gel Electrophoresis and Gel Extraction

AIM:

Prepare the gel, run the gel electrophoresis, and extract the DNA from the gel-electrophoresis band.

Materials for the gel:

- Agarose
- TAE buffer
- Gel red dye (caution: carcinogenic, make sure never to touch the dye without gloves and discard the gloves properly)
- 6x loading dye (blue)

Materials for gel extraction:

- Yellow spin column
- Gel pieces from gel-electrophoresis
- NT1 buffer
- NT3 buffer
- MilliQ

PROTOCOL:

For the gel:

- For 1%, weigh 1 g of agarose in a Schott bottle, then add 100 mL of TAE buffer.
- Heat it in the microwave: swirl it when it starts boiling, then reheat it. (Repeat till the agarose dissolves entirely in the buffer).
- For the casting chamber, ensure it is assembled correctly and stable when closed.
- Add 5 µL of the red dye to the middle of the chamber.
- Pour the agarose gel into the chamber and mix it with the red dye using the comb.
- Let it sit for 20 min.
- NOTE: after working in the fume hood, DO NOT TOUCH anything with the gloves and discard them immediately.

Electrophoresis:

- Add 10 µL of blue loading dye (6x LD) to the PCR tubes, flick, vortex, and centrifuge quickly.
- Release the clamp on the chamber (pull up by the handles and move it to the back to free the gel, remove the entire casting chamber and slide it into the running chamber covered with buffer).
- Remove the comb.
- Ad 5 µL of 1 kb marker to the first well.
- NOTE: When pipetting into the wells, try not to puncture the gel. Alternatively, wiggle the pipet tip without moving the gel (if you have steady hands).
- Slowly add 30 µL of PCR product (with the blue loading buffer) to wells 2 and 3 so it does not splash into the other wells.

NOTE: If the bands are thick, use less of the PCR product for precise resolutions.

- Run gel: red=plus, black=minus.
- Voltage: 90 V, press run.
- Let it run for 40 min.

NOTE:

- If the PCR reaction gel gave a single band, clean up the PCR reaction according to the gel extraction part below.
- If more than one band is present or there is an intense low molecular weight smear on the gel, clean up the PCR reaction according to the gel extraction part below and consider doing a gradient PCR (different annealing temperatures).

Gel extraction:

- Add 1 mL of NT1 buffer to the gel tube and incubate at 75C for 5-10 minutes until completely dissolved. Flick the tube a few times to ensure the gel is fully submerged.
- Put an Eppendorf with MiliQ on the heat block.
- When dissolved, transfer all liquid to one of the yellow spin columns.
- Apply vacuum.
- Wash with 700 µL NT3 buffer and apply vacuum.
- A second wash is recommended (repeat the same step).
- Spin down the column at 11'000g for 1 min.
- Transfer the spin column to a clean, labeled Eppendorf tube.
- Add 15 µL MilliQ (from the heat block) to the middle of the membrane (DO NOT touch the membrane).
- Incubate for 3-5 minutes at 42°C.
- Centrifuge at 11'000g for one min.
- Discard the spin column.
- Check concentration and purity with Nanodrop.

Project Specific Protocols

M9 Minimal Liquid Medium

AIM:

To make a transparent medium for the read-out in the plate reader to achieve low autofluorescence and low absorbance.

MATERIALS:

- 10 mL M9 salt (5x)
- 100 µL $MgSO_4$ (1M)
- 50 µL $CaCl_2$ (0.1M)
- 1.5 mL Cas Aa (2%)
- 1 mL Glucose (20%)
- ddH_2O

Preparing the materials:

- Weigh 2.5 g of $MgSO_4$ in a 50 mL glass bottle for a 10 mL solution (1M x 246 g/mol x 0.01 L = 2.46 g).
- Weigh 0.15 g of $CaCl_2$ in a 50 mL glass bottle for a 10 mL solution (0.1 M x 147.02 g/mol x 0.01 L = 0.15 g).
- NOTE: The molar weight is written on the jars of the chemicals.
- Weigh 14.1 g of the M9 salt in a 500 mL glass bottle for a 250 mL solution.
- Add ddH_2O to all three bottles till the volume for each solution is reached.
- Autoclave the three bottles overnight.
- Weigh 10 g of Glucose, then add ddH_2O till the 50 mL mark (20%).
- Weigh 1.5 g Cas Aa, then add dd H_2O till the 50 mL mark (2%).
- Store them both in the fridge.

PROTOCOL:

- Mix everything according to the amounts written in materials in descending order.
- NOTE: work under sterile conditions (flame...).
- Add ddH_2O till 50 mL.
- NOTE: antibiotics should be added in a 1:1000 ratio (1 µL of antibiotic for 1 mL M9 medium).
- Store the medium in the fridge.

Nitric Oxide Induction Using DETA/NO

AIM:

To measure the sensitivity of our circuit to NorR in a dynamic manner and under different concentrations of the inducer DETA/NO.
NOTE: You may need to adjust the amounts according to your conditions.

MATERIALS:

- Fresh overnight cultures
- DETA/NO
- ddH2O
- M9-Amp
- 96 well plate

PROTOCOL:

The day before each experiment:

- Set up overnight cultures of all strains needed, grow at 37 C, 250 rpm.

The day of the experiment:

- Dilute all overnight cultures at a 1:10 dilution to reach OD600=0.5 according to the following calculations:

Vc = Volume of the culture, VM9 = Volume of M9 (V1=Vc, C1=OD600, V2=1mL, C2=0.5):

Sample	OD600	Vc (uL)	VM9(uL)
piGEM 1	2.2	227	773
piGEM 2	3.0	167	833
piGEM 3	2.7	185	815

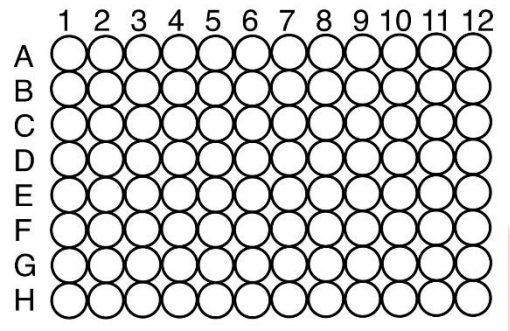


Figure 1: 96 Well Plate Layout

- Set up the PCR tubes in 8 columns and 12 rows.
- Pipette 187 µL M9-Amp into each PCR tube.
- Pipette 22 µL culture into each tube according to the 96 well-plate scheme above. (Fill the layout according to the experiments you want to do and pipette accordingly)
- Gently invert all tubes and spin them down quickly.

1:4 Dilution series:

- Set up 6 Eppendorf tubes and label them Stock Solution (SS), 2, 3, 4, 5, and water(w).
- Fill tubes 2-5 with 210 µL ddH2O.
- Put the tubes on ice to make sure they stay cold.
- Into the SS tube, measure approximately 1.83 mg DETA/NO, then keep on ice.
- After measuring the DETA/NO, calculate the volume of ddH2O needed to add to the stock solution to reach the concentration of 40 mM. Vortex to dissolve the DETA/NO in the water:

Tube	Water	5	4	3	2	SS
Concentrations of the serial dilutions	0 mM	0.15625 mM	0.625 mM	2.5 mM	10 mM	40 mM
DETA/NO concentration inside the wells	0 µM	7.8 µM	31.25 µM	125 µM	500 µM	2000 µM

- From the stock solution, pipette 70 µL into tube 2, mix through pipetting up and down.
- Repeat for tubes 3-5. Tube 5 should contain 70x4=280 µL at the end.

Starting the Assay - work as fast as possible:

- Pipette 11 µL from the respective dilution Eppendorf tubes into all PCR tubes from the lowest to the highest concentration.
- Mix gently by inverting the PCR tubes and spinning them down quickly.
- From the PCR tubes, pipette 200 µL into each well of the 96 well-plate according to the designed layout.
- After pipetting, cover the plate with a membrane.
- Put the 96 well-plate into the plate reader and start the program under constant shaking.

Flow Cytometry

AIM:

To measure the behavior of our circuits at the single-cell scale.

MATERIALS:

- Fresh overnight cultures
- DETA/NO
- ddH2O
- M9-Amp
- Flow tubes (flow cytometry tubes)
- Cold PBS solution (on ice)
- BD FACSCanto II flow cytometry

PROTOCOL:

Preparations:

- Prepare overnight cultures in LB medium
- Prepare overnight cultures in M9 medium:
    - Add 20µL of the cultures in 2 mL M9 medium for each culture tube.
    - Add the different concentrations of DETA/NO (including ddH2O for zero DETA/NO concentration).

Starting the Assay:

- Label the tubes according to your bacterial cultures.
- Add 1 mL of PBS solution in each flow tube.
  NOTE: Both the flow tubes and the PBS solution should be placed on ice.
- Add 20 µL of bacterial cultures into the flow tubes.
- Leave the flow tubes on ice when going to the flow cytometry machine.
- Vortex each flow tube before measuring with the BD FACSCanto II flow cytometry.

Genome Integration (Clonetegration)[1]

AIM:

To avoid horizontal gene transfer and reduce metabolic burden.

MATERIALS:

- pOSIP backbone
- Gene fragments (to be integrated into the genome)
- Primers for the inserts
- LB-Amp and LB-Kan
- pFLP plasmid

PROTOCOL:

Part 1:

- Gibson assembly of the inserts into the pOSIP backbone.
- Transformation of cells:
    1. Add 5 µL Gibson product to an aliquot of competent cells.
    2. Incubate on ice for 30 min.
    3. Heat shock 45 s at 42°C.
    4. Immediately add 1 mL LB.
    5. Incubate for 1 h at 37°C (this starts the integrase expression) at 150 rpm.
- Plate 200 µL of the mixture on an LB-KAN plate. Incubate overnight at 30°C (inhibits integrase expression again).
- Restreak colonies and incubate overnight at 30°C.
- Set up colony PCR to check for correct insertion.

Part 2: Removing the pOSIP-backbone:

This step removes the antibiotic resistance and the integrase from the plasmids:

- Grow a fresh overnight culture of cells containing pFLP in LB-AMP at 30°C.
- Make cells competent with the TSS method and use them directly or store them at -80°C.
- Transform the chemically competent strain with 20 ng of the pE-FLP plasmid. Let the cell recover for at least 30 min at 30°C.
- Spread the transformed cells on LB-AMP agar plates and incubate at 30°C overnight. (The FLP-mediated excision occurs during the growth of the colony overnight).

Part 3: Checking for Successful Excision:

This step checks for successful excision of the integration/selection module:

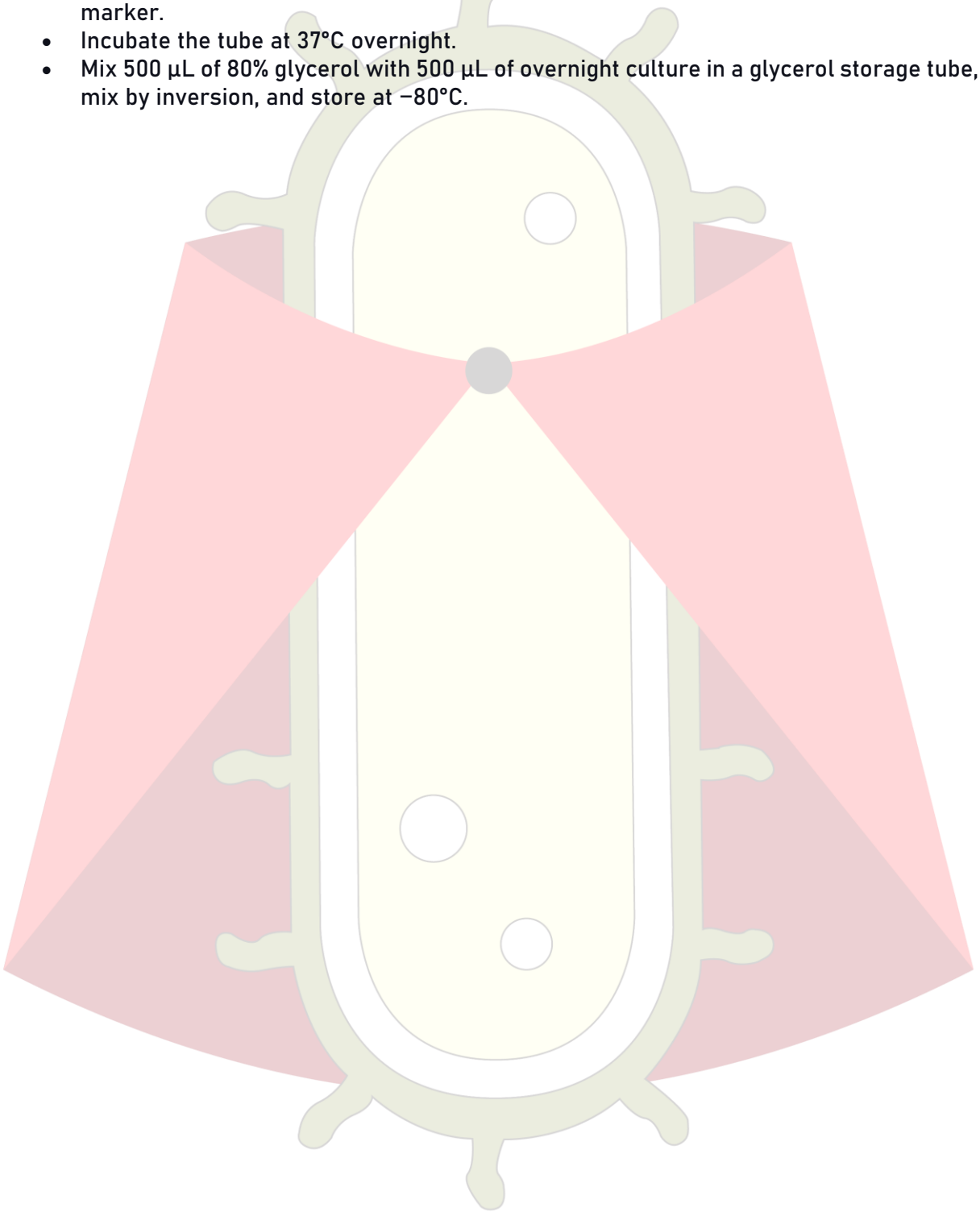
- Restreak a single isolated colony onto three different plates:
    - LB-Kan plate.
    - LB-Amp plate.
    - LB (no antibiotic) plate.
- Incubate the three plates at 42°C for 2 h, then move them to a 37°C incubator for overnight growth. (Several colonies can be restreaked onto each plate).

[1] Randall A. Hughes (ed.), Synthetic DNA: Methods and Protocols, Methods in Molecular Biology, vol. 1472, DOI 10.1007/978-1-4939-6343-0_11, © Springer Science+Business Media New York 2017

Next day:

If the pOSIP-KO backbone were successfully removed, cells would not grow on the kanamycin plates but on the antibiotic-free LB plates:

- Make an overnight culture of the “successfully flipped” strains. Ensure the use of a sterile technique at this stage, as the strains no longer carry any antibiotic resistance marker.
- Incubate the tube at 37°C overnight.
- Mix 500 µL of 80% glycerol with 500 µL of overnight culture in a glycerol storage tube, mix by inversion, and store at −80°C.



Colony PCR

AIM:

To test if the desired inserts were integrated into the bacterial genome.
NOTE: This is the exact protocol that we followed. You may adjust the amounts according to your requirements.

MATERIALS:

- PCR master mix according to PCR protocol
- Primers:
  - 1 Primer on the backbone, pointing towards the insert
  - 1 Primer on the insert (in the opposite direction)
- 1% Agarose gel
- Gel red dye
- 1 kb ladder
- Loops for restreaking
- LB-Kan plates

PROTOCOL:

- Label the LB plates for restreaking (multiple can be restreaked on the same plate if sectioned correctly).
- Set up a PCR master mix (calculate the amount needed).
- Prepare the PCR tubes with the PCR master mix.
- From each culture plate, pick 5 colonies with a sterile loop and resuspend the colony in the PCR master mix. When resuspended, directly restreak on a new LB-Kan plate (multiple colonies can be restreaked on the same plate).
- Run PCR with all tubes.
- Cast agarose gel in the meantime.
- Run a gel with a 1 kb ladder and 10 µL per PCR tube when the PCR is done.
- Analyze the gel.
- Pick colonies from cultures with insert and restreak on a new plate or set up liquid culture (depending on future use).

NOTE: A single band should be visible on the gel if the integration worked.

FX cloning of Nanobodies into pSB_init

AIM:
Insert nanobody constructs into the pSB_init expression plasmid to be purified and tested

PROTOCOL:

- Add the following components to a PCR tube for each reaction

Component	Amount per reaction
10x NEB 3.1 buffer	1 µl
PSb_init	Volume corresponding to 100ng -> 0.75 µl
Insert DNA	Volume corresponding to 300ng -> 1 µl (2 µl for biv. NBs)
BspQI / SapI	1 µl
Water	Fill to 10 µl

- Incubate at 50 °C in PCR cycler for 1 h. Deactivate restriction enzyme at 80 °C for 20 min. Cool reaction to room temperature.
- Add 1.2 µl 10mM ATP and 1 µl T4 DNA Ligase
- Incubate at 37 °C for 1 h. Deactivate T4 DNA ligase at 65 °C for 10 min
- Thaw one 50 µl aliquot of *E. coli* MC1061 on ice per reaction
- Add 2.5 µl of reaction to cells and incubate for 10 mins
- Transform by heat shock at 42 °C for 45 s
- Rest cells on ice for 5 min
- Add 500 µl LB and recover at 37 °C shaking at 600 r.p.m. for 30 min
- Plate 100 µl of transformation reaction onto agar plates
- Incubate overnight at 37 °C

Preculture and Glycerol Stocks

AIM:

Grow successfully transformed colonies overnight and make stock to be used for later experiments

PROTOCOL:

- In 50 ml conical flask, add 5 ml LB and 1:1000 dilution of antibiotic
- Pick one colony from plate and supplement into conical flask
- Incubate conical flask in shaker for 6 h/overnight at 37 °C
- For glycerol stocks, add 900 ul glycerol and 900 ul preculture to tube and shock freeze with liquid nitrogen to be stored at -80 °C

Purification of Nanobodies

AIM:
Induce expression and purify nanobodies from cultures to test target binding

PROTOCOL:

- In a 100 ml conical flask add 20 ml LB and 1:1000 dilution of antibiotic
- Scrape sample from glycerol stock
- Incubate in shaker at 37 °C for 6 hours
- In a 2 L conical flask add 540 ml TB, 60 ml phosphate buffer, 1:1000 dilution of antibiotic and12 ml bacterial culture
- Place in shaker at 37 °C until an OD_{600} of 0.4-0.7 is reached
- Induce expression by adding 0.02% L-arabinose
- Incubate in shaker for 16 h at 22 °C
- Spin cells at 4,500 r.p.m. for 20 min at 4 °C and discard supernatant
- Resuspend cells in 25 ml periplasmic extraction buffer, incubate at 4 °C for 30 min
- Add 100 ml TBS and 1 mM $MgCl_2$
- Debris pelleted at 4400 r.p.m. for 15 min at 4 °C
- Supernatant transferred to bottle with 20 nM imidazole pH 7.5
- Batch binding using 5 ml Ni-NTA resin incubated for 2 h while shaking
- Resin poured into gravity flow columns and washed with TBS pH 7.5, 30 mM imidazole
- Elution with 10 ml TBS pH 7.5, 300 mM imidazole and collected into fractions
- Fraction concentrations measured with nanodrop
- Fractions pooled and concentrated using concentration columns. Spun at 2500 g in 10 kDa concentrators)
- Loaded on Sepax in TBS pH 7.5

Biotinylation of TNF-α

AIM:
Biotinylate TNF-α to be used for ELISA and western blot

PROTOCOL:

- Prepare NHS biotin reagent in anhydrous DMSO (10mM)
- Mix with TNF
- Incubate 0.5-1 h at room temperature or 4 °C overnight
- Remove excess biotin via SEC, PD10 column or dialysis

ELISA

AIM:
Quantify the binding abilities of nanobodies against TNF-α

PROTOCOL:

- On the night before, prepare a 96-well Nunc Maxicorp immunoplate with 100 µl 1:1000 dilution of protein A stock solution in PBS. Seal plate an d incubate overnight at 4 °C
- Wash the plate with 250 µl TBS per well
- Block the plates with 250 µl TBS-BSA. Incubate for 30 min.
- Wash the plates three times with 250 µl of TBS per well.
- Add 100 µl of a 1:2,000 dilution of the monoclonal anti-c-Myc antibody in TBS-BSA-D to each well. Incubate for 20 min
- Wash the plates three times with 250 µl of TBS-D per well
- Add samples to be tested in TBS-BSA-D (if supernatant or periplasmic extract then 20 µl in 80 µl solvent, if purified nanobodies than final concentration of ca. 50 nM)
- Wash the plates three times with 250 µl of TBS-D per well
- Add 100 µl of 50 nM biotinylated TNF-α in TBS-BSA-D (dilute 5 µl aliquots in 1.5 ml TBS-BSA-D)
- Wash the plates three times with 250 µl of TBS-D per well
- Add 100 µl of 1:5,000 diluted streptavidin–peroxidase polymer in TBS-BSA-D. Incubate for 20 min
- Wash the plates three times with 250 µl of TBS-D per well
- Add 100 µl of the ELISA developing buffer. Wait until individual wells turn blue, which takes ca. 5–15 min
- Measure the absorbance at 650 nm in a plate reader. ELISA signals as small as 1.5-fold above background can indicate a high-affinity binder

Induced Secretion with Arabinose

AIM:

Induce secretion of nanobodies in MC1061 and *E. coli Nissle 1917* cells using arabinose to test the secretion system

PROTOCOL:

- Make a 5 ml preculture with 1:1000 antibiotic dilution using colony on plate or glycerol stock, incubate overnight at 37 °C while shaking at 120 r.p.m
- The next day, transfer to 10 ml TB with 1:1000 antiboitic dilution, grow at 37 °C while shaking until a OD_{600} of 0.6 is reached
- To induce secretion, add L-arabinose to a final concentration of 0.02%
- Secrete overnight at 37 °C while shaking
- The next day, spin down the cells and collect 2 ml of supernatant for testing via western blot or ELISA
- If cell lysate is to be tested:
    - Resuspend cells in TBS
    - Add one PCR tube of glass beads to a screw-lid microcentrifuge tube
    - Add the cell mixture to tube containing the glass beads
    - Lyse the cells by using maxiprep machine at 4 m/s for 20 s
    - Rest cells on ice for 5 min
    - Repeat shaking twice, resting cells on ice for 5 min in between

Induced Secretion with Nitric Oxide

AIM:

Induce secretion of nanobodies in MC1061 and *E. coli Nissle 1917* cells using nitric oxide to mimic the elevated levels found in IBD patients

PROTOCOL:

- Make a 5 ml preculture with 1:1000 antibiotic dilution using colony on plate or glycerol stock, incubate overnight at 37 °C while shaking at 120 r.p.m
- The next day, transfer to 10 ml TB with 1:1000 antiboitic dilution, grow at 37 °C while shaking until a OD_{600} of 0.6 is reached
- To induce secretion, add 0 mM, 1 mM or 2 mM DETA NO
- Secrete overnight at 37 °C while shaking
- The next day, spin down the cells and collect 2 ml of supernatant for testing via western blot or ELISA
- If cell lysate is to be tested:
    - Resuspend cells in TBS
    - Add one PCR tube of glass beads to a screw-lid microcentrifuge tube
    - Add the cell mixture to tube containing the glass beads
    - Lyse the cells by using maxiprep machine at 4 m/s for 20 s
    - Rest cells on ice for 5 min
    - Repeat shaking twice, resting cells on ice for 5 min in between

Western Blot

AIM:

To quantify the presence of nanobodies in supernatant from secretion experiements

PROTOCOL:

(amounts for 2 gels/membranes)

- Run SDS PAGE
  - Prepare samples: 50 ul supernatant/lysate + 12.5 ul 5x Protein loading dye
  - Run 20 µl sample on 4-20% gradient gel in MOPS buffer (50 mins at 140 V)
- Prepare 200 ml Transfer buffer
  - 20 ml 10x transfer buffer
  - 20 ml 100% methanol
  - 0.2g SDS
- Trim gel, and wet in transfer buffer
- Wet blotting paper in transfer buffer
- Wet membrane in methanol then transfer buffer
- Blot assembly (from top to bottom)
  - Blotting paper
  - Gel
  - Membrane
  - Blotting paper
- Transfer for 0.5-1 h at 12 V in Trans-blot SD semi dry transfer cell
- Prepare ca. 800 ml PBS-T (PBS + 0.05% Tween 20)
- Prepare 250 ml blocking buffer (250 ml PBS-T, 7.5 g BSA)
- Block for 0.5-1hr at room temperature
- Primary antibody incubation
  - Myc tag: 5 ml Blocking buffer + 1 ul anti-myc
  - His tag: 5 ml blocking buffer + 5 ul anti his
  - Roll into 50 ml falcon tube (No bubbles, no overlap)
  - Incubate for 0.5-1 hr while rotating
- 3x Wash with PBS-T for 5 mins on shaker
- Secondary antibody incubation
  - 25 ml blocking buffer + 1 ul anti-mouse antibody
  - Incubate for 0.5-1 h while shaking
- 3x Wash with PBS-T for 5 mins on shaker
- Turn imaging machine on to allow for cooldown time
- Development and imaging (Image Quant 800)
  - 1:1 ratio of immobilon western blot HRP substrate peroxide solution and immobilon western blot HRP substrate luminol reagent (1 ml per membrane) in 2 ml eppendorf tube
  - Cut film and insert membrane
  - Slowly pipette 1:1 ratio substance over membrane
  - Image with 1 s exposure (Chemiluminescence setting with colorimetric marker on for ladder)

PCR (Q5)

AIM:

Change the resistance of the secretion system plasmid from chloramphenicol to kanamycin in an attempt to increase transformation in *E. coli Nissle 1917*

PROTOCOL:

- Prepare the following
  - 5X Q5 Reaction Buffer 10µl
  - Primer fwd 2.5 µl
  - Primer rev 2.5 µl
  - 2mM DNTPs 5 µl
  - Template DNA 1 ng-1 µg
  - Q5 High-Fidelity Polymerase 0.5 µl
  - Nuclease free water add to 50 µl
- Begin thermocycling after calculating temperature on NEBTm calculator

Step	Temperature	Time
Initial denaturation	98 °C	30 s
25-35 cycles	98 °C 50-72 °C 72 °C	5–10s 10–30s 20–30s/kb
Final Extension	72 °C	2 min
Annealing	4-10 °C	

PCR (Phusion Plus)

AIM:

Transfer nanobody constructs into the plasmid containing the nitric oxide sensor

PROTOCOL:

- Take 10ul primer and add 90ul water to it -> stock solution
- Prepare the following:
  - 5X Phusion Plus Buffer 10µl
  - Primer fwd 2.5µl (from stock)
  - Primer rev 2.5µl (from stock)
  - 2mM DNTPs 5µl
  - Phusion Plus Polymerase 0.5µl
  - Water add to 50µl

a. 3-step protocol

Cycle step	Temp.	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation Annealing Extension	98°C 60°C 72°C	5–10 s 10 s 15–30 s/kb	25–35
Final extension	72°C 4°C	5 min Hold	1 Hold

- Add 1ul of DpnI to PCR products
- Incubate reaction at 37°C for 30min

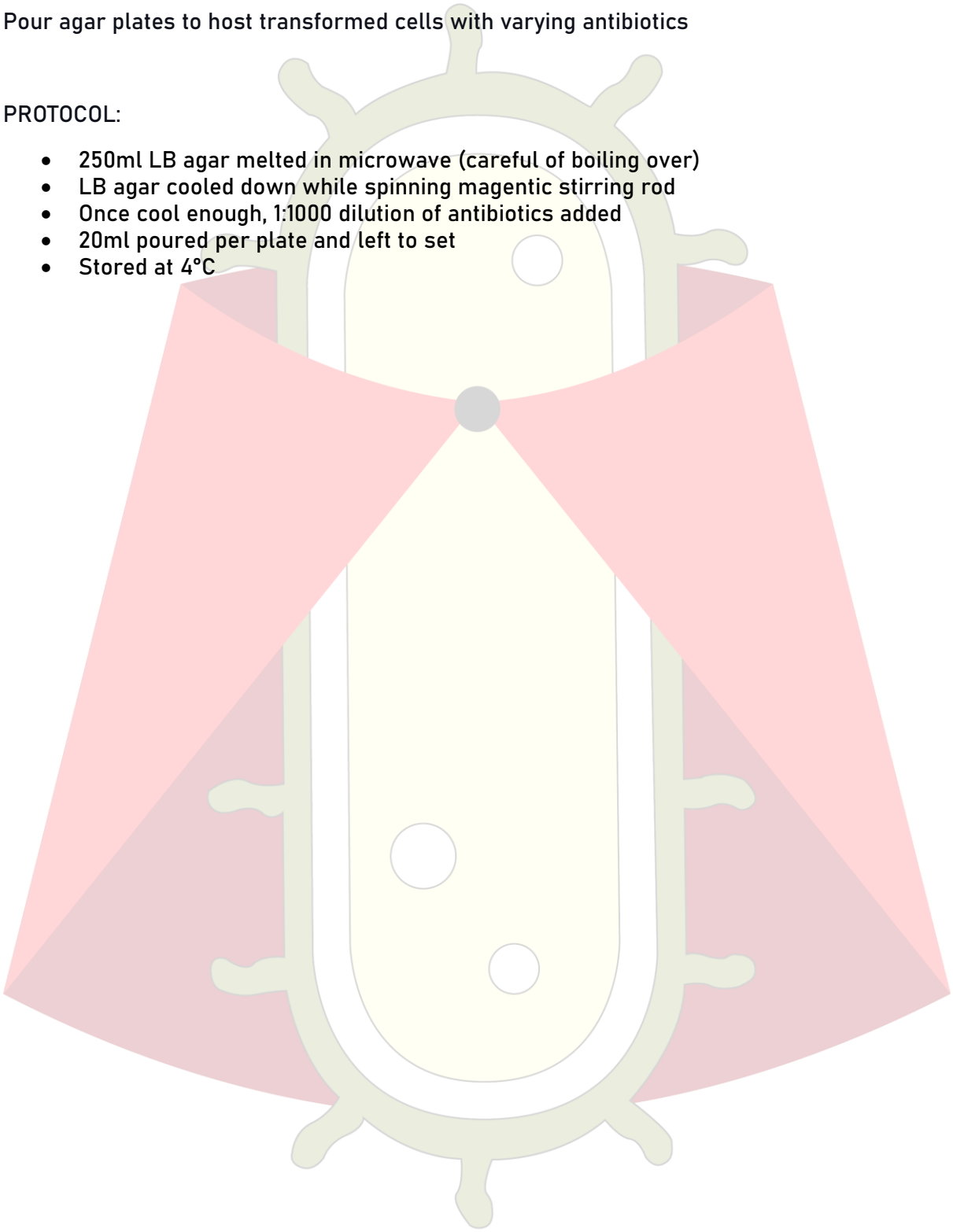
Pouring Agar Plates for Bacterial Growth

AIM:

Pour agar plates to host transformed cells with varying antibiotics

PROTOCOL:

- 250ml LB agar melted in microwave (careful of boiling over)
- LB agar cooled down while spinning magentic stirring rod
- Once cool enough, 1:1000 dilution of antibiotics added
- 20ml poured per plate and left to set
- Stored at 4°C



Cultivation of Non-Adherent Monocytic Cell Line THP1

AIM:

Grow and cultivate cells to be used in assay to show the inhibitory effect of nanobodies

PROTOCOL:

Media preparation

- 90% RPMI 1640 (Gibco)
- 10% Fetal Bovine Serum (FBS)

Subculture

- Maintain cells at 0.1 - 1.0*10^6 cells/ml
- Seed cells at ca. 0.5*10^6 cells/ml
- Split 1:2 or 1:3 every 3-4 days
- Incubate at 37°C with 5% CO2
- Doubling time: ca. 35-50 hours

Splitting cells

- Centrifuge cells at 1700 rpm for 5 minutes
- Resuspend cell pellet with fresh media
- Split the cells

Cell Assay

AIM:

Demonstrate the ability of nanobodies to inhibit inflammatory signals by binding TNF-α

PROTOCOL:

Preparation

- Centrifuge cells and resuspend in starvation media (RPMI 1640 without FBS)
- Seed cells at a density of ca. 1*10^6 cells/ml in a 96-well plate (Volume 200µl)
- Incubate cells for 24 hours at 37°C with 5% CO2

Stimulation

- Prepare dilution series for TNF (100ng/ml, 50ng/ml, 10ng/ml, 5ng/ml, 1ng/ml, 0.5ng/ml, 0.1ng/ml).
- Dilute nanobodies and positive/negative control to a concentration of approximately 100nM.
- First, add the correct volumes of nanobodies and positive/negative control to cells. Shake the plate well!
- Place the cells in the incubator for 30 minutes.
- Stimulate cells with TNF and shake the plate well! Alternatively, mix wells with a pipette.
- Incubate cells for 24 hours at 37°C with 5% CO2

Harvest

- Transfer each well to a separate Eppendorf tube
- Centrifuge tubes at 3.5G for 10 minutes at 4°C
- Shock-freeze cell pellet by placing the tubes in liquid nitrogen
- Store cell pellets at -80°C

RNA isolation using Maxwell RCS (Promega)

- Add 200µl of homogenizing buffer to the cell pellet
- Lyse cells by adding 200µl of lysis buffer
- Vortex each sample
- Prepare Maxwell RCS cartridges according to company's protocol
- Measure RNA yields with nanodrop

Reverse transcription

1x master mix:

10x RT Buffer	1 µl
25x dNTPs Mix 100mM	0.4 µl
10x Random Primers	1 µl
Reverse Transcriptase	0.5 µl
RNA	7.1 µl
Total volume	10 µl

- Dilute RNA to reach 1µg in a volume of 7.1µl

Thermocycler:

25°C	10min
37°C	2hrs
85°C	5min
4°C	∞

Quantitative RT-PCR

- Dilute cDNA obtained from reverse transcription to ca. 25ng/µl
- Prepare the following master mix

1x master mix:

ABI Fast Polymerase mix (Applied Biosystems)	5ul
Primer GOI (FAM)	0.5ul
Primer HKG (VIC)	0.5ul
Water	3ul
Total (per well)	9ul
cDNA added	1ul

- "Gene of interest" = GOI
- "House-keeping gene" = HKG -> human GAPDH
- Pipette samples into a 384-well plate
- Place a foil on top and centrifuge plate
- Set up RT-PCR machine
- Analyze fluorescence readout