iGEM MIT_MAHE

Composite Bio-Brick 2 Handbook

Acknowledgements

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1. Introduction to Composite Bio-Brick 2

A. List of standard biological parts and their source organisms:

Part/Bio-Brick	Species name (including strain)	iGEM registry
Constitutive Promoter (J23100) [Present in Composite Bio-Brick 1]	No natural comparator	http://parts.igem.org/Part:BBa_J23100
SoxR transcription factor (K554003) [Present in Composite Bio-Brick 1]	*Escherichia coli K-12*	http://parts.igem.org/Part:BBa_K554003
SoxS promoter (K554000)	*Pseudomonas nitroreducens*	http://parts.igem.org/Part:BBa_K554000
HlyB/ Hemolysin B (K554007)	*Escherichia coli O157 H7 strain EDL 933*	http://parts.igem.org/Part:BBa_K554007
HlyD/ Hemolysin D (K554008)	*Escherichia coli O157 H7 strain EDL 933*	http://parts.igem.org/Part:BBa_K554008
TolC (K554009)	*Escherichia coli O157 H7 strain EDL 933*	http://parts.igem.org/Part:BBa_K554009
IL-10/ Interleukin 10 (K554004)	Human (Th1 cell)	http://parts.igem.org/Part:BBa_K554004
HlyA/ Hemolysin A (K554002)	*Escherichia coli O157 H7 strain EDL 933*	http://parts.igem.org/Part:BBa_K554002
GFP/ Green Fluorescent Protein (E0040)	*Aequorea victoria*	http://parts.igem.org/Part:BBa_E0040
Double terminator (B0015)	*Escherichia coli K-12*	https://parts.igem.org/Part:BBa_B0015
RBS (B0034)	No natural comparator	http://parts.igem.org/Part:BBa_B0034

Note: The circuits are to be assembled on pSB4C5 vector*.

* iGEM registry: http://parts.igem.org/Part:pSB4C5

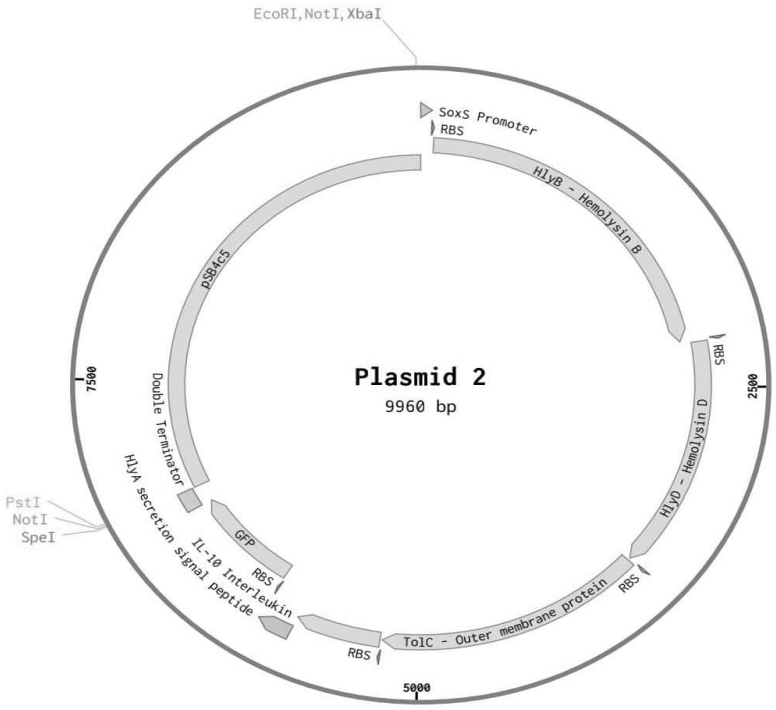
B. Working principles of each part:

Part/Bio-Brick	Working principle:
Constitutive promoter	A constitutive promoter is an unregulated promoter that continuously transcribes the genes downstream to it. It can be used to tune the expression of parts.
SoxR transcription factor	SoxR is a transcription factor, activated in the conditions of oxidative stresses (presence of NO; pro-inflammatory signal) by reversible one-electron oxidation of its [2Fe-2S] cluster. In the active state, it activates the SoxS promoter by untwisting the promoter DNA by binding to the long spacer DNA (19- or 20-bp spacer between the -35 and -10 operator elements) [12] [17].
SoxS promoter	SoxS is a conditional promoter, regulated by the SoxR transcription factor (In Composite Bio-Brick 1). In the active state (when it is bound to NO; pro-inflammatory signal), SoxR activates the SoxS promoter and starts the transcription of the genes downstream to it [12].
HlyB	HlyB acts as an ATP-binding cassette and recognizes the substrate via its secretion signal peptide (HlyA). It exports any protein (like IL-10) fused to the secretion signal, produced inside the bacteria that must act on targets outside and is responsible for the specificity of the secretion system process [1] [7] [10] [22].
HlyD	HlyD consists of a short cytoplasmic domain at the N-terminus, a membrane anchor and a large periplasmic domain. It acts as a membrane fusion or adaptor protein by linking the HlyB and TolC domains of the Hemolysin transport system and helps in the transportation of proteins [1] [7] [10] [22].
HlyA	HlyA is a signal sequence that interacts with the cytoplasmic region of the pre-formed HlyB–D complex. After the binding of the HlyA secretion signal by the HlyB–D complex, HlyD induces the interaction with TolC which then transports it out of the cell [10] [11].
TolC	TolC is a specific outer membrane protein which forms a long channel throughout the periplasm and the outer membrane, largely open towards the extracellular matrix for export of required components to targets outside the bacteria [1] [7] [10] [22].

[1] Bravo et al., 2008
[7] Delepelaire, 2004
[10] Gentschev et al., 2002
[11] Hess et al., 1990
[12] Hidalgo et al., 1998
[17] Miki et al., 2008
[22] Schmitt et al., 2003

IL-10	Interleukin 10 (IL-10) is an anti-inflammatory cytokine which plays a role in limiting the host immune response so that the host does not harm its own cells and maintains normal tissue homeostasis. It is also responsible for enhancing B-cell survival, proliferation, and antibody secretion [9] [23] [25].
GFP	Green Fluorescent Protein (GFP) acts as a critical reporter gene to assess the functioning of plasmid by emitting green fluorescence when excited by light in the blue-UV spectrum. Typically, the excitation wavelength measurement range is 430-520 (nm) and the emission wavelength measurement range is 490-580 (nm) [21].
RBS	A ribosomal binding site, or ribosome binding site (RBS), is a short sequence of nucleotides present upstream of the start codon of a gene in mRNA. It is involved in the recruitment of the ribosome during translation initiation [24].
Double terminator	It functions to signal the termination of transcription.
pSB4C5 plasmid vector	A vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed. pS43C5 is a low to medium copy number plasmid carrying chloramphenicol resistance.

Plasmid 2 (9960 bp)



[9]Elenkov & Chrousos, 1999 [21]Remington, 2011 [23]Steidler et al., 2000 [24]G. D. Stormo, 2000
[25]Sung et al., 2006

2. Working design of Composite Bio-Brick 2

Circuit:

SoxS promoter – RBS – **HlyB** – RBS – **HlyD** – RBS – **TolC** – RBS - **IL-10+HlyA** – RBS – **GFP** - **Double Terminator**

Working:

Mercury-induced inflammation is one of the most important side effects of mercury poisoning. Exposure to mercuric chloride (MC) causes damage to many organelles such as lysosomes, etc. [3]. Long exposure to low doses of mercury in vascular smooth muscle cells activates the MAPK (Mitogen-activated protein kinase) signaling pathway (responsible for regulating fundamental cellular processes involved in cell growth and survival) which results in the activation of pro-inflammatory proteins [13]. Other studies also showed that mercury exposure affects gut ecology and increases inflammation [27] [8] [26]. It is also proposed that the inflammation following exposure to mercury, likely arises as a consequence of tissue damage leading to the availability of damage-associated molecular patterns (DAMPs), resulting in the engagement of innate sensors including nucleic acid sensing TLRs, the production of inflammatory cytokines and the induction of chronic inflammation [15].

SoxR transcription factor which is required for the transcription initiation of Composite Bio-Brick 2 (Plasmid 2) is present on Composite Bio-Brick 1 (Plasmid 1). Plasmid 1 is mercury responsive, regulated by the MerR gene which binds to mercury and initiates transcription of PmerT promoter by causing a structural distortion, allowing the RNA polymerase tight contacts to be made, leading to the expression of downstream genes [2] [18] [19] [20]. Thus, SoxR transcribes only in the presence of mercury.

The SoxR then gets activated in the presence of oxidative stress (NO). In its active state, it initiates the transcription of SoxS promoter and all the genes downstream to it by untwisting the promoter DNA by binding to the long spacer DNA (19- or 20-bp spacer between the -35 and -10 operator elements) [12]. The IL-10 (an anti-inflammatory cytokine) is fused to HlyA signal peptide and thus is transported out into the extracellular medium [9] [11] [23] [25].

The HlyA signal peptide is recognized by the HlyB and transport occurs through the HlyB-D-TolC complex and IL-10 is released out only in the condition of mercury-induced inflammation. During experimentation, this is checked using the reporter gene GFP (Green Fluorescent Protein) which is present downstream to all these genes [1] [7] [10] [21] [22]. The plasmid vector for the Bio-Bricks is pSB4C5 which has chloramphenicol resistance.

[1] Bavro et al., 2008
[2] Brown et al., 2003
[3] Buelna-Chontal et al., 2017
[7] Delepelaire, 2004
[8] Eaton et al., 2017
[9] Elenkov & Chrousos, 1999
[10] Gentschev et al., 2002
[11] Hess et al., 1990
[12] Hidalgo et al., 1998
[13] Johnson & Lapadat, 2002
[15] Pollard et al., 2019
[18] Nakaya et al., 1960
[19] Park et al., 1992
[20] Ralston & O'Halloran, 1990
[21] Remington, 2011
[22] Schmitt et al., 2003
[25] Sung et al., 2006
[26] Toomey et al., 2014
[27] Stejskal, 2013

3. General protocols

Note: For safety instructions of the complete handbook refer to **iGEM MIT_MAHE safety handbook**

A. Preparation of media:

i) Preparation of Luria-Bertani broth (LB broth) (1L)

Aim: To prepare a medium to grow bacteria.

Principle:

Luria-Bertani (LB) broth is the most widely used medium for the growth of bacteria. The contents are usually peptone, yeast extract and sodium chloride (NaCl). The peptone and yeast extract in the broth supply essential growth factors that the bacteria would otherwise have to synthesize. The NaCl provides essential electrolytes for transport and osmotic balance.

Materials required:

Conical flasks**,** Test tubes, Ultra-pure water, LB broth powder, Cotton, Autoclave, Weighing machine, Spatula, Chloramphenicol- 30mg/mL in 70% Ethanol, stored at -20°C – This is a 1000X stock solution.

Procedure:

- Weigh 25g of LB broth powder and add it to 1L of ultra-pure water in an autoclavable flask.
- Swirl to mix (The powder may not dissolve completely).
- Close the flask with a cotton plug and autoclave (121°C, 20 mins).
- Cool on benchtop to about 50°C before use.
- In the laminar flow chamber, add 100 µL of the chloramphenicol stock solution to 100 mL of broth. (The broth should not be too hot).
- Mix vigorously and pour it into required test tubes.

Precautions:

- The broth should not be too hot while adding chloramphenicol solution since the heat will degrade the antibiotics.

Note: **If non-selective broths (without antibiotic) are required for the experiments it would be mentioned.**

Protocol source: Luria-Bertani broth

ii) Preparation of Luria-Bertani agar (LB agar) (1L)

Aim: To prepare a medium to culture bacteria.

Principle:

Luria-Bertani (LB) agar is the most widely used medium for the culturing of bacteria. The contents are usually peptone, yeast extract, agar and sodium chloride (NaCl). The peptone and yeast extract in the broth supply essential growth factors that the bacteria would otherwise have to synthesize. The NaCl provides essential electrolytes for transport and osmotic balance. The agar is the solidifying agent that creates a gel to plate the bacteria.

Materials required:

Conical flasks**,** Petri plates, Ultra-pure water, LB broth powder, Agar, Cotton, Autoclave, Weighing machine, Heating apparatus (microwave oven), Spatula, Chloramphenicol- 30mg/mL in 70% Ethanol, stored at -20°C - This is a 1000X stock solution.

Procedure:

- Weigh 25g of LB broth Powder and 15g of agar and add it to 1000mL Ultra-pure water in an autoclavable flask.
- Microwave till the agar dissolves (Do not overheat it).
- Put the cotton plug and autoclave (120°C, 20 min).
- Cool it on benchtop and keep it in the laminar flow chamber.
- Wait until the agar is warm, but not too hot, and add the chloramphenicol. For 100mL of the LB agar mix, add 100µL of the 1000X stock solution.
- Swirl vigorously and immediately pour the plates - about 15 to 20mL of the mixture per plate.
- Allow the plates to solidify and store at 4°C for several weeks.

Precautions:

- The mixture should not be too hot while adding chloramphenicol solution since the heat will degrade the antibiotics.
- Do not overheat the mixture in the microwave as it will overflow.
- Pouring into plates can be done next to a flame to maintain sterility.

Note: If non-selective plates (without antibiotic) are required for the experiments it would be mentioned

Protocol source: Luria-Bertani agar, Luria-Bertani agar 2

B. Polymerase Chain Reaction (PCR)

Aim: To conduct a Polymerase Chain Reaction of the DNA fragments (parts) before the Gibson Assembly Reaction.

Principle:

The principle of Polymerase Chain Reaction (PCR) is based on the temperature variations of heating and cooling-thermocycling reaction divided into three steps:

Denaturation: The hydrogen bonds between the nitrogen bases are broken and the double-stranded DNA is broken to single-stranded DNA at a high temperature. This occurs at 98°C.

Annealing: The primer (short RNA sequence) attaches to the DNA (complementary to it). The primer provides a site for the initiation of synthesis. This occurs at 60-72°C.

Extension: DNA polymerase uses the 3' end of the primer and starts DNA synthesis by adding nucleotides to the growing DNA strand. This occurs at 72°C.

These steps make-up 1 cycle and each cycle is repeated for about 25-30 times resulting in amplification of the desired DNA. This results in an exponential increase/amplification of DNA.

Materials required:

Micropipette, PCR tubes, Phusion HF DNA Polymerase, dNTP mix (10mM) (deoxyribonucleotide triphosphate), Nuclease-free Water, Insert DNA (template DNA), 10µM forward primers, 10µM reverse primers, Vortex, Ice bath, Thermal cycler, Tips, Gloves and 5X Phusion HF Buffer.

Procedure:

Note: Maintain aseptic conditions throughout.

PCR primers used to amplify the Bio-Brick Parts:

Primers	Description	Sequence
VR	Reverse primer for sequencing/amplifying Bio-Brick parts (VR)	attaccgcctttgagtgagc (R)
VF2	Forward primer for sequencing/amplifying Bio-Brick parts (VF2)	tgccacctgacgtctaagaa (F)

Reaction preparation:

- Prepare a Master Mix with 10µL of 5x Phusion HF buffer, 5µL of 10mM dNTP, and 2.5µL of forward and reverse primers each.
- Aliquot 8µL of the Master Mix into each PCR tube. Prepare Master Mix for 5 PCR tubes.
- Mix 2.5µL of the template DNA in 10µL of nuclease-free water.
- Take 1µL of the sample in each PCR tube with the help of a micropipette.
- Mix 2.5µL of Phusion HF DNA polymerase in 10µL of nuclease-free water, taking 1µL of the sample in each PCR tube.
- Add 15µL of nuclease-free water into each tube. Hence the total volume in each tube came up to be 25µL.
- Mix the contents gently with the help of a vortex.

Thermocycler:

Initial Denaturation	98°C	30 seconds	1 cycle
Amplification	98°C. Primer Tm (60-70°C). 72°C	10 seconds 20 seconds 30 seconds per kb	25-30 cycles
Final Extension	72°C	5 minutes	1 cycle
Hold	4°C	-	1 cycle

Precautions:

- Proportions of different reagents should be carefully monitored to get the desired output.
- Handle with gloves and maintain aseptic conditions to prevent contamination by unwanted DNA.

Protocol source: Polymerase chain reaction

C. Construction of plasmids

i) Restriction digestion of the Bio-Bricks using XbaI and SpeI and Gel Electrophoresis

Aim: To perform the restriction digestion (double digestion) of the amplified Bio-Bricks with XbaI & SpeI enzymes and visualization in Agarose Gel Electrophoresis.

Principle:

Digesting a DNA substrate with two restriction endonucleases simultaneously is called double digestion. These restriction enzymes are nucleases that cleave the sugar-phosphate backbone of DNA, in a specific site called restriction site to generate a smaller set of fragments. These restriction sites are palindromic sequences. The enzyme usually cuts within the molecule and are hence called restriction endonucleases. These enzymes act as a defense system in bacteria by cleaving foreign DNA.

Restriction sites:

XbaI: 5'…T | CTAG A…3'

3'…A GATC | T…5'

SpeI: 5'…A | CTAGT…3'

3'…TGATC | A…5'

Materials required:

Distilled water, DNA (Bio-Bricks), Control DNA, 2X Assay buffer, Restriction enzymes (XbaI, SpeI), 0.5ml Microfuge tubes (vials), Micropipettes and tips, Dry bath incubator, Vortex, Crushed ice, Beakers, Gel loading buffer and dye, Conical flask, Measuring cylinder and Staining tray.

Procedure:

- Place the vials containing restriction enzyme (XbaI and SpeI) on ice.
- Thaw the vials containing substrate (DNA) and assay buffer.
- Prepare the reaction mixture using the following constituents.

Reaction mix (XbaI and SpeI double digestion) (10 µL):

DNA (substrate)	0.5-1µg
2X assay buffer	2µL
XbaI	1µL
SpeI	1µL
Distilled water	Rest

- Gently mix by tapping. Briefly spin for a few seconds.
- Incubate the vial at 37°C for 1 hour on a dry bath.
- Meanwhile, prepare agarose gel for electrophoresis.
- After an hour, incubate at room temperature for 10 minutes.
- Add 5 µL of gel loading buffer to vials.
- Load the digested samples, 10 µL of Control DNA, and 5 µL of the gel loading dye; note down the order of loading.
- Electrophorese the samples at 50-100 V for 1-2 hours.
- Using UV illuminator visualize the gel.

Precautions:

- Make sure that the restriction enzyme does not exceed more than 10% of the total reaction volume, otherwise, the glycerol and the EDTA in the enzyme storage buffer may inhibit the digestion process.

Note: Other restriction sites that can be used are EcoR1, Pst1, and Not1 (Bio-Brick compatibility).

Protocol source: Restriction digestion
For more info on Bio-Brick Compatibility refer here.

Agarose gel electrophoresis: Protocol

ii) Agarose Gel Electrophoresis

Aim: To visualize and analyze the restriction digested fragments.

Principle:

DNA is a negatively charged molecule at neutral pH. Gel electrophoresis apparatus is used to separate charged molecules under the influence of a constant current. The DNA molecules migrate towards the positive charge with the separation depending on the molecular size of DNA, strength of the electric field applied, hydrophobicity of the DNA, the concentration of agarose, ionic strength of the buffer, the temperature of buffer, and conformity of the DNA.

The agarose gel is a linear polymer extracted from seaweeds. It (purified powder) is insoluble in water and buffers at room temperature but dissolves on boiling. On pouring into a mold and cooling, it undergoes polymerization acting like a molecular sieve. The DNA moves through this on the application of current. Pore size depends on the concentration of agarose used.

Small fragments can pass through the pores easily whereas the larger ones find it harder to do so and separation occurs. A visible tracking dye is applied to determine the progress of the DNA through the gel, and Serva DNA stain G is used for visualizing the DNA in the transilluminator.

Materials required:

Agarose, Micropipettes and tips, Beakers, Distilled water, 2.5X Gel loading buffer, Gel loading dye (bromophenol blue), 6X Staining dye (Serva DNA stain G), TAE 10X Buffer, Heating apparatus (microwave oven), Gel electrophoresis apparatus (Basic Unit, Gel casting tray, Gel running tray, Comb, Comb stand, Electrodes, and Power cord), UV transilluminator.

Procedure:

Gel preparation:

- Dilute the appropriate amount of 50X TAE buffer and prepare 1X TAE.
- Prepare 1% agarose by weighing 0.5g of agarose and adding it to 50ml of 1X TAE.
- Boil the agarose in the microwave till it dissolves completely and a clear solution is obtained.
- Add 1 µL of Serva DNA stain G (staining dye) to the clear agarose solution.
- Meanwhile, place the combs of electrophoresis set approximately 2 cm away from the cathode (black).

- Pour the agarose + Serva DNA stain G solution in the central part of the tank when the temperature reaches approximately 60°C. Make sure no air bubbles are generated. The thickness of the gel should be around 0.5 to 0.9 cm.
- Keep the gel undisturbed at room temperature for the agarose to solidify.
- Pour 1 X TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
- Gently lift the combs, ensuring that wells remain intact and no air bubbles are formed.

Ladder & samples:

- Mix 2 µL of the control DNA with 2 µL loading dye (bromophenol blue) and 1 µL Ultra-pure water for the preparation of the ladder.
- Mix 10 µL of PCR samples with 2 µL loading dye.

Electrophoresis conditions:

- Move the solidified gel to the electrophoresis set up and connect the wires appropriately (Red (anode), Black (cathode)).
- Load the samples (and ladder) in each well carefully and run it for 90 minutes at 100 V.
- Visualize the sample in UV transilluminator.

Precautions:

- The comb/well side should face the cathode (Black).
- Make sure there aren't any air bubbles when the agarose is in its liquid form.

Protocol source: Agarose gel electrophoresis

iii) Gibson Assembly Protocol (E5510)

Aim: To ligate and assemble one or more inserts into a vector to create a circular vector containing the inserts of interest.

Principle:

Gibson assembly is a molecular cloning technique used to ligate fragments of DNA, and is named after its creator Daniel G. Gibson.

Three enzymatic activities: exonuclease, polymerase and ligase are carried out in isothermal conditions. The 5' exonuclease generates long overhangs by which the 5' end sequences are chewed back. This exposes the complementary sequence for annealing to which the primer attaches followed by filling of gaps of the annealed single-stranded regions by the polymerase. The nicks of the annealed and filled-in gaps are then sealed and ligated (covalently linked) by DNA ligase.

Materials required:

Milli-Q water (dH2O), Bucket with ice, PCR tubes, gBlock(s), Linearized vector, 2X Gibson Assembly Master Mix (NEB), Thermal cycler, Fiber-free tissues, NanoDrop spectrophotometer, Labels (Cryo-tags), Pipettes and tips, Pre-prepared primers.

Procedure:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps.
- Determine the concentration of fragments using agarose gel electrophoresis, a Nanodrop™ instrument, or another method.

Nanodrop protocol:

- Start the NanoDrop spectrophotometer and select DNA measurement 'Nucleic Acid' in the NanoDrop menu.
- Clean the surface of the NanoDrop with dH2O and a fiber-free tissue.
- Perform calibration and blank measurement by entering one drop of 2 µL autoclaved dH2O.
- Clean the surface again with dH2O and a fiber-free tissue. Place 2 µL per sample on the NanoDrop and measure the concentration. Note the concentration on appropriate labels (Cryo-tags).

- Calculate how much insert is needed for the reaction.
- NEB recommendations:
    - When 1-3 inserts are being assembled, a total of 0.02 – 0.5 pmol of DNA fragments is needed.
    - When 4-6 inserts are being assembled, a total of 0.2-1.0 pmol of DNA fragments is needed.

Formula: $pmol = \frac{(weight\ in\ nanogram)\times 1000}{number\ of\ bp\ \times 650\ da}$

Online Tools: NEBioCalculator

(***Note:*** Yields will be best when the different inserts are present in equimolar concentrations)

- Prepare an icebox, put every material on ice.

Set up the following reaction in a PCR tube on ice:

Assembly type	**2-3 Fragment Assembly (vector + 1-2 inserts)**	**4-6 Fragment Assembly (vector + 3-5 inserts)**	**Positive Control****
Total Amount of Fragments	0.02–0.5 pmols* (x µL)	0.2–1 pmols* (x µL)	10 µL
2X Gibson Assembly Master Mix	10 µl	10 µl	10 µl
Milli-Q water	10-Xµl	10-Xµl	0
Total Volume	20 µl***	20 µl***	20 µl***

* Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if the size is less than 200 bp. The total volume of unpurified PCR fragments in the Gibson Assembly reaction should not exceed 20%.

** Control reagents are provided for 5 experiments.

*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

- Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled.
- Following incubation, store samples on ice or at –20°C for subsequent transformation.
- Transform competent cells with 2µL of the assembly reaction, following the transformation protocol.

Precautions:

- Reaction time less than 15 minutes are generally not recommended. Extended incubation time (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the reaction overnight.
- The efficiency of assembly decreases as the number or length of inserts increases. Hence use the optimal amount only.
- Inserts to be assembled should not have a stable single-stranded DNA secondary structure, such as a hairpin or a stem-loop, or repeated sequences, as this would directly compete with the required single-stranded annealing/priming of neighboring assembly fragments.

D. Competent cell preparation

Aim: To prepare fresh competent cells of *Escherichia coli* Nissle 1917.

Principle:

Competent cells are cells that have altered cell walls which allow DNA to easily pass through. The most widely used method to artificially induce competency in cells is the calcium chloride heat shock method. The exact mechanism of how this process works is still unknown. One of the hypothesis states that the calcium ions act as a cation bridge between the negatively charged phosphorylated lipid in lipopolysaccharide (LPS) of the cell membrane and the phosphate backbone of DNA. The ice-cold $CaCl_2$ solution facilitates the binding of DNA to the surface of the cell which enters after a short period of heat shock. Competent cells are then identified by selection or screening markers such as drug resistance (Chloramphenicol in our case) etc.

Materials required:

Petri plates with LB, LB liquid medium, Wild type *Escherichia coli* Nissle 1917, Incubator, Laminar flow chamber, Autoclave, Micropipette and tips, Sterile loop, 500ml flask, Shaker incubator, Ice, $CaCl_2$ solution, Glycerol solution, Centrifuge, Micro-centrifuge tubes.

Procedure:

Note: All the steps should be conducted aseptically (Using sterile/autoclaved labware and in laminar flow chamber)

- Culture the wild type *Escherichia coli* Nissle 1917 on LB plate overnight (Do not add the antibiotic).
- Using a sterile loop inoculate one colony from the LB plate into 2 ml LB liquid medium. Shake at 37 °C overnight (Do not add antibiotic).
- Inoculate 1-ml overnight cell culture into 100 ml LB medium (in a 500 ml flask).
- Shake vigorously at 37 °C to OD600 ~0.25-0.3.
- Chill the culture on ice for 15 min. Also, make sure the 0.1M $CaCl_2$ solution and 0.1M $CaCl_2$ plus 15% glycerol are on ice.
- Centrifuge the cells for 10 min at 5000*g at 4°C.
- Discard the medium and resuspend the cell pellet in 30-40 ml cold 0.1M $CaCl_2$. Keep the cells on ice for 30 min.
- Centrifuge the cells as above.

- Remove the supernatant and resuspend the cell pellet in 6 ml 0.1 M $CaCl_2$ solution plus 15% glycerol.
- Pipet 0.4-0.5 ml of the cell suspension into sterile 1.5 ml micro-centrifuge tubes.

Storage:

- Prepare a glycerol solution (65% glycerol, 0.1 M $MgSO_4$, 0.025 M Tris/HCl pH 8).
- Store in 1 ml aliquots at -80°C.

Precautions:

- Stock freezing can decrease the transformation efficiency, hence if possible, prepare fresh competent cells.

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E. Transformation

Aim: To introduce the composite Bio-Bricks into the competent *Escherichia coli* Nissle 1917 (or any other bacteria)

Principle:

Genetic alteration of a cell due to direct uptake and incorporation of genetic material from its surroundings is called Transformation. It occurs through the cell membrane. It is a key step in molecular cloning. Transformation occurs in nature due to conditions such as starvation, cell density, etc. It can also be induced in the lab. The bacteria then produce multiple copies of foreign DNA which can then be utilized according to the application.

Materials required:

Competent cells (*Escherichia coli* Nissle 1917*)*, Plasmid DNA, Competent cells, Sterile LB broth, LB agar plate (supplemented with chloramphenicol), Microcentrifuge tubes, L-rod, Ice bath, Dry bath or water bath, Incubator with shaker and Laminar airflow chamber.

Procedure:

Note: All the steps should be performed aseptically using sterile equipment and experiment conditions.

Use media without antibiotics.

- If the competent cells were stored, take it out of -80°C and thaw on ice (approximately 20-30 mins).
- Keep LB agar plates ready (Incubate at 37° C (optional)).
- Add 1-5 µl of plasmid DNA and 20 -50 µl of competent cells into a microfuge tube and gently mix by flicking.
- Incubate the tube on ice for 20-30 mins.
- Place the tube (1/2 to 2/3 of it) in a 42°C water bath or a dry bath for 30-60 secs (heat shock treatment).
- Put the tubes back on ice for 2 min.
- Add 250-1,000 µl LB broth (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.

- Plate the transformed bacteria onto a 10 cm LB agar plate containing the appropriate antibiotic (Chloramphenicol).
- Incubate plates at 37°C overnight.

Precautions:

- If the culture volume is high, centrifuge and gently collect and resuspend the cells in a smaller volume of LB broth so that there isn't too much of this media on the plates. If too much media is present, the bacteria diffuse through the liquid and will not grow colonies. Invert the plates after the liquid is absorbed.
- Avoid the thawing of cells before use.
- The cells should not be kept on ice for longer than 3 hours.
- The cells that have been on ice should not be used again.

F. Cell lysis using sonication

Aim: To perform cell lysis using Sonication.

Principle:

Sonication is the procedure of lysing cells utilizing sound waves. During sonication, thousands of microscopic vacuum bubbles are formed in the solution due to cycles of pressure. The bubbles then collapse into the solution (Cavitation). This leads to an enormous energy release due to the powerful waves of vibration in the cavitation field which ultimately causes the cell to rupture.

The energy from these waves also causes friction in the solution which in turn creates heat. Hence the tube is kept in ice and is carefully placed such that it is not touching the Sonicator.

Materials required:

Microcentrifuge tubes, Cells (used in the experiment), Centrifuge, Ice, MQ grade water, Lysozyme, Lysis buffer (Tris-buffered saline, EDTA, deionized water, NP-40), Sonicator, Micropipette and tips.

Procedure:

Pre-preparation:

- Transfer the cells to be lysed into a microcentrifuge tube (From the LB broth).
- Centrifuge the cells for 10 mins at 5000rpm or 5 mins at 6000 rpm.
- Resuspend the cells in 2mL of MQ grade water and transfer into another sterile microcentrifuge tube.
- Incubate on ice for 30 mins and at 30°C for 15 minutes (or till the solution becomes viscous) after the addition of lysozyme.

Sonication:

- Place the sonicator probe at a frequency of 20 kHz.
- Place the tube on ice and gently move under the tip of the probe (Make sure the tube is not touching the probe).

- Sonicate the sample on ice using three (or two depending on the sample and its viscosity) 10-second bursts at high intensity and let the mixture cool down for 30 seconds on ice between each burst. At the end of this step, the sample should lose its viscosity as the DNA is sheared.
- Prepare the lysis buffer priorly.

Reagent	**Amount**	**Final concentration**
Tris-buffered saline (TBS, 10x, pH 7.5)	5 mL	1x
EDTA (0.5 M)	100 µL	0.05 M
NP (nonionic polyoxyethylene) -40 (10%)	5 mL	1%
Deionized water	39.9 mL	

- Using the prepared lysis buffer, dilute the lysate to approximately 50 ml.
- Centrifuge at 35,000 rpm for 30 minutes at 4°C to pellet the remaining cellular debris in the lysate.
- Transfer the supernatant to new tubes and store at -20 °C.

Precautions:

- Avoid extended periods of sonication and make sure the microtip is immersed by about 1 cm in the mixture to prevent frothing which may lead to protein denaturation.
- Make sure to observe the tubes because when the ice melts due to the heat, the tube might move and touch the probe.

Protocol source: Cell lysis using sonication LB broth: protocol

G. Calibration

Aim: To calibrate instruments to ensure accurate reading

Principle: Instrument calibration is an essential stage in most measurement procedures. It is a set of operations that establish the relationship between the output of the measurement system (e.g., the response of an instrument) and the accepted values of the calibration standards (e.g., the amount of analyte present).

Materials:

Spectrophotometer, fluorometer, 187 µg FITC, 10ml 1xPBS (phosphate buffered saline), cuvettes.

Procedure:

Note: The calibrations should be done before each experiment utilizing these methods.

GFP:

- By measuring these in all standard modes in your plate reader or fluorimeter, you will generate a standard curve of fluorescence for FITC concentration.
- You will be able to use this to correct your cell based readings to an equivalent fluorescein concentration.
- You will then be able to convert this into a concentration of GFP.

Prepare the FITC stock solution:

- Spin down FITC stock tube to make sure pellet is at the bottom of tube.
- Prepare 10x FITC stock solution by resuspending FITC in 1 mL of 1xPBS.
- Incubate the solution at 42°C for 4 hours.
- Dilute the 10x FITC stock solution in half with 1xPBS to make a 5x FITC solution and resulting concentration of FITC stock solution 2.5 µM.

[***Note:*** it is important that the FITC is properly dissolved. To check this after the incubation period pipetted up and down – if any particulates are visible in the pipette tip continue to incubate overnight.]

Note: Use 2.0mL tubes for cuvette dilutions, and then transfer 1.0mL into your cuvettes.

- Add 1 mL of PBS into tubes 2-11. ! Add 2.0 mL of FITC 5x stock solution tube 1.
- Transfer 1.0 ml of FITC stock solution tube 1 into tube 2.
- Mix tube 2 by pipetting up and down 3x and transfer 1 mL into tube 3…
- Mix tube 3 by pipetting up and down 3x and transfer 1 mL into tube 4…
- Mix tube 4 by pipetting up and down 3x and transfer 1 mL into tube 5…
- Mix tube 5 by pipetting up and down 3x and transfer 1 mL into tube 6…
- Mix tube 6 by pipetting up and down 3x and transfer 1 mL into tube 7…
- Mix tube 7 by pipetting up and down 3x and transfer 1 mL into tube 8…
- Mix tube 8 by pipetting up and down 3x and transfer 1 mL into tube 9…
- Mix tube 9 by pipetting up and down 3x and transfer 1 mL into tube 10…
- Mix tube 10 by pipetting up and down 3x and transfer 1 mL into tube 11…
- Mix tube 11 by pipetting up and down 3x and transfer 1 mL into liquid waste

Measurement:

- Measure the plate (or cuvettes) in your plate reader (or fluorimeter).
- Setup the machine with the standard GFP settings (filters or excitation and emission wavelengths) that you will use when measuring your cells (if you change them you will not be able to use this standard curve).
- Repeat the measurement a number of times with different settings. You will then have a series of standard curves to choose from. Most important it is necessary to use a number of settings that affect the sensitivity (principally gain and/or slit width).

OD_{600}:

- Calibration curve can be determined by comparing measured OD_{600} to expected OD_{600}.
- Determine the expected OD_{600} by counting cell number using an alternative technique (for example microscope slide method).
- Convert to OD_{600} using the rule of thumb that 1 OD_{600} = 8 x 108 cells/ml for *Escherichia coli*.

Note: Clumping together of cells will also affect readings, so the medium they are suspended in will also make a difference.

Protocol source: GFP OD600

4. Experimentation

A. Characterization of SoxR and SoxS

i) Experiment 1:

Aim: To analyze the relationship between the amount of oxidative stress and transcription rates of SoxR and SoxS promoter.

Principle:

Circuit:

Constitutive Promoter – RBS – **SoxR** - **Double terminator** - **SoxS promoter** – **GFP** - **Double Terminator**

The working of each part is given in detail above.

Note: Assemble circuit using Gibson assembly.

Materials required: Micropipettes, Pipette tips, Test tubes, Cotton, Parafilm, Competent transformed bacteria, Sterile loop, LB broth with chloramphenicol, Paraquat (Methyl viologen dichloride hydrate), To adjust the pH of the medium (1 N NaOH to raise the pH or 1 N HCL to lower the pH (add dropwise)), Fluorometer and Incubator.

Procedure:

Note: The experiment is performed at gut pH (6-7), temperature (37°C), and in aseptic conditions.

- Using a sterile loop inoculate the transformed *Escherichia coli* Nissle 1917 into different test tubes containing LB broth medium and incubate overnight (5+5 (+duplicates if needed)).
- Add the paraquat in different concentrations in the respective test tubes (0 µM (control), 5 µM, 10 µM, 20 µM, 30 µM and 40 µM*) and incubate at 37°C.
- Check the GFP intensities using the fluorometer (with excitation in 500 nm and emission spectra from 508-550 nm) every hour. Plot a graph to analyze.

Expected observation:

- The GFP intensity should increase with time for all the concentrations except the control and stabilize after a few hours.
- The culture fluorescence will be more for a higher concentration of paraquat, with the highest being for the 40µM.

Expected result:

- The intensity of GFP increases with time indicating that even if the concentration of the oxidative stress inducer is constant, the rate of activation of SoxR and thus the rate of transcription of SoxS promoter increases and then stabilizes to a constant rate.
- The more the concentration of the inducer, the more is the transcription rate of SoxS promoter.

Unexpected outcomes:

The most likely unexpected result would be that the GFP intensity for the 40µM concentration is not the highest which could be the result of a high amount of oxidative stress on the bacteria which reduces the growth rate. This is checked by the experiment (Measurement of growth rate of *Escherichia coli* Nissle 1917: Under oxidative stresses) and the dosage of the probiotic can be modified accordingly.

Appendix:

* The concentrations used is determined according to results from Team:UNICAMP-EMSE_Brazil 2011 and Team:Stanford 2009.

Gibson assembly: Protocol Transformation: protocol LB broth: protocol
To know more about determination of concentration refer here

ii) Experiment 2:

Aim: To check the amount of oxidative stress required to activate SoxR and in turn activate SoxS promoter.

Principle:

Circuit:

To be tested:

Constitutive Promoter – RBS – **SoxR** – **GFP** - **Double terminator** - **SoxS promoter** - **Double Terminator**

Control:

Constitutive Promoter – RBS – **SoxR** - **Double terminator** - **SoxS promoter** – **GFP** - **Double Terminator**

The working principle of each part is given above.

Note: Assemble circuit using Gibson assembly.

Materials required: Micropipettes, Pipette tips, Test tubes, Cotton, Parafilm, Competent transformed bacteria, LB broth, Chloramphenicol, Paraquat (Methyl viologen dichloride hydrate), Sterile loop, to adjust the pH of the plates or broth (1 N NaOH to raise the pH or 1 N HCL to lower the pH (add dropwise)), Fluorometer and Incubator.

Procedure:

Note: The experiment is performed at gut pH (6-7), temperature (37°C), and in aseptic conditions.

- Using a sterile loop inoculate the transformed *Escherichia coli* Nissle 1917 into different test tubes containing LB broth medium and incubate overnight (5+5 (+duplicates if needed)).
- Add the paraquat in different concentrations in the respective test tubes (0 µM (control*), 1 µM, 2 µM, 3 µM, 4 µM and 5 µM) for each of the circuits and incubate at 37°C.
- Check the GFP intensities using the fluorometer (with excitation in 500 nm and emission spectra from 508-550 nm) every hour. Plot a graph and compare the results of the two circuits.

Expected observation:

- On comparison of the two graphs, we should be able to map the amount of SoxR produced compared to SoxS promoter transcribed and thus map the amount of SoxR required to activate SoxS promoter.

Expected result:

- The amount of NO required to activate SoxR is low.
- The amount of activated SoxR required to activate SoxS promoter is also low.
- Thus, the anti-inflammatory response activation of composite Bio-Brick 2 occurs even at low oxidative stress, preventing any unwanted inflammation due to our probiotic.

Gibson assembly: Protocol Transformation: protocol LB broth: protocol
To know more about determination of concentration refer here

iii) Experiment 3:

Aim: To check the basal level of transcription of SoxS promoter.

Principle:

In the absence of SoxR, the transcription of SoxS is its basal level of transcription. This occurs with the help of transcription factors called the Basal, or general, transcription factors.

Circuit:

SoxS promoter – RBS – **GFP** - **Double Terminator**

Control:

Constitutive promoter – RBS – **GFP** - **Double Terminator**

The working principle of each part is given above.

Note: Assemble circuit using Gibson assembly.

Materials required: Micropipettes, Pipette tips, Test tubes, Cotton, Competent transformed bacteria, LB broth, Sterile loop, to adjust the pH of the medium (1 N NaOH to raise the pH or 1 N HCL to lower the pH (add dropwise)), Fluorometer and Incubator.

Procedure:

Note: The experiment is performed at gut pH (6-7), temperature (37°C) and in aseptic conditions.

- Using a sterile loop inoculate the transformed *Escherichia coli* Nissle 1917 into different test tubes containing LB broth medium and incubate overnight (2 + (duplicates if necessary)).
- Check the GFP intensities using a fluorometer (with excitation in 500 nm and emission spectra from 508-550 nm) every hour. Plot graphs to analyze and compare the graphs of the two circuits.

Expected observation:

- The transcription of SoxS promoter without SoxR should not be comparable to the transcription of the constitutive promoter (Should be much lesser).

Expected result:

- The basal level of transcription of SoxS is very low. Thus, unwanted anti-inflammatory substances will not be released without the presence of oxidative stress.

Gibson assembly: Gibson assembly. Transformation: protocol LB broth: protocol

B. Characterization of the IL-10 transport system

Aim: To check the efficiency of IL-10+HlyA transport system.

Principle:

Circuit:

To be tested:

Constitutive Promoter – RBS – **HlyB** – RBS – **HlyD** – RBS – **TolC** – RBS - **GFP+HlyA** - **Double Terminator**

Control:

Constitutive Promoter – RBS - **GFP+HlyA** - **Double Terminator**

The working principle of each part is given above.

Note: Assemble circuit using Gibson assembly.

Materials required: Petri plates, Micropipettes, Pipette tips, Sterile loop, Microfuge tubes, Competent transformed bacteria, LB Agar, LB broth, Chloramphenicol, Dropper, 1N NaOH and 1N HCl (depending on pH), Fluorometer, Centrifuge and Sonicator.

Procedure:

The experiment is performed at gut pH (6-7), temperature (37°C), and in aseptic conditions.

- Using a sterile loop, inoculate the transformed cells to be tested on LB broth and incubate for 3 hours at 37°C.
- Then transfer the same into a microfuge tube hourly and centrifuge at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant (Supernatant 1) in another microfuge tube and centrifuge again at 13000 rpm for 10 minutes to remove the remaining cells.
- Sonicate the sample and control to get Supernatant 2 and Supernatant 3 (control) respectively.
- Measure the supernatant fluorescence in a fluorometer with excitation in 500 nm and emission spectra from 508-550 nm at specific time intervals.

- Plot culture absorbance vs time graphs to analyze that -

    - Supernatant 1- represents the HlyA+GFP transported outside the cell
    - Supernatant 2- represents the HlyA+GFP remaining inside the cell
    - Supernatant 3- represents the total HlyA+GFP produced.

Expected observation:

- The plot of supernatant 1 should determine the amount of HlyA+GFP transported outside. Hence it should be ideally equal to the plot of supernatant 3 (control).
- Ideally, the plot of supernatant 2 should not exist.

Expected result:

- This shows that the transport system is extremely efficient in getting the anti-inflammatory cytokines outside the cell to the target site.

C. Working of Composite Bio-Brick 2

Aim: To check the safety and working of composite Bio-Brick 2

Principle:

Circuit used:

To be tested and control:

SoxS promoter – RBS – **HlyB** – RBS – **HlyD** – RBS – **TolC** – RBS - **IL-10+HlyA** – RBS – **GFP** - **Double Terminator**

(Constitutive Promoter – RBS – **MerR** - **PmerT promoter** – RBS – **MerT** – RBS – **MerP** – RBS – **MerE** – RBS – **MerC** – RBS – **MerA** – RBS – **MerB** – RBS – **SoxR** - **Double Terminator)**

The working design of composite Bio-Brick 2 is given above.

Note: Assemble circuit using Gibson assembly.

Note: The GFP position is changed to be expressed after each of the gene so that the overall efficiency of each gene in the whole system can be tested.

Materials required: Micropipettes, Pipette tips, Test tubes, Cotton, Parafilm, Gloves, Gasmask, Competent transformed bacteria, LB broth, Sterile loop, Methylmercury chloride, to adjust the pH of the medium (1 N NaOH to raise the pH or 1 N HCL to lower the pH (add dropwise)), Fluorometer and Incubator.

Procedure:

Note: The experiment is performed at gut pH (6-7), temperature (37°C), and in aseptic conditions.

- Using a sterile loop inoculate the transformed *Escherichia coli* Nissle 1917 into different test tubes containing LB broth medium and incubate overnight. (5, 5, 5+1)
- *Case 1:*
  Add only methylmercury chloride (0.3µM, 2µM, 5µM, 10µl and 20µl) in one set of the LB broth medium.
  Case 2:
  Add only paraquat in another set (5 µM, 10 µM, 20 µM, 30 µM, 40 µM).

Case 3 (control set):
Add both (0.3µM +5 µM, 2µM +10 µM, 5µM +20 µM, 10µM +30 µM, 20µM +40 µM (0.1M methylmercury chloride + paraquat)) (control* will be one plate and will have no methylmercury or paraquat).

- Check the GFP intensities using the fluorometer (with excitation in 500 nm and emission spectra from 508-550 nm) every hour.
- Plot graphs to analyze and compare the graphs of the different medium.

Expected observation:

- The graph of control and the tested circuit should not be comparable (first 2 cases) and the tested circuit should have minimum or no GFP intensity (in the first two cases).
- In the third case, GFP intensity should be significantly higher than the control*.

Expected result:

- Thus, composite Bio-Brick 2 will work only in the conditions of methylmercury induced inflammation.

Gibson assembly: Protocol Transformation: protocol LB broth: protocol
To know more about determination of concentration refer here

D. Measurement of the growth rate of *Escherichia coli* Nissle 1917

i) Under oxidative stresses

Aim: To check the limits of oxidative stress the bacteria can be subjected to and the effect of oxidative stress on its growth rate.

Principle:

Oxidative stress occurs due to an imbalance of free radicals and antioxidants. Reactive oxygen species usually involved in oxidative stress is also required for various physiological functions, but its imbalance can cause a variety of problems. Bacteria can tolerate some amount of oxidative stress using enzymes such as catalase, superoxide dismutase, etc. [29].

The ODs of the bacteria culture grown in different conditions can be compared and analyzed to determine the effects of oxidative stress.

Mathematically, an OD of 2 would correspond to 2 x (8 x 108) cells/ml = 1.6 x 109 cells/ml. That is assuming that the OD is directly proportional to the number of cells over that range of OD.

Thus, A graph of lnX vs time to determine the specific growth rate (μ) for cells in the control (0 μM) and experimental conditions can be drawn where, X is the cellular concentration per volume.

Specific growth rate (μ) is equal to the slope of the plot lnX vs time, described by the equation

$$LnX= lnX_0 + \mu t.$$

Circuit:

Transformed *Escherichia coli* Nissle 1917 with both Composite Bio-Brick 1 and 2.

Materials required: Micropipettes, Pipette tips, Test tubes, Cotton, Parafilm, Sterile loop, Competent transformed bacteria, LB broth, Paraquat (Methyl viologen dichloride hydrate), To adjust the pH of the medium (1 N NaOH to raise the pH or 1 N HCL to lower the pH (add dropwise)), Fluorometer and Incubator.

[29]Z.N.Kashmiri and S.A.Mankar, 2014

Procedure:

Note: The experiment is performed at gut pH (6-7), temperature (37°C), and in aseptic conditions.

- Using a sterile loop inoculate the transformed *Escherichia coli* Nissle 1917 into different test tubes containing LB broth medium and incubate overnight at 37°C.
- Add the paraquat in different concentrations in the respective test tubes (0 µM (control*), 20 µM, 30 µM, 40 µM, 60µM and 80 µM) for each of the circuits and incubate at 37°C.
- Check the OD at 600nm hourly. Plot the graph to determine the specific growth rate of the bacteria using the OD.

Expected observation:

- There should be no significant difference in growth rate or OD till 40 µM after which the growth rate should decrease.

Expected result:

- The bacteria can tolerate up to 40 µM (experimental condition) of Paraquat without any significant change in growth rate.
- Even though growth inhibition seems to occur at concentrations above 40 µM, the system seems to be stable for a large range of concentration for a long period of time.

Unexpected outcomes:

Any unexpected outcome of this experiment such as low growth rates at paraquat concentrations lower than 40 µM can be troubleshot by determining the proper dosage of the probiotic.

Gibson assembly: Protocol Transformation: protocol LB broth: protocol
To know more about determination of concentration refer here

ii) In different pH conditions

Aim: To check for the robustness of our modified probiotic under various pH conditions to get an idea of its growth rate

Principle:

Probiotic was originally used to describe substances produced by one microorganism that stimulated the growth of others and was later used to describe tissue extracts that stimulated microbial growth and animal feed supplements exerting a beneficial effect on animals by contributing to their intestinal flora balance. Until recently the most widely used definition was that of Fuller: "probiotics are live microbial feed supplements which beneficially affect the host animal by improving microbial balance." The definition used at present was given by the Food and Agriculture Organization of the United Nations World Health Organization, according to which probiotics are redefined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [16].

In order for a potential probiotic strain to be able to exert its beneficial effects, it is expected to be pH tolerant. This experiment helps to get an idea of the range of pH tolerance the probiotic can offer.

Growth curve calculations:

Mathematically, an OD of 2 would correspond to 2 x (8 x 108) cells/ml = 1.6 x 109 cells/ml. That is assuming that the OD is directly proportional to the number of cells over that range of OD.

Thus, A graph of lnX vs time to determine the specific growth rate (μ) for cells in the control (0 μM) and experimental conditions can be drawn where, X is the cellular concentration per volume.

Specific growth rate (μ) is equal to the slope of the plot lnX vs time, described by the equation

$$LnX = lnX_0 + \mu t.$$

***Circuit*:**

To be tested and control: ***Escherichia coli*** **Nissle 1917** with both the Bio-Bricks assembled using Gibson assembly.

Materials required: Petri plates, Micropipettes, Pipette tips, Test tubes, Cotton, Sterile loop, L-rod, Competent transformed bacteria, LB Agar, LB broth, Chloramphenicol, Dropper, 1N NaOH and 1N HCl (to adjust the pH).

[16] Maria Kechagia et. al, 2013

Procedure:

Note: The experiment is performed at temperature (37°C) and in aseptic conditions.

- Using a sterile loop, inoculate the transformed cells to be tested and the control in their respective LB broths overnight.
- Then transfer the same to LB agar using a micropipette and spread using a sterile L-rod and let it grow for two hours at pH 7. Repeat for 7 plates.
- Change the medium of the 7 plates to respective pH (pH from 1 to 7) and let it grow for 3 hours.
- Plot *Escherichia coli* Nissle 1917 growth curve in LB at 37°C for different pH levels.
- Change the medium again after 3 hours to pH 7 to check for recovery.

Expected observation:

- There should be usual growth for pH 5 and pH 6.
- There might be no growth in both cases (test and control) for pH 1, 2 and 3 (according to results from a previous team).
- Control is kept at pH 7.

Expected result:

- This shows that *Escherichia coli* Nissle 1917 can withstand some amount of stress, having a pH range of 6 to 8 and is robust enough to survive the journey through our gut.

Unexpected outcomes:

The most likely unexpected outcome of this experiment is the reduced growth of the bacteria at higher pH. This can be tackled by choosing appropriate coatings to protect the bacteria from high pH stresses of the gut after ingestion.

Gibson assembly: Protocol Transformation: protocol LB broth: protocol LB agar: protocol
To know more about determination of concentration refer here

E. Safety of *Escherichia coli* Nissle 1917

Aim: To indicate that our genetically modified probiotic bacteria cannot survive and spread in the ecosystem.

Principle:

Genetically modified organisms (GMOs) are organisms whose genetic material has been artificially modified to change their characteristics in some way or another. One of the most important concerns regarding the ethics of GMOs is the spread of GMOs in the environment which might cause unknown diseases as well as cause ecological and economic damage. To tackle this concern with respect to our probiotic we can show that our modified organism would not be able to compete with the wild type for long and spread in the environment and even show that the wildtype did not undergo conjugation with the *Escherichia coli* Nissle 1917.

While constructing of GMOs, it is necessary to understand the burden of the synthetic constructs on the bacteria. This burden is mostly caused by competition between the host genes and the construct for the resources used in gene expression. This causes slower cell growth and productivity [5] [14]. For any given gene, its burden is in the first instance the resource cost of expressing the gene and if the gene encodes a function, for example, an enzyme, the impact of this can further cause a more specific role-based metabolic burden that adds to the expression burden, e.g., by consuming host cell metabolites and co-factors [6] [28].

Research primarily in the model bacteria *Escherichia coli* has demonstrated that a lack of understanding of the burden of expressing additional genes affects our ability to predictively engineer cells [4]. This is the reason why our modified probiotic will be weaker than the wild type and hence indicates that our genetically modified probiotic bacteria cannot survive and spread in the ecosystem.

Circuit:

To be tested: ***Escherichia coli*** **Nissle 1917** with both the Bio-Bricks assembled using Gibson assembly.

Control: **Wild type *Escherichia coli* Nissle 1917** (we can also use a mixed culture)

Materials required: Petri plates, Micropipettes, Pipette tips, Test tubes, Cotton, Sterile loop, L-rod, Competent transformed bacteria, Wild type (*Escherichia coli* Nissle 1917), LB Agar, LB broth, Chloramphenicol, Sterile water, Dropper, 1N NaOH and 1N HCl (depending on pH).

[4] Carbonell- Ballstero M et al., 2015 [5] Cardinale S. et al., 2013 [6] Ceroni F et al., 2015 [14] Kafri M. et al., 2016 [28] Wu G. et al., 2016

Procedure:

Note: The experiment is performed at gut pH (6-7), temperature (37°C), and in aseptic conditions.

- Using a sterile loop, inoculate the transformed cells to be tested and the control in their respective LB broths overnight.
- Then transfer the same to LB agar using a micropipette and spread using a sterile L-rod with one plate having both wild type and transformed cells (non-selective plate) (1:1) and one plate with only transformed cells (selective plate).
- Change the medium everyday (for about 5-6 days) and maintain the culture by a thousand-fold dilution and let it grow overnight.
- Plate a dilution of the culture on both selective, and non-selective plates (without antibiotic) to track the fraction of each population over time.
- Plate the cells from the final non-selective plate on a selective medium.

Expected observation:

- The number of colonies on the selective plates gives us the number of cells carrying the Bio-Brick plasmids, while the number of colonies on the non-selective plates gives us the total amount of cells.
- The fraction of cells carrying the plasmid should decline rapidly, and after few days (maybe 5-6 days), there should be no traces of the *Escherichia coli* Nissle 1917 with the Bio-Bricks on the selective plates, while the wild type should still grow on the non-selective plates.
- The wildtype from the non-selective plate, plated on selective one should not grow to show that no transfer of genetic material has taken place.

Expected result:

- This shows that the wild type *Escherichia coli* Nissle 1917 *(*or any other wild type) outcompetes our modified probiotic due to genetic burden. Hence, if released into the environment, they would not spread in the natural ecosystem.
- No conjugation takes place showing that the modified probiotic has not imparted the plasmid to the wild type.

Unexpected outcomes:

- The modified probiotic outcompetes the wild type *Escherichia coli* Nissle 1917. To combat this issue, we will not be disclosing important aspects such as activation mechanisms of our product, to prevent any possible contamination of the environment.

Gibson assembly: Protocol Transformation: protocol LB broth: protocol LB agar: protocol
Protocol source: Team Uppsala 2013

5. Glossary

Term	Definition	Ref
2X Assay buffer	Buffer is a solution that resists changing its pH level even when acidic or basic components are added to it. For assays, this makes it very useful for maintaining a desired pH (often physiological pH, but not always) even during extended or multi-step experiments.	Link
Agarose gel electrophoresis	Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar.	Link
Amplification	The production of multiple copies of a sequence of DNA. Repeated copying of a piece of DNA.	Link
Annealing	The process of two strands of DNA rejoining is called annealing.	Link
Antibiotics	Antibiotics are medicines that help stop infections caused by bacteria. They do this by killing the bacteria or by keeping them from copying themselves or reproducing. The word antibiotic means “against life.” Any drug that kills germs in your body is technically an antibiotic.	Link
Anti-inflammatory cytokine	The functional definition of an anti-inflammatory cytokine is the ability of the cytokine to inhibit the synthesis of IL-1, tumor necrosis factor (TNF), and other major pro-inflammatory cytokines.	Link
Antioxidants	Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that may damage the cells of organisms.	Link
Aseptic	Asepsis or aseptic means the absence of germs, such as bacteria, viruses, and other microorganisms that can cause disease or contamination.	Link
ATP binding cassette	ATP-binding cassette (ABC) transporters are a large superfamily of membrane proteins with diverse functions.	Link
Autoclave	An autoclave is a machine used to carry out industrial and scientific processes requiring elevated temperature and pressure in relation to ambient pressure/temperature.	Link

Bio-Brick	Bio-Brick parts are DNA sequences which conform to a restriction-enzyme assembly standard.	Link
Bio-Brick compatibility	The assembly standard enables two groups of synthetic biologists in different parts of the world to re-use a Bio-Brick part without going through the whole cycle of design and manipulation.	Link
Bromophenol blue	Bromophenol blue is used as a pH indicator, an electrophoretic color marker, and a dye.	Link
Cell	Basic unit of a living organism	Link
Cell suspension	A cell suspension or suspension culture is a type of cell culture in which single cells or small aggregates of cells are allowed to function and multiply in an agitated growth medium, thus forming a suspension.	Link
Characterization	The discernment, description, or attributing of distinguishing traits of a biological part.	Link
Chloramphenicol	Chloramphenicol is an antibiotic useful for the treatment of a number of bacterial infections.	Link
Cleaving	A reaction that severs one of the covalent sugar-phosphate linkages between NUCLEOTIDES that compose the sugar phosphate backbone of DNA. It is catalyzed enzymatically, chemically or by radiation. Cleavage may be exonucleolytic - removing the end nucleotide, or endonucleolytic - splitting the strand in two.	Link
Codon	A codon is a sequence of three DNA or RNA nucleotides that corresponds with a specific amino acid or stop signal during protein synthesis.	Link
Competent cells	Cell competence refers to a cell's ability to take up foreign (extracellular) DNA from its surrounding environment.	Link
Conjugation	Conjugation is the process by which one bacterium transfers genetic material to another through direct contact.	Link
Control DNA	Control DNAs are powerful tools in the investigation of genomic DNA methylation and epigenomic research. They can be utilized in many protocols where they can serve as comparisons to sample DNA.	Link

Critical reporter	Reporter genes are those genes that when introduced into target cells (e.g., brain tissues, cancer, and circulating white cells) produce a protein receptor or enzyme that binds, transports, or traps a subsequently injected imaging probe.	Link
Cytoplasm	Cytoplasm is a thick solution that fills each cell and is enclosed by the cell membrane. It is mainly composed of water, salts, and proteins. In eukaryotic cells, the cytoplasm includes all of the material inside the cell and outside of the nucleus.	Link
Deionized water	Deionized water (DI water, DIW or de-ionized water), often synonymous with demineralized water / DM water, is water that has had almost all of its mineral ions removed, such as cations like sodium, calcium, iron, and copper, and anions such as chloride and sulfate.	Link
Denaturation	Denaturation is a process in which proteins or nucleic acids lose the quaternary structure, tertiary structure, and secondary structure which is present in their native state, by application of some external stress or compound such as a strong acid or base, a concentrated inorganic salt, an organic solvent, etc.	Link
dNTP	dNTP stands for deoxyribonucleotide triphosphate. Each dNTP is made up of a phosphate group, a deoxyribose sugar and a nitrogenous base. There are four different dNTPs and can be split into two groups: the purines and the pyrimidines.	Link
Downstream	In molecular biology and genetics, upstream and downstream both refer to relative positions of genetic code in DNA or RNA. ... When considering double-stranded DNA, upstream is toward the 5' end of the coding strand for the gene in question and downstream is toward the 3' end.	Link
EDTA	Ethylene-diamine-tetra-acetic acid, also known by several other names, is a chemical used for both industrial and medical purposes.	Link
Electrolytes	Electrolytes are minerals that carry an electrical charge when dissolved in water.	Link
Emission spectra	The emission spectrum of a chemical element or chemical compound is the spectrum of frequencies of electromagnetic radiation emitted due to an atom or molecule making a transition from a high energy state to a lower energy state.	Link

Equimolar	The definition of equimolar is having the same amount of moles.	Link
Escherichia coli DH5alpha	*DH5-Alpha* Cells are *Escherichia coli* cells engineered to maximize transformation efficiency.	Link
Exponential	An exponential rate of increase becomes quicker and quicker as the thing that increases becomes larger.	Link
Extension	In PCR, The extension step, also referred to as the elongation step, is the PCR step in which Taq polymerase adds nucleotides to the annealed primer.	Link
Extracellular matrix	Extracellular matrix (ECM) is a three-dimensional network of extracellular macromolecules, such as collagen, enzymes, and glycoproteins, that provide structural and biochemical support to surrounding cells.	Link
Fluorescence	Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation.	Link
Fluorometer	A fluorometer or fluorimeter is a device used to measure parameters of visible spectrum fluorescence: its intensity and wavelength distribution of emission spectrum after excitation by a certain spectrum of light.	Link
Free radicals	Free radicals are natural by-products of ongoing biochemical reactions in the body, including ordinary metabolic processes and immune system responses.	Link
Frothing	A mass of bubbles in or on a liquid; foam.	Link
Gel loading buffer	DNA gel loading buffer is intended to be mixed with samples containing DNA in order to facilitate loading of the samples into the wells of horizontal and vertical agarose and polyacrylamide.	Link
Genetic burden	In synthetic biology, the term "metabolic burden" describes the influence of heterologously expressed genes on the distribution availability of resources in the host cell.	Link
Gibson assembly	Gibson Assembly is a molecular cloning method which allows for the joining of multiple DNA fragments in a single, isothermal reaction.	Link

Homeostasis	Homeostasis is the ability to maintain a relatively stable internal state that persists despite changes in the world outside. All living organisms, from plants to puppies to people, must regulate their internal environment to process energy and ultimately survive.	Link
Immune response	The immune response is how your body recognizes and defends itself against bacteria, viruses, and substances that appear foreign and harmful.	Link
Initiation	Initiation is the beginning of transcription. It occurs when the enzyme RNA polymerase binds to a region of a gene called the promoter.	Link
Intestinal flora balance	Gut flora or gut microbiota are the microorganisms including bacteria, archaea and fungi that live in the digestive tracts of humans and other animals including insects.	Link
Laminar air flow	A laminar flow cabinet or tissue culture hood is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive materials. Air is drawn through a HEPA filter and blown in a very smooth, laminar flow towards the user.	Link
LB Agar	LB agar is LB broth that contains agar and is typically prepared in petri dishes.	Link
LB Broth	Lysogeny broth is a nutritionally rich medium primarily used for the growth of bacteria. Its creator, Giuseppe Bertani, intended LB to stand for lysogeny broth, but LB has also come to colloquially mean Luria broth, Lennox broth, or Luria–Bertani medium.	Link
Ligase	ligase is an enzyme that can catalyze the joining of two large molecules by forming a new chemical bond, usually with accompanying hydrolysis of a small pendant chemical group on one of the larger molecules or the enzyme catalyzing the linking together of two compounds.	Link
Lipopolysaccharide	Lipopolysaccharides (LPS), also known as endotoxins, are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria.	Link
Long spacer DNA	Spacer DNA is a region of non-coding DNA between genes.	Link

Lysosomes	A lysosome is a membrane-bound cell organelle that contains digestive enzymes. Lysosomes are involved with various cell processes. They break down excess or worn-out cell parts. They may be used to destroy invading viruses and bacteria.	Link
Lysozyme	Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase, is an antimicrobial enzyme produced by animals that forms part of the innate immune system.	Link
Mercury poisoning	Mercury poisoning is a type of metal poisoning due to exposure to mercury.	Link
Mitogen-activated protein kinase	A mitogen-activated protein kinase is a type of protein kinase that is specific to the amino acids: serine and threonine.	Link
Molecular cloning	Molecular cloning is a set of techniques used to insert recombinant DNA from a prokaryotic or eukaryotic source into a replicating vehicle such as plasmids or viral vectors.	Link
NanoDrop spectrometer	The NanoDrop Spectrophotometer from NanoDrop Technologies is designed for measuring nucleic acid concentrations in sample volumes of one microliter.	Link
Nitrogen bases	A nitrogenous base, or nitrogen-containing base, is an organic molecule with a nitrogen atom that has the chemical properties of a base.	Link
NP-40	NP-40 is an ethoxylated nonylphenol for non-ionic surfactants and can act as emulsifier and demulsifier agent.	Link
N-terminus	The N-terminus (also known as the amino-terminus, NH2-terminus, N-terminal end or amine-terminus) is the start of a protein or polypeptide referring to the free amine group (-NH2) located at the end of a polypeptide.	Link
Nucleic acid sensing TLRs	Toll-like receptors (TLRs) play an important role in innate immune responses against pathogenic microorganisms or tissue damage.	Link
Osmotic balance	Osmoregulation is the process of maintenance of salt and water balance (osmotic balance) across membranes within the body's fluids, which are composed of water, plus electrolytes and non-electrolytes.	Link

Oxidation	Oxidation is the loss of electrons during a reaction by a molecule, atom or ion.	Link
Oxidative state	The oxidation state describes loss of electrons of an atom	Link
Oxidative stress	Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.	Link
Palindrome	A palindromic sequence is a nucleic acid sequence in a double-stranded DNA or RNA molecule wherein reading in a certain direction on one strand matches the sequence reading in the same direction on the complementary strand.	Link
Paraquat	Paraquat, or N, N'-dimethyl-4,4'-bipyridinium dichloride, also known as Methyl Viologen, is a chemical herbicide, or weed killer, that's highly toxic and used all over the world.	Link
Peptide	Peptides are short chains of between two and fifty amino acids, linked by peptide bonds.	Link
Peptone	Peptone water is a microbial growth medium composed of peptic digest of animal tissue and sodium chloride.	Link
pH	pH is a scale used to specify the acidity or basicity of an aqueous solution.	Link
Phosphorylated	Phosphorylation of a molecule is the attachment of a phosphoryl group. Together with its counterpart, dephosphorylation, it is critical for many cellular processes in biology.	Link
Physiological	Physiology is the branch of biology relating to the function of organs and organ systems, and how they work within the body to respond to challenges.	Link
Plasmid	A plasmid is a small, extrachromosomal DNA molecule within a cell that is physically separated from chromosomal DNA and can replicate independently.	Link
Polymerase	A polymerase is an enzyme that synthesizes long chains of polymers or nucleic acids. DNA polymerase and RNA polymerase are used to assemble DNA and RNA molecules, respectively, by copying a DNA template strand using base-pairing interactions or RNA by half ladder replication.	Link

Polymerase chain reaction	Polymerase chain reaction is a method widely used to rapidly make millions to billions of copies of a specific DNA sample, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail.	Link
Polymerization	Polymerization is a process through which a large number of monomer molecules react together to form a polymer.	Link
Primers	A primer is a short single-stranded nucleic acid utilized by all living organisms in the initiation of DNA synthesis.	Link
Probiotic	Probiotics are live microorganisms promoted with claims that they provide health benefits when consumed, generally by improving or restoring the gut flora. Probiotics are considered generally safe to consume but may cause bacteria-host interactions and unwanted side effects in rare cases.	Link
Pro-inflammatory signal	An inflammatory cytokine or proinflammatory cytokine is a type of signaling molecule (a cytokine) that is secreted from immune cells like helper T cells (Th) and macrophages, and certain other cell types that promote inflammation.	Link
Proliferation	Cell proliferation is the process that results in an increase of the number of cells and is defined by the balance between cell divisions and cell loss through cell death or differentiation.	Link
Promoter	In genetics, a promoter is a sequence of DNA to which proteins bind that initiate transcription of a single RNA from the DNA downstream of it.	Link
pSB4C5	pSB4C5 is a Bio-Brick standard vector with low to medium copy p15A replication origin.	Link
Reagents	A reagent is a substance or compound added to a system to cause a chemical reaction or added to test if a reaction occurs.	Link
Restriction endonuclease	A restriction enzyme, restriction endonuclease, or restrictase is an enzyme that cleaves DNA into fragments at or near specific recognition sites within molecules known as restriction sites. Restriction enzymes are one class of the broader endonuclease group of enzymes.	Link
Secretion signal peptide	Signal peptides are short amino-terminal parts of exported precursor proteins that direct the respective proteins to the protein export systems in the cytoplasmic membrane.	Link

Selective plates	Selective media used for the growth of only selected microorganisms. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium in order to prevent other cells, which do not possess the resistance, from growing.	Link
Serva DNA stain G	SERVA DNA Stain G is a safer alternative to traditional ethidium bromide stain for detecting nucleic acids in agarose gels.	Link
Sonication	Sonication is the act of applying sound energy to agitate particles in a sample, for various purposes such as the extraction of multiple compounds from plants, microalgae and seaweeds. Ultrasonic frequencies are usually used, leading to the process also being known as ultrasonication or ultra-sonication.	Link
Specificity	Specificity relates to the test's ability to correctly reject healthy patients without a condition. Specificity of a test is the proportion of healthy patients known not to have the disease, who will test negative for it.	Link
Staining tray	Staining Trays are designed specifically for incubation and storage of immunohistochemistry slides.	Link
Sterile loop	An inoculation loop, also called a smear loop, inoculation wand or microstreaker, is a simple tool used mainly by microbiologists to pick up and transfer a small sample from a culture of microorganisms, e.g. for streaking on a culture plate.	Link
Sterility	Sterility is the physiological inability to effect sexual reproduction in a living thing, members of whose kind have been produced sexually. Sterility has a wide range of causes.	Link
Sterilization	Sterilization refers to any process that removes all forms of life.	Link
Stock solution	A stock solution is a concentrated solution that will be diluted to some lower concentration for actual use. Stock solutions are used to save preparation time, conserve materials, reduce storage space, and improve the accuracy with which working lower concentration solutions are prepared.	Link
Supernatant	The soluble liquid reaction of a sample after Centrifugation or precipitation of insoluble solids.	Link
Template DNA	A template strand is the term that refers to the strand used by DNA polymerase or RNA polymerase to attach complementary bases during DNA replication or RNA transcription, respectively.	Link

Termination	Termination requires that the progress of the DNA replication fork must stop or be blocked. Termination at a specific locus, when it occurs, involves the interaction between two components: (1) a termination site sequence in the DNA, and (2) a protein which binds to this sequence to physically stop DNA replication.	Link
Thermocycling	The use of a thermocycler to amplify DNA segments.	Link
Transcription	Transcription is the process by which the information in a strand of DNA is copied into a new molecule of messenger RNA (mRNA). DNA safely and stably stores genetic material in the nuclei of cells as a reference, or template.	Link
Transcription factor	In molecular biology, a transcription factor (TF) (or sequence-specific DNA-binding factor) is a protein that controls the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence.	Link
Transport system	In biology a mass transport system is an arrangement of physical structures by which materials are moved in the form of a fluid containing particles of those materials travelling in one direction [through a system of tubes] from one or more exchange surface(s) within an organism to cells located throughout the organism.	Link
Translation	In molecular biology and genetics, translation is the process in which ribosomes in the cytoplasm or endoplasmic reticulum synthesize proteins after the process transcription of DNA to RNA in the cell's nucleus. The entire process is called gene expression.	Link
Tris-buffer saline	Tris-buffered saline is a buffer used in some biochemical techniques to maintain the pH within a relatively narrow range. Tris has a slightly alkaline buffering capacity in the 7–9.2 range.	Link
Vector	In molecular cloning, a vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed. A vector containing foreign DNA is termed recombinant DNA.	Link
Yeast extract	Yeast extracts consist of the cell contents of yeast without the cell walls; they are used as food additives or flavorings, or as nutrients for bacterial culture media.	Link
Wild type	Wild type refers to the phenotype of the typical form of a species as it occurs in nature.	Link

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Preparation of media:

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- http://mcb.berkeley.edu/labs/krantz/protocols/agar_plates.pdf

PCR amplification:

- https://www.neb.com/protocols/0001/01/01/pcr-protocol-m0530

Restriction digestion:

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Agarose gel electrophoresis

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