# iGEM MIT_MAHE

## COMPOSITE BIOBRICK 1 HANDBOOK

# Acknowledgement

The curation of this handbook by the wet lab team of iGEM MIT_MAHE would not have been possible without the exceptional support of our Principal Investigator (PI), Dr. Ritu Raval. Her enthusiasm and knowledge kept the information on track from start to end. Soumodeep Sarkar and Varun Udaya Kumar, our fellow teammates, have also given us insight into the documentation and answered with unfailing patience numerous questions about the content. We thank Shravan Balasubramaniam for writing, editing, safety and experimentation, Lavanya Karinje for experimentation, safety and writing, Naidu Shameshwar Muralidarran for writing, Beejady Adithi Somayaji for writing, editing, experimentation and safety and Bhumika Narang for experimentation and writing. The expertise and skills of everyone involved has improved this handbook in innumerable ways.

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# 1. Introduction to Composite BioBrick 1

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

| Part/Biobrick | Species name (including strain) | iGEM registry |
|---|---|---|
| Constitutive Promoter (J23100) | No natural comparator | http://parts.igem.org/Part:BBa_J23100 |
| MerR + RBS (K346001) | *Shigella flexneri* | http://parts.igem.org/Part:BBa_K346001 |
| PmerT promoter (K346002) | *Shigella flexneri* | http://parts.igem.org/Part:BBa_K346002 |
| MerT (K1420005) | *Serratia marcescens* | http://parts.igem.org/Part:BBa_K1420005 |
| MerP (K1420003) | *Serratia marcescens* | http://parts.igem.org/Part:BBa_K1420003 |
| MerC + RBS (K346088) | No natural comparator | http://parts.igem.org/Part:BBa_K346088 |
| RBS with MerE (K1471002) | No natural comparator | http://parts.igem.org/Part:BBa_K1471002 |
| MerA (K1420001) | *Serratia marcescens* | http://parts.igem.org/Part:BBa_K1420001 |
| MerB (K1420002) | *Serratia marcescens* | http://parts.igem.org/Part:BBa_K1420002 |
| SoxR (K554003) | *Escherichia coli K-12* | http://parts.igem.org/Part:BBa_K554003 |
| RBS (B0034) | *Escherichia coli K-12* | http://parts.igem.org/Part:BBa_B0034 |
| Bright Yellow Fluorescent Protein SYFP2 (K864100) | *Aequorea victoria* | http://parts.igem.org/Part:BBa_K864100 |
| Double Terminator (B0015) | *Escherichia coli K-12* | http://parts.igem.org/Part:BBa_B0015 |

***Note:*** The circuits are to be assembled on the pSB3K5 vector*.

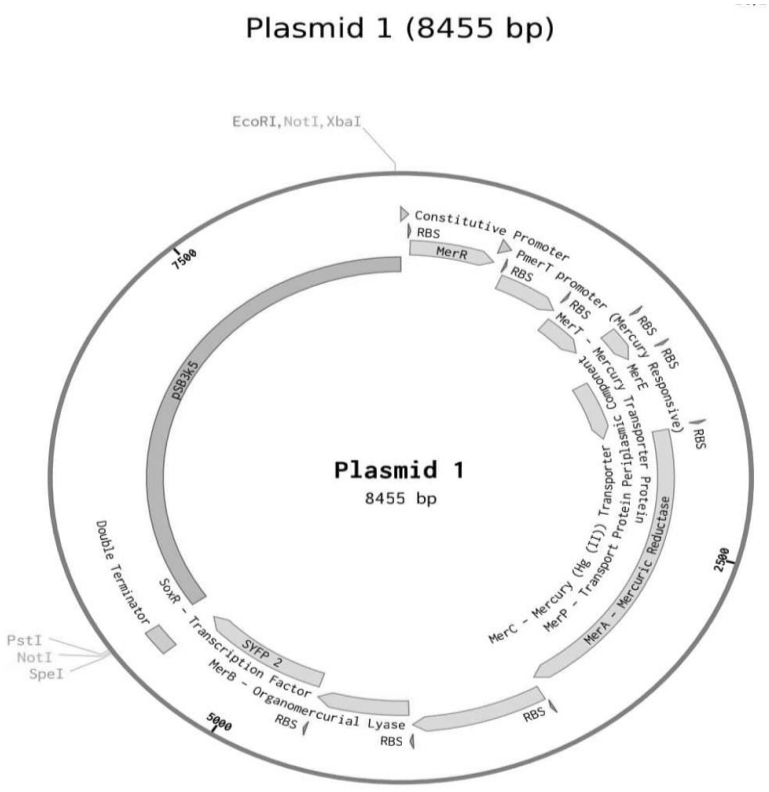
* iGEM registry: http://parts.igem.org/Part:pSB3K5

# B. Working principles of each part:

| Part/BioBrick | Working principle: |
|---|---|
| Constitutive Promoter | Constitutive promoter helps tune the expression levels of constitutively (continuously) expressed parts. |
| MerR + RBS | MerR is a homodimer and the regulatory factor of the mer operon. It acts as an activator in the presence of Hg (II) and a weak repressor in its absence, maintaining its expression at a certain level. It binds to the operator site between –35 and –10 elements of mercury inducible promoter PmerT, independent of the presence of mercury. The Hg- bound MerR dimer causes a structural distortion of PmerT, which allows the RNA polymerase to bind, leading to the transcription of genes downstream to it [2] [5]. |
| PmerT | PmerT is the mercury inducible promoter of mer operon. It has an unusually long spacer DNA of 19bp to which MerR binds, inhibiting the RNA polymerase. When the MerR binds to Hg it causes a distortion in the structure of PmerT allowing the polymerase to bind and transcribe the genes downstream to it. The threshold of PmerT depends on the expression level of MerR [9]. |
| MerT | MerT is a transmembrane protein which receives mercury from MerP, at its first transmembrane helix and transports it into the cytoplasm of the bacterial cell [1] [14]. |
| MerP | MerP is the periplasmic component of the mer transport system which helps in the uptake of mercury inside the cell. It binds to a single Hg (II) ion using its two conserved cysteine residues, which define its metal-binding motif. It removes any attached ligands before passing the Hg (II) on to MerT transmembrane protein. It is the most abundantly synthesized protein in the mer operon due to its role in scavenging of Hg (II) in the periplasm [1] [17]. |
| MerC + RBS | MerC is a transmembrane component of the mer transport system which helps in the uptake of mercury inside the cell. It can function without the help of MerP and has been hypothesized to be required in cases of high mercury concentration [16]. |
| RBS + MerE | MerE is a transmembrane component of the mer transport system which helps in the uptake of mercury inside the cell. It helps in the transport of organo-mercury compounds [16]. |
| MerA | MerA encodes the mercury reductase enzyme. It reduces Hg (II) to relatively inert and volatile Hg (0) in an NADPH dependent reaction [12]. |

[1] T. Barkay et al., 2003 [2] Brown et al., 2003 [5] Hobman et al., 2005 [9] Nakaya et al., 1960 [12] Parks et al., 2009
[17] Steele & Opella, 1997 [14] Rossy E et al., 2004 [16] Sone et al., 2013

| | |
|---|---|
| MerB | MerB encodes the organomercurial lyase enzyme and is usually found immediately downstream to MerA. It catalyzes breaking the bond between carbon and mercury through the protonolysis of compounds such as methylmercury. This produces the less mobile Hg (II) which is then reduced to Hg (0) by MerA [1] [8]. |
| SoxR | 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) [4] [8]. |
| 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. |
| Double Terminator | It functions to signal the termination of transcription. |
| SYFP2 | SYFP2 is a GFP based monomeric protein with optimized folding, maturation, and a narrow fluorescence emission spectrum with a maximum of 515 nm. [6]. |
| pSB3K5 | 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. pSB3K5 is a low copy number plasmid carrying kanamycin resistance. |




[1] T. Barkay et al., 2003 [4] Hidalgo et al., 1998 [6] Kremers et al., 2006 [7] Mathema et al., 2011 [8] Miki et al., 2008

# 2. Working design of Composite BioBrick 1

## Circuit:

**Constitutive Promoter -** RBS **- MerR - PmerT promoter –** RBS **- (MerT –** RBS **– MerP –** RBS **– MerE –** RBS **– MerC -** RBS**)* - MerA –** RBS **– MerB –** RBS **– SoxR -** RBS **- SYFP2** – **Double Terminator**

*The final transport system will be determined after experimentation.

## Working:

Methylmercury is a highly toxic organometallic compound. The primary source of exposure of this heavy metal for humans is the consumption of food (primarily but not limited to fish) contaminated with it [18]. Mercury is a vital component in industries, used to manufacture electrical devices and electronics such as batteries, industrial chemicals, etc. [10]. Unfortunately, improper disposal techniques create massive problems that can lead (and has previously led) to catastrophes.

Mercury in water bodies (from different sources) is converted by aquatic anaerobic bacteria (such as sulphate-reducing bacteria and iron-reducing bacteria) and fungi into methylmercury and other organic mercury compounds via methylation, which makes the mercury circulating in the environment bio-available [10] .Plasmid 1 will be responsible for the transport of methylmercury inside the bacterial system, production of mercury (II) reductase enzyme and alkylmercurial lyase, along with its regulation, and the production of SoxR which controls the release of IL-10 from Plasmid 2. It features a modified Mer operon with an SYFP2 reporter downstream to all coding regions and control elements in a pSB3K5 vector.

A constitutive promoter ensures the continuous transcription of MerR [5]. When the constitutive promoter is translated, MerR produces a weak repressor molecule that can bind to the PmerT region, preventing the transcription of genes downstream to it [2] [9] [11] [13]. In the presence of Hg (II) cation, the repressor molecule would not bind to PmerT but instead, bind to mercury (II) cation and reactivate the transcription of all downstream elements - MerP, MerT, MerE and MerC to deal with the production of transport proteins that will help transport of methylmercury inside the bacterial system [1] [3] [14] [15] [16] [17]. MerA and MerB produce our dual enzymes – mercuric (II) reductase and alkylmercurial lyase required for the conversion of MeHg to elemental Hg [7] [12].
However, since the release of elemental mercury has the potential to disturb the gut microbiota and induce inflammation, we also explore a mechanism to tackle that problem. Composite BioBrick 2 will have the anti-inflammatory cytokine, IL-10 and the associated transport and regulatory system.

The plasmid will also contain the gene for the transcription factor SoxR, which will be useful for activating the anti-inflammatory system present in Plasmid 2 hence allowing for the anti-inflammatory signals to be produced only during the presence of a Hg (II) compound [4] [8]. SYFP2 is to assess the functioning of Plasmid 1 components, mainly the MerR regulation [6] .


[1] T. Barkay et al., 2003
[2] Brown et al., 2003
[3] Hamlett et al., 1992
[4] Hidalgo et al., 1998
[5] Hobman et al., 2005
[6] Kremers et al., 2006
[7] Mathema et al., 2011
[8] Miki et al., 2008
[9] Nakaya et al., 1960
[10] Nogara et al., 2019
[11] Park et al., 1992
[12] Parks et al., 2009
[13] Ralston & O'Halloran, 1990
[14] Rossy E et al., 2004
[15] Sone, Nakamura, Pan-Hou, Sato et al., 2013
[16] Sone et al., 2013
[17] Steele & Opella, 1997
[18] Thackray & Sunderland, 2019

# 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, Kanamycin- 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 Kanamycin 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 Kanamycin 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, Kanamycin- 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 Kanamycin. For 100mL of the LB agar mix, add 100uL 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 Kanamycin 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 the 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 BioBrick Parts:

| Primers | Description | Sequence |
|---|---|---|
| VR | Reverse primer for sequencing/amplifying BioBrick parts (VR) | attaccgcctttgagtgagc (R) |
| VF2 | Forward primer for sequencing/amplifying BioBrick 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.<br>Primer Tm (60-70°C).<br>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 BioBricks using XbaI and SpeI and Gel Electrophoresis

**Aim:** To perform the restriction digestion (double digestion) of the amplified BioBricks 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 defence 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 (BioBricks), 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 (BioBrick Compatibility).

Protocol source: Restriction digestion
Agarose gel electrophoresis: Protocol
For more info on BioBrick Compatibility refer here.

# 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, the strength of the electric field applied, hydrophobicity of the DNA, the concentration of agarose, ionic strength of the buffer, the temperature of the 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) and 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 100V.
- 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 as 'Nucleic Acid' in the NanoDrop menu.
- Clean the surface of the NanoDrop with dH2O and a fibre-free tissue.
- Perform calibration and blank measurement by entering one drop of 2 µL autoclaved dH2O.
- Clean the surface again with dH2O and a fibre-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 bps. 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 neighbouring assembly fragments.

# D. Competent cell preparation

**Aim:** To prepare fresh competent cells of *Escherichia coli* DH5alpha.

**Principle:**
Competent cells are cells that have altered cell walls that 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 (Kanamycin in our case) etc.

**Materials required:**
Petri plates with LB, LB liquid medium, Wild type *Escherichia coli* DH5alpha, Incubator, Laminar flow chamber, Autoclave, Micropipette, tips, Sterile loop, 500mL flask, Shaker incubator, Ice, $CaCl_2$ solution, Glycerol solution, Centrifuge and Micro-centrifuge tubes.

**Procedure:**
***Note:*** All the steps should be performed aseptically using sterile equipments and experiment conditions.

- Culture the wild type *Escherichia coli* DH5alpha 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.

# E. Transformation

**Aim:** To introduce the composite BioBricks into the competent *Escherichia coli* DH5alpha (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 be utilized according to the application.

**Materials required:**
Competent cells (*Escherichia coli DH5α)*, Plasmid DNA, Competent cells, Sterile LB broth, LB agar plate (supplemented with Kanamycin), 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 equipments 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 the 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 (Kanamycin).
- 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 to study their contents.

**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 5000 rpm 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 at 30°C for 15 minutes (or till the solution becomes viscous) after 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 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. Method for the determination of methylmercury by Gas Chromatography

**Aim:** To determine methylmercury using Gas Chromatography method.

**Principle**:
Methylmercury bromide is extracted from the sample into toluene and is selectively adsorbed on the cysteine paper. Interfering compounds are washed from the paper with toluene. The isolated methylmercury is set free with sulphuric acid containing bromide, extracted into benzene and determined by GC. The extraction procedure results in good recovery and producibility for various biological and environmental samples, good sensitivity with a detection limit of 0.1 ng/g, avoidance of difficulties arising from emulsion formation, cleaner chromatograms and faster analysis. It is particularly suitable for determination of low levels of MeHg.

They are based on addition of acid (hydrochloric, hydrobromic or hydriodic) to a homogenized sample, extraction of the MeHg halide into an organic solvent (benzene or toluene), purification by stripping with a thiol compound (cysteine, glutathione) or thiosulphate, and re-extraction into benzene. The MeHg is then determined by GC with various detection systems (electron-capture, atomic absorption, mass spectrometry, microwave emission spectrometry). The main difficulties are the formation of emulsions (often persistent) during the extraction or stripping and loss of volatile MeHg.

**Materials Required:**

*Reagents:*
L(+)-Cysteine hydrochloride solution (1%) prepared daily in 20% sodium citrate solution, Sulphuric acid (2M) saturated with cupric sulphate, Potassium bromide (4M), Hydrochloric acid (4M), Mercuric chloride as a saturated solution in benzene, Benzene and toluene, Chromatographic grade (Merck (name of the company) methylmercury and ethylmercury standard solutions (1 mg/mL).

*Note*:
Dissolve 116.3 mg of $CH_3HgCl$ or 115.4 mg of $C_2H_4HgCl$ (Merck) in 100 mL of toluene. Make working standard aqueous solutions of methylmercury and ethylmercury by appropriate dilution with benzene to cover the range 0.02-0.10 ng/µL. Dissolve the same amounts of $CH_3HgCl$ and $C_2H_4HgCl$ as above in 1-2 mL of acetone and dilute to 100 mL with 0.1 M hydrochloric acid. The stock and working standard solutions must be kept in darkness to prevent decomposition of MeHg by ultraviolet light.

*Apparatus:*
*Gas chromatograph*: A Hewlett Packard model 5890 instrument connected to an HP 3390. An integrator was used, with an electron capture detector (63Ni radioactive source). The chromatographic conditions were as follows:

- Column temperature: 160°C
- Injector temperature: 190°C
- Detector temperature: 280°C

*Carrier gas*: $N_2$ with a flowrate of 60 mL/min.

*Glass columns*: Length 1.6 m, inner diameter 2 mm, packed with 5% DEGS-PS on Supelcoport 100-120 mesh (commercially available from Supelco).

*Filter papers*: A narrow strip of 2 mm in width, cut from the circumference of a circular filter paper (Schleicher and Schiill, No. 58g3, 7 cm diameter) is saturated with cysteine solution and dried at room temperature in an acid-free atmosphere, just before use. It is inserted as a spiral into the extraction tube.

**Procedure:**
***Note:*** All the steps should be performed aseptically using sterile equipments and experiment conditions.

***Note:***

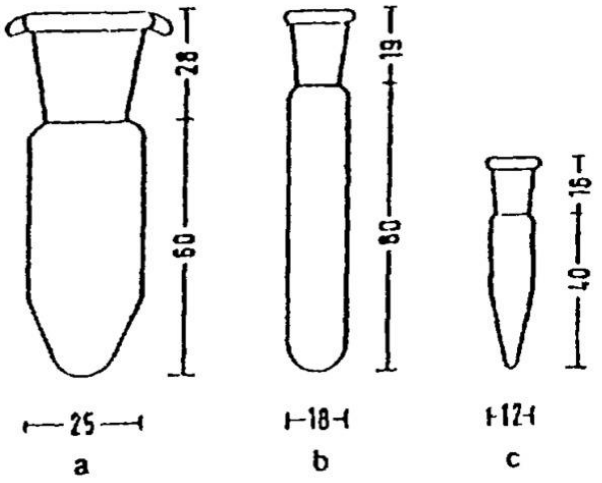
- First create a calibration curve by preparing standard solutions and measuring known concentrations of MeHg (For every experiment involving gas chromatography).

*Method 1:*

- Shake 1-3 g of fresh sample (0.1-1.5 g of dry sample) + 2 mL of $H_2S0_4$ saturated with $CuSO_4$, 2 mL of KBr solution + 3 mL of toluene in test-tube “a”, Fig. 1. Equilibrate for 10 min then centrifuge for 5 min at 6000 rpm. Repeat the extraction with 2 mL of toluene and transfer the toluene phases quantitatively.
- Shake toluene phase + 1 mL of cysteine solution in test-tube “b”, Fig. I. Centrifuge at 6000 rpm. Transfer the aqueous phase quantitatively. Repeat the extraction with cysteine.
- Shake aqueous phase + 1 mL of KBr solution + 1 mL of H2SO4+ 0.5-1.0 mL of benzene in test-tube “c”, Fig. 1. Centrifuge.
- Transfer the benzene phase into test-tube “c”, Fig. 1.
- Inject 1-5 µL of the organic phase into GC column.

*Method 2:*

- Shake 1-3 g of fresh sample (0.1-1.5 g of dry sample) + 2 mL of $H_2SO_4$ saturated with $CuSO_4$, 2 mL of KBr solution + 3 mL of toluene in test-tube "a", Fig. 1. Equilibrate for 10 min then centrifuge for 5 min at 6000 rpm. Repeat the extraction with 2 mL of toluene and transfer the toluene phases quantitatively.
- Shake toluene phase + cysteine paper in test-tube "b", Fig. 1, for 10 min. Decant organic phase and wash the cysteine paper with three 5-mL portions of toluene. Dry the paper.
- Transfer the paper into test-tube "c", Fig. 1, and add 0.1 mL of HBr + 0.1 mL of H2SO4, + 0.2-1.0 mL of benzene (0.2 mL). Equilibrate and then centrifuge.
- Transfer the benzene phase into test-tube "c", Fig. 1.
- Inject 1-5 µL of the organic phase into GC column.




Test tubes for the decomposition of samples and extraction of methylmercury: 25ml (a), 10ml (b), 3ml (c).

Fig 1: Image source

**Precautions**:

- It is very important to transfer only clear organic phase.
- MeHg is extracted into aqueous cysteine solution only at neutral pH.

Protocol source: Gas chromatography

# H. 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:**
Refer here for required materials in GC, Spectrophotometer, fluorometer, 187 µg FITC, 10mL 1xPBS (phosphate buffered saline), cuvettes.

**Procedure:**

***GFP:***
***Note:***

- 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.

***Gas Chromatography:***

- Use known amount of Methylmercury as standard solutions following the protocol:

| **Concentration (µM)** | **Measurement in GC (µM)** | | |
|---|---|---|---|
| 0.00052 | | | |
| 0.3 | | | |
| 2 | | | |
| 5 | | | |
| 10 | | | |

**Table 1: Calibration data for residual standard error**

| Conc $x_i$ | Absorbance $y_i$ | $(x_i - \bar{x})^2$ | Predicted $\hat{y}_i = mx_i + c$ | Residuals $y_i - \hat{y}_i$ | Residuals$^2$ $(y_i - \hat{y}_i)^2$ |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| $\bar{x}$ | $\bar{y}$ | $\sum_{i=1}^{n}(x_i - \bar{x})$ | | | $\sum_{i=1}^{n}(y_i - \hat{y}_i)^2$ |
| | | | | | |

**Table 2: Data required to calculate a prediction interval**

If deviations are observed

- The residual standard deviation (also known as the residual standard error) is a statistical measure of the deviation of the data from the fitted regression line.

We use the equation given below to calculate the residual standard deviation,

$$s(r) = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}}$$

where, $\bar{y}i$ is the observed value of y for a given value of $x_i$, $\bar{y}i$ is the value of y predicted by the equation of the calibration line for a given value of $x_i$, and n is the number of calibration points.

The predicted interval can be calculated using the equation,

$$s_{x_0} = \frac{s(r)}{m}\sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(\bar{y}_o - \bar{y})^2}{m^2\sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

where, s(r) is the residual standard deviation, n is the number of paired calibration points ($x_i$,$y_i$), m is the calculated best-fit gradient of the calibration curve, N is the number of repeat measurements made on the sample (this can vary from sample to sample and can equal 1), $\bar{y}_0$ the mean of N repeat measurements of y for the sample, $\bar{y}i$ is the mean of the y values for the calibration standards, $x_i$ is a value on the x-axis and $\bar{x}$ is the mean of the $x_i$ values.

The uncertainty (in % relative) can be calculated using the equation mentioned below,

$$x_{pred} = \frac{\bar{y}_o - c}{m}$$

where, $\bar{y}_o$ is the mean of N repeat measurements of y for the sample, c is the intercept of the best-fit gradient of the calibration curve and m is the calculated best-fit gradient of the calibration curve.

The uncertainty in predicted values can be reduced by increasing the number of replicate measurements (N) made on the test sample.

| N | $S_{xo}$ (µM) | Uncertainty (% relative) |
|---|---|---|
| | | |
| | | |

Table 3: Residual standard error for different values of N

# 4. Experimentation

## A. Preliminary Experiment

### i) Experiment 1:

**Aim**: To check the threshold of mercury required by the transformed bacteria to generate a response.

**Principle:**

*Circuit:*

To be tested:
**Constitutive Promoter –** RBS **– MerR - PmerT promoter –** RBS - **MerT –** RBS **– MerP –** RBS **– MerE –** RBS - **MerC –** RBS **– GFP - Double Terminator**

Control:
**Constitutive 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, Gloves, Gasmask, Transformed bacteria, LB broth, Sterile loop, Kanamycin (34µg/mL), Methylmercury chloride, Luria-Bertani medium, TN Buffer (0.15M NaCl + 10mM Tris-HCl), Fluorometer, and Incubator.

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

- Inoculate bacteria (*Escherichia coli DH5α* transformed with BioBrick 1) in Luria-Bertani (LB) liquid + Kanamycin 34 µg/mL.
- Incubate in the shaker at 37°C overnight.
- Transfer 1 mL of bacterial suspension to 50 mL of LB medium without antibiotics.
- Incubate in 37°C shaker until the absorbance at 600nm spectrophotometer measures 0.4 to 0.6.

- Before starting the induction with methylmercuric chloride, measure fluorescence on a spectrofluorometer (excitation-350nm, emission-500nm wavelength).
- Aliquot 500µL of bacterial suspension in 20 tubes (2mL).
- Add the amount of methylmercury chloride to achieve concentrations: 0.00052µM, 0.15µM, 0.3µM, 1µM, 2µM and 5µM** and a tube without mercury as a negative control.
- Add 100µL of each tube in the black plate of the spectrofluorometer to measure the intensity of GFP and white board to measure optical density on the spectrophotometric simultaneously. Do not forget to add the negative control (*Escherichia coli DH5α* with BioBrick 1 without induction of mercury).
- Measure O.D and GFP intensity at intervals of 60 minutes until 4.5 hours. Between intervals, incubate at 37°C in the shaker.

*To check for the accumulation of mercury inside the cell:*

- Transfer the cells into microcentrifuge tubes.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell and collect the supernatant (of the two different circuits).
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- A plot of OD in function of time at different methylmercury concentrations.
- A plot of GFP fluorescence intensity in function of time keeping methylmercury concentration constant.

**Expected Result:**

- The threshold of response is noted and used as the minimum limit for the characterization experiments.

****Note:***

The concentrations used in the experiment are chosen through extensive literature survey and have physiological importance. However, the lower dosage limit of methylmercury cannot be precisely determined due to differences of absorption/ effect in different individuals.

**Precautions**:

- Perform all steps in sterile conditions under a laminar flow chamber.

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

## ii) Experiment 2:

**Aim**: To check the limits of tolerance of our bacteria to high levels of methylmercury chloride.

**Principle:**

*Circuit:*

To be tested:
**Constitutive Promoter –** RBS **– MerR - PmerT promoter –** RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS - **MerC –** RBS **– GFP - Double Terminator**

Control:
**Constitutive 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, Gloves, Gasmask, Competent transformed bacteria, LB broth, Sterile loop, Kanamycin (34µg/ml), Methylmercury chloride, Luria-Bertani + Mercury (LM) medium, TN Buffer (0.15M NaCl + 10mM Tris-HCl), Fluorometer and Incubator.

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

- Inoculate bacteria (*Escherichia coli DH5α* transformed with BioBrick 1 in Luria-Bertani (LB) liquid + Kanamycin 34 µg/mL.
- Incubate in the shaker at 37°C overnight.
- Transfer 1mL of bacterial suspension to 50 mL of LB medium without antibiotics.
- Incubate in 37°C shaker until the absorbance at 600nm spectrophotometer measures 0.4 to 0.6.
- Before starting the induction with mercuric chloride, measure fluorescence on a spectrofluorometer (excitation-350nm, emission-500nm wavelength).
- Aliquot 500µL of bacterial suspension in 20 tubes (2mL).
- Add the amount of methylmercuric chloride to achieve concentrations: 30µM, 40µM, 50µM, 60µM, 70µM and 80µM** and a tube without mercury as a negative control.
- Add 100µL of each tube in the black plate of the spectrofluorometer to measure the intensity of GFP and white board to measure optical density on the

spectrophotometric simultaneously. Do not forget to add the negative control (*Escherichia coli DH5α* with BioBrick 1 without induction of mercury).

- Measure O.D and GFP intensity at intervals of 60 minutes until 4.5 hours. Between intervals, incubate at 37°C in the shaker.

*To check the maximum accumulation of mercury inside the cell:*

- Transfer the cells into a microcentrifuge tube.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell and collect the supernatant (of the two different circuits).
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- A plot of OD in function of time at different methylmercury concentrations.
- A plot of GFP fluorescence intensity in function of time keeping methylmercury concentration constant.

**Expected Result:**

- The limit of tolerance of the bacteria is noted and used as the maximum limit for the characterization experiments.

****Note:***

The concentrations used in the experiment are chosen through extensive literature survey and have physiological importance. However, the upper dosage limit of methylmercury cannot be precisely determined due to differences of absorption/ effect in different individuals.

# B. Characterization of MerR

**Aim:** To test the efficiency of and characterize MerR.

**Principle:**

*Circuit:*

To be tested:
Circuit 1: **Constitutive Promoter –** RBS **– MerR - PmerT promoter – GFP - Double Terminator**

Control:
Circuit 2: **Constitutive Promoter – GFP - Double Terminator**
Circuit 3: **PmerT promoter – GFP - Double Terminator**

The working of each part is given in detail above.

The circuit assembled 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 (1N 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 DH5α* into different plates containing LB agar medium and incubate overnight (2 + (duplicates if necessary)).
- Then transfer the same to LB broth using a sterile loop. (5 + 5 + (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM, and 20µM of $CH_3HgCl_2$ (respectively to the two different circuits).
- According to the results of the preliminary experiment, let it grow for the required time and check hourly.

- Check the GFP intensities using a fluorometer (with excitation in 500 nm and emission spectra from 508-550 nm) every hour. Plot graphs to analyse and compare the graphs of the three circuits.
- Then transfer 1 mL of the mixtures into the respective set of microfuge tubes and centrifuge at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set of microfuge tubes.
- Measure the amount of mercury in each of the tubes.

***Note:*** Steps 5-8 to be repeated 3-4 times hourly.

- Plot a graph of fluorescence vs concentration of methylmercury (μg) and analyse.

**Expected observation:**

- Exponential increase in fluorescence with an increase in methylmercury concentration in the case of circuit 1.
- In case of control with PmerT i.e. circuit 3, transcription is initiated but at a lower level when compared to circuit 1.
- PmerT from circuit 3 behaves like a constitutive promoter. Hence the fluorescence intensity is the same for both the controls.

**Expected result:**

- The plot indicates that MerR contributes significantly in methylmercury response.
- Without MerR, even in the absence of methylmercury, transcription of downstream genes may occur with the help of PmerT.

# C. Characterization of the transport system

## i) Experiment 1:

**Aim:** To check the efficiency of different transport systems and determine the final transport circuit design of plasmid 1.

**Principle:**

*Circuit:*

To be tested:
Circuit 1: **Constitutive Promoter –** RBS **– MerP –** RBS **– MerT –** RBS **– MerE –** RBS **– MerC –** RBS **- Double Terminator**
Circuit 2: **Constitutive Promoter** – RBS **– MerC** – RBS **– MerE** – RBS **- Double Terminator**
Circuit 3: **Constitutive Promoter -** RBS **– MerP -** RBS **- MerT -** RBS **- MerE -** RBS **- Double Terminator**

Control:
Circuit 4: **Wild type *Escherichia coli* DH5alpha**

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

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

**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 control on LB agar overnight (1+1+1+1).
- Then transfer the same to LB broth using a sterile loop. (5+5+5+5 (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM, and 20µM of $CH_3HgCl_2$ (respectively to the four different circuits).

- According to the results of the preliminary experiment, let it grow for the required time.
- Then transfer 1 mL of the mixtures into the respective set of microfuge tubes and centrifuge again at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set of microfuge tubes.
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |
| *Supernatant set 3/ conc** | Represents the mercury remaining outside of the cell containing the third circuit set. |
| *Supernatant set 4/ conc** | Represents the mercury remaining outside of the cell containing the fourth circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- Compare the graphs with each other and determine the most efficient transport system.

**Expected result:**

- The most efficient transport system is the final transport circuit design.

**Unexpected result:**

- If there are two transport system circuits with similar efficiency, the one with the least genetic burden will be selected.

## ii) Experiment 2*:

**Aim**: To test the efficiency of and characterize MerP.
*This experiment is performed only if the final circuit design has MerP.

**Principle:**

*Circuit*:

To be tested:
Circuit 1: **Final transport system design**
Circuit 2: **Constitutive Promoter -** RBS **– MerP –** RBS **– Double Terminator**

Control:
Circuit 3: **Final circuit design without MerP**
Circuit 4: **Wild type *Escherichia coli* DH5alpha**

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

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

**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 control on LB agar overnight (1+1+1+1).
- Then transfer the same to LB broth using a sterile loop. (5+5+5+5 (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM, and 20µM of $CH_3HgCl_2$ (respectively to the four different circuits).
- According to the results of the preliminary experiment, let it grow for the required time.
- Then transfer 1 mL of the mixtures into the respective set of microfuge tubes and centrifuge again at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set of microfuge tubes.
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |
| *Supernatant set 3/ conc** | Represents the mercury remaining outside of the cell containing the third circuit set. |
| *Supernatant set 4/ conc** | Represents the mercury remaining outside of the cell containing the fourth circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- Compare the graphs as given:

| | |
|---|---|
| Plot of set 1 vs set 3 | Set 1 transported more mercury than Set 3. |
| Plot of set 2 vs set 4 | Set 2 transported significantly higher mercury than set 4. |

**Expected result:**

- Plot of set 1 vs set 3 shows that MerP contributes significantly in the presence of other transport system elements.
- Plot of set 2 vs set 4 shows the efficiency of MerP in transporting methylmercury, which should be higher than the natural transport (without mer operon transporters).

## iii) Experiment 3*:

**Aim:** To test the efficiency of and characterize MerT.
*This experiment is performed only if the final circuit design has MerT.

**Principle:**

*Circuit*:

To be tested:
Circuit 1: **Final transport system design**
Circuit 2: **Constitutive Promoter -** RBS **– MerT -** RBS **– Double Terminator**

Control:
Circuit 3: **Final circuit design without MerT**
Circuit 4: **Wild type *Escherichia coli* DH5alpha**

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

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

**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 control on LB agar overnight (1+1+1+1).
- Then transfer the same to LB broth using a sterile loop (5+5+5+5 (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM, and 20µM of $CH_3HgCl_2$ (respectively to the four different circuits).
- According to the results of the preliminary experiment, let it grow for the required time.
- Then transfer 1 mL of the mixtures into the respective set of microfuge tubes and centrifuge again at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set of microfuge tubes.
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |
| *Supernatant set 3/ conc** | Represents the mercury remaining outside of the cell containing the third circuit set. |
| *Supernatant set 4/ conc** | Represents the mercury remaining outside of the cell containing the fourth circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- Compare the graphs as given:

| | |
|---|---|
| Plot of set 1 vs set 3 | Set 1 transported more mercury than Set 3. |
| Plot of set 2 vs set 4 | Set 2 transported significantly higher mercury than set 4. |

**Expected result:**

- Plot of set 1 vs set 3 shows that MerT contributes significantly in the presence of other transport system elements.
- Plot of set 2 vs set 4 shows the efficiency of MerT in transporting methylmercury, which should be higher than the natural transport (without mer operon transporters).

## iv)Experiment 4*:

**Aim:** To test the efficiency of and characterize MerC.
*This experiment is performed only if the final circuit design has MerC.

**Principle:**

*Circuit:*

To be tested:
Circuit 1: **Final transport system design**
Circuit 2: **Constitutive Promoter -** RBS **– MerC -** RBS **– Double Terminator**

Control:
Circuit 3: **Final circuit design without MerC**
Circuit 4: **Wild type *Escherichia coli* DH5alpha**

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

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

**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 control on LB agar overnight (1+1+1+1).
- Then transfer the same to LB broth using a sterile loop (5+5+5+5 (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM and 20µM of $CH_3HgCl_2$ (respectively to the four different circuits).
- According to the results of preliminary experiment, let it grow for the required time.
- Then transfer 1 mL of the mixtures into the respective set of microfuge tubes and centrifuge again at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set microfuge tube.
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |
| *Supernatant set 3/ conc** | Represents the mercury remaining outside of the cell containing the third circuit set. |
| *Supernatant set 4/ conc** | Represents the mercury remaining outside of the cell containing the fourth circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- Compare the graphs as given:

| | |
|---|---|
| Plot of set 1 vs set 3 | Set 1 transported more mercury than Set 3. |
| Plot of set 2 vs set 4 | Set 2 transported significantly higher mercury than set 4. |

**Expected result:**

- Plot of set 1 vs set 3 shows that MerC contributes significantly in the presence of other transport system elements. It shows that MerC is the most efficient tool as it is sufficient for mercurial transport into cells.
- Plot of set 2 vs set 4 shows the efficiency of MerC in transporting methylmercury, which should be higher than the natural transport (without mer operon transporters).

## v) Experiment 5:

**Aim:** To test the efficiency of and characterize MerE.

**Principle:**

*Circuit:*

To be tested:
Circuit 1: **Final transport system design**
Circuit 2: **Constitutive Promoter -** RBS **– MerE –** RBS **– Double Terminator**

Control:
Circuit 3: **Final circuit design without MerE**
Circuit 4: **Wild type *Escherichia coli* DH5alpha**

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

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

**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 control on LB agar overnight (1+1+1+1).
- Then transfer the same to LB broth using a sterile loop. (5+5+5+5 (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM and 20µM of $CH_3HgCl_2$ (respectively to the four different circuits).
- According to the results of preliminary experiment, let it grow for the required time.
- Then transfer 1mL of the mixtures into the respective set of microfuge tubes and centrifuge again at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set of microfuge tubes.
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |
| *Supernatant set 3/ conc** | Represents the mercury remaining outside of the cell containing the third circuit set. |
| *Supernatant set 4/ conc** | Represents the mercury remaining outside of the cell containing the fourth circuit set. |

*Different concentrations used.

***Note:*** Steps 5-6 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- Compare the graphs as given:

| | |
|---|---|
| Plot of set 1 vs set 3 | Set 1 transported more mercury than Set 3. |
| Plot of set 2 vs set 4 | Set 2 transported significantly higher mercury than set 4. |

**Expected result:**

- Plot of set 1 vs set 3 shows that MerE contributes significantly in the presence of other transport system elements but less efficient than MerT in presence of other transport system elements.
- Plot of set 2 vs set 4 shows the efficiency of MerE in transporting methylmercury, which should be higher than the natural transport (without mer operon transporters).

# D. Characterization of MerA and MerB:

## i) Experiment 1:

**Aim:** To check for methylmercury resistance in the presence as well as the absence of MerA

**Principle:**

*Circuits:*

To be tested:
**1. Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerA –** RBS **– MerB –** RBS **– GFP - Double Terminator**

**2. Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerB –** RBS **– GFP - Double Terminator (Deletion of MerA)**

Control:
**3. Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– GFP - Double Terminator**

* The transport system is subject to change according to results of experiment 1 of characterization of transport system.

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

**Materials required:**
Sterile agar plates, Methylmercury chloride, E. coli with plasmid not containing MerA, E. coli with the control plasmid (no MerA and MerB), E. coli with both MerA and MerB, Incubator, Cork borer, Forceps, L-rod, Gloves and Gas mask.

**Procedure:**

*Preparation of MeHg standard solution (1mg/mL):*

Dissolve 116.3mg of $CH_3HgCl_2$ in 1-2mL of acetone and dilute to 100mL with 0.1M HCl. The stock and working standard solutions have to be kept in darkness to prevent decomposition of MeHg by UV light. This standard solution can be diluted to the required concentration using distilled water.

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

- Take 3 sterile LB agar plates and spread 70 microliters of fresh *Escherichia coli* DH5alpha cells containing plasmid circuit 2, using a sterile L-Rod.
- Punch one hole each in all three plates using a sterile cork borer. Carefully remove the agar piece and ensure that it does not come into contact with the rest of the agar.
- Inject 60-70 microliters of 30mg/mL methylmercury into one of the wells, 60-70 microliters of 300mg/mL methyl mercury into another well, and 30 microgram/mL into the last well.
- Incubate at 37°C for 24hrs. Extend to 48hrs if needed.

***Note:*** Also perform the above method for control plates and bacteria containing plasmid circuit 1.

**Expected observation:**

- Control plates will have the largest zone diameter as they do not contain MerA or MerB. Hence, methylmercury will not be converted to elemental mercury.
- Plates containing plasmid circuit 3 (deletion of MerA) will have the same result as the control plate. This is because even though MerB can break the carbon-mercury bond, the plasmid is unable to convert Hg (II) to the less toxic Hg (0).
- Plates containing plasmid 1 (presence of both MerA and MerB) will have a smaller zone diameter compared to the other three circuits, as methylmercury is successfully converted to elemental mercury.
- In all the 3 plates, the zone diameter reduces as the dilution of the methylmercury solution increases.

**Expected Result:**

- The introduction of MerB and MerA increases the Mer spectrum. Hence the addition of the two genes confers better resistance to methylmercury.

**Precautions:**

- Perform all steps in sterile conditions under a laminar flow chamber.

## ii) Experiment 2:

**Aim**: To check for methylmercury resistance in the presence as well as the absence of MerB.

**Principle**:

*Circuits:*

To be tested:
**1. Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*- RBS – MerA –** RBS **– MerB –** RBS **– GFP - Double Terminator**

**2. Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerA –** RBS **– GFP - Double Terminator (Deletion of MerB)**

Control:
**3. Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– GFP - Double Terminator**

* The transport system is subject to change according to results of experiment 1 of characterization of the transport system.

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

**Materials required:**
Sterile agar plates, Methylmercury chloride, E. coli with plasmid not containing MerB, E. coli with the control plasmid (no MerA and MerB), E. coli with both MerA and MerB, Incubator, Cork borer, Forceps, L-rod, Gloves and Gasmask.

**Procedure:**

*Preparation of MeHg standard solution (1mg/mL):*

Dissolve 116.3mg of $CH_3HgCl_2$ in 1-2mL of acetone and dilute to 100mL with 0.1M HCl. The stock and working standard solutions must be kept in darkness to prevent decomposition of MeHg by UV light. This standard solution can be diluted to the required concentration using distilled water.

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

- Take 3 sterile LB agar plates and spread 70 microliters of fresh *Escherichia coli* DH5alpha cells containing plasmid circuit 2, using a sterile L-Rod.
- Punch one hole each in all three plates using a sterile cork borer. Carefully remove the agar piece and ensure that it does not come into contact with the rest of the agar.
- Inject 60-70 microliters of 30mg/mL methylmercury into one of the wells, 60-70 microliters of 300mg/L methylmercury into another well, and 30 microgram/mL into the last well.
- Incubate at 37 C for 24hrs. Extend to 48hrs if needed.

***Note:*** Also perform the above method for control plates and bacteria containing plasmid circuit 1.

**Expected Observation:**

- Control plates will have the largest zone diameter as they do not contain MerA or MerB. Hence, methylmercury will not be converted to elemental mercury.
- Plates containing plasmid circuit 2 (deletion of MerB) will have the same result as the control plate because MerA cannot function without MerB. MerB has to break the carbon-mercury bond for MerA to convert Hg (II) to Hg (0).
- Plates containing plasmid 1 (presence of both MerA and MerB) will have a smaller zone diameter compared to the other three circuits, as methylmercury is successfully converted to elemental mercury.
- In all the plates, the zone diameter reduces as the dilution of the methylmercury solution increases.

**Expected Result:**

- The above experiment proves that MerA and MerB are necessary for efficient conversion of methylmercury to elemental mercury.

## iii) MTT assay to check for methylmercury resistances:

**Aim:** To check for methylmercury resistance in the presence as well as the absence of MerA and MerB

**Principle:**
The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium salt MTT into insoluble formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells.

The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540 and 720 nm. For drug sensitivity measurements, the OD values of wells with cells incubated with drugs are compared to the OD of wells with cells not exposed to drugs [19].

*Circuits:*

To be tested:
Circuit 1**: Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerA –** RBS **– MerB –** RBS **– GFP - Double Terminator**

Circuit 2**: Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerB –** RBS **– GFP - Double Terminator (Deletion of MerA)**

Circuit 3**: Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerA –** RBS **– GFP - Double Terminator (Deletion of MerB)**

Control:
Circuit 4: **Constitutive Promoter** - RBS - **MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– GFP - Double Terminator**

* The transport system is subject to change according to results of experiment 1 of characterization of transport system.

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

[19] Van Meerloo, Kaspers and Cloos, 2011

**Materials required:**
Incubator, Methylmercury solution, Centrifuge tubes, Centrifuge, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) stock solution, LB broth, Spectrophotometer, Shaker, Transformed *Escherichia coli* DH5α*,* Micropipette and tips, Test tubes, DMSO(Dimethyl sulphoxide).

**Procedure:**

- Inoculate bacteria (*Escherichia coli* DH5α transformed with BioBrick 1 in Luria-Bertani (LB) liquid + Kanamycin 34 µg/ml.
- Incubate in the shaker at 37°C overnight.
- Transfer 1mL of bacterial suspension to 50 mL of LB medium without antibiotics.
- Take four 1.5-mL centrifuge tubes (for one sample), the LB medium, and MTT stock solution.
- Determine the OD at 600nm using a spectrophotometer of the *Escherichia coli DH5α* culture. Collect the cells by centrifugation (10,000 g, 30 s) and resuspend these in the same volume of LB + methylmercury (2µM, 5µM, 10µM, 15µM and 20µM of $CH_3HgCl_2$) medium.
- Dilute the cell suspension with LB medium to an OD600 of 0.01 – 0.18.
- Transfer precisely 200 µL of the cell suspension into the centrifuge tubes at 37°C. Initiate the reduction reaction by adding 20 µL of the MTT stock solution and manually mixing the tube for a few seconds. (Four parallel tests are recommended by the study (per conc. of methylmercury)).
- Incubate the mixtures at 37°C for 20 min with the tube cap open.
- Collect the bacteria–formazan crystal complexes by centrifugation (10,000 g, 30 s) and remove the supernatant using a pipette. Try to keep the volume of residual liquor less than 20 µL to reduce its interference with formazan dissolution.
- Transfer the pellets of the cell–formazan crystal complexes into glass test tubes using 2500 µL DMSO. Manually mix the tubes to accelerate the dissolution process. Record the $A_{550}$ of the mixtures at 10–15 min using a plate reader.
- Using this protocol, the MTT reduction activity of *E. coli* cultures per OD600 and per millilitre can be calculated by the following equation:

  $$MRU = A_{550} \times (2500\mu L/220\mu L) \times (1000\mu L/200\mu L) \times K/OD600$$

  Thus, $MRU=56.8 \times A_{550} \times K/OD600$, where,
  - K is the dilution rate of E. coli samples with LBG medium to maintain the cell densities at OD600 0.01–0.18.
  - 200 µL is the volume of the E. coli cell dilution.
  - 220 µL is the total volume of the mixture of 200 µL cell dilution and 20 µL MTT stock.

- Plot a graph of MTT reduction vs $A_{550}$, Cell density vs $A_{550}$, Methylmercury chloride conc. vs $A_{550}$ and Time vs $A_{550}$.

**Expected observation:**

- We can quantitatively map the resistance provided by each gene using the graphs.
- The resistance provided should be in the order Control<Circuit 3< Circuit 2< Circuit 1.

**Expected result:**

- The introduction of MerB and MerA increases the Mer spectrum. Hence the addition of the two genes confers better resistance to methylmercury.

**Precautions:**

- Wear protective laboratory clothing and equipment (gloves, laboratory coat, and safety glasses) be worn when handling kit reagents.

Gibson assembly: Protocol  Transformation: protocol  LB broth: protocol
Protocol source: MTT assay  Final circuit design experiment: protocol
To know more about determination of concentration refer here

# E. Estimation of MerA and MerB activity

**Aim:** To purify the MerA and MerB proteins, measure its activity and find the relation between number of cells and enzyme concentration.

**Principle:**
A his-tag, or polyhistidine tag, is a string of histidine residues at either the N or C terminus of a recombinant protein. There can be from four to ten residues in a string, although commonly there are six histidine residues — a hexahistidine tag. Some recombinant proteins are engineered to have two hexahistidine tags.

His-tag purification uses the purification technique of immobilized metal affinity chromatography, or IMAC. In this technique, transition metal ions are immobilized on a resin matrix using a chelating agent such as iminodiacetic acid. The most common ion for his-tag purification of a recombinant protein is $Ni^{2+}$, though $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ are also used. The his-tag has a high affinity for these metal ions and binds strongly to the IMAC column. Most other proteins in the lysate will not bind to the resin, or bind only weakly. The use of a his-tag and IMAC can often provide relatively pure recombinant protein directly from a crude lysate.

***Circuit:***

**Constitutive Promoter –** RBS **– MerA-His-tag - Double Terminator**

**Constitutive Promoter –** RBS **– MerB-His-tag - Double Terminator**

**Constitutive Promoter –** RBS **– MerA-His-tag –** RBS **- MerB-His-tag - Double Terminator**

**Materials Required:**
Transformed cells, nutrient broth, centrifuge,
Protein samples, Charge Buffer (80ml), Wash buffer (300ml), $H_2O$ tubes, TCEP,
pipettes, Elution Buffer (150mL), Protein samples, Filter for on top of the resin Eppendorf tubes, Strip Buffer (150 mL), Columns Ni-NTA HisBand resin.

***For harvesting proteins:***
Centrifuge , Centrifuge tube, Centrifuge bottles , Lids , Balance , Bugbuster, Benzonase Nuclease (enzyme 25U/µl) , Agarose gel electrophoresis system , DNA ladder , Pipettes and tips , Prepared samples , Solidified agarose gel.

***For Column loading:***
Charge buffer 100 mM $NiSO_4$, T14-3-3cΔC wash buffer B, T14-3-3cΔC wash buffer A , T14-3-3cΔC elution buffer , Assay buffer, strip buffer

***For buffer exchange:***

Amicon Ultra tube with filter, Filter sizes: 3 kDa, 10 kDa, 30 kDa, 50 kDa, or 100 kDa , Volumes: 0.5ml,2ml,4ml,or15ml , Centrifuge, Protein Buffer (values depend on the protein and the purpose) ,Salt: 150mM NaCl ,Ph:7.5 , Tris: 25mM, Pipette ,Pipette tips.

**Procedure:**

***Note:*** Perform the procedure for each circuit.

- Inoculate LB broth with transformed *E coli* DH5alpha (of each circuit) and incubate at 37°C overnight (For the bacteria containing the third circuit perform a dilution series).
- Estimate the amount of bacteria present using a haemocytometer in the dilution containing the 3rd circuit.

***Purification of proteins using His-tag***

Estimated bench time: 2 hours

Estimated total time: 3 hours, depending on the amount of samples

**Purpose:** To wash, elute and regenerate the column

***For harvesting proteins***

- Precool the centrifuge, 5 minutes at 2500 rpm.
- Weight the bottles when they are empty, so that you can later on determine the weight of the cell pellet. 3.
- Harvest the cells by centrifuging at 4°C, 8000 rpm for 10 minutes.
- Keep the cell pellet and discard the supernatant.

***Note:*** Optional, snap freeze the cell pellet and store in -80°C and continue later. Weight the bottles and calculate the weight of the cell pellet.

- Use 5 mL of bugbuster per gram cell pellet
- Add 1 µl Benzonase Nuclease per 1 ml BugBuster
- Centrifuge the cell lysate at 4°C, 20000 rpm for 20 minutes.
- Store the protein solution on ice.

***For column loading***

- Load a column with Ni-NTA HisBind resin (5-10 mg protein per mL resin). For example load 6 mL of resin for a 3 mL column.
- Add a filter on top of the resin. Make sure that no air gets trapped in the resin or between filter and resin.
- Equilibrate the column with 6 column volumes H2O (~18 mL).
- Add 3 column volumes of Charge Buffer (~9 mL). Collect the waste flow-through separately as this is heavy metal waste (has to be thrown away in specific jerry can in fume hood)
- Add 3 column volumes of Wash Buffer B (~9 mL) to remove unbound nickel. Collect the waste flow-through again separately (has to be discarded in halogen rich waste).
- Load the protein samples (supernatant from Bugbuster cell lysis) onto the column. Collect a drop of the flow-through for SDS-PAGE analysis.

- Add 3 column volumes of wash buffer A and 3 column volumes of wash buffer B (~18 ml total). Collect a drop of the flow-through for SDS-PAGE analysis.
- Elute the protein by adding 3 column volumes of elution buffer (~9 mL).
- Collect the coloured elution sample in 0.5-1 mL fractions in Eppendorf tubes. Store a drop of the flow-through for SDS-PAGE analysis
- Keep the protein elution fractions on ice in a closed bucket
- Strip the Ni2+ off the column using 2 column volumes of strip buffer (~12 mL). Once more it is necessary to use a separate waste disposal container.
- Wash with 5 column volumes of H2O
- To preserve the column it is necessary to add 2 column volumes (~6 mL) of 20% ethanol.
- Store the column in the fridge at 4 °C

**For Buffer Exchange:**
Estimated bench time: 1 hour
Estimated total time: 1 hour
Purpose: changing the buffer (wash) of the protein to the desired buffer

- Load the protein sample on the filter.
- Place the filter tube in the centrifuge (minispin or table centrifuge, depending upon the filter tube volume), balance well.
- Set the spin-time and rpm. A good first spin time is 4-5 minutes. Afterwards, you can test how much of the buffer went through the filter and how much longer you want to continue. It is also good to mix the protein solution, as it can form a layer at the surface of the filters. The necessary rpm depends on the tube, for 15 ml an rpm of 4000 is sufficient, while for the tube of 0.5 mL rpm of 13500 is better.
- Repeat the loading of the protein buffer till the original protein sample buffer is sufficient diluted. A dilution of at least 30x is recommended.
- After the buffer exchange, measure the protein concentration with nanodrop and perform a QTOF to see if the protein had the right size.

***To estimate the activity***

- Prepare the required concentration of MerB and MerA (2.225mg/mL of MerB and 49.45mg/mL of MerA).
- Mix 5 mL of the two into different test-tube containing 2.9µM (7.278* 10^-7 grams of $CH_3HgCl$ to be added per ml) and 3.8µM (9.537 * 10^-7 g of $CH_3HgCl$ to be added per ml) of methylmercury (chloride) (for each concentration of enzyme) in 5mL water and incubate in a shaker incubator at 37°C.
- At 60 min intervals, pipette 1mL of the mixture and check the concentration of mercury using gas chromatography.
- Plot mercury concentration vs time graph and measure the activity keeping enzyme concentration constant.
- Plot enzyme concentration vs activity graph.
- Plot no of cells vs enzyme concentration graph for the third circuit.

**Observation:**

- Graph of mercury concentration vs time is obtained for constant conc. of enzyme.
- Graph of enzyme concentration vs activity is obtained.
- Graph of no of cells vs enzyme concentration graph is obtained.

**Result:**

- Using the graphs we can estimate the optimal amount and enzyme required to be produced for optimal activity.
- We can estimate the optimum CFU/mL to be used to get maximum activity.

**Dry lab results:**

According to the results obtained in the MATLAB model, the optimal concentration of enzymes should be in the order $10^{-4}$ (Refer to iGEM MIT_MAHE Model handbook for more information)

# F. Working of Composite BioBrick 1

**Aim**: To test the efficiency of overall working of composite BioBrick 1.

**Principle**:

*Circuits:*

To be tested:
**Constitutive Promoter –** RBS **– MerR - PmerT promoter - (**RBS **– MerT –** RBS **– MerP –** RBS **– MerE –** RBS **- MerC)*-** RBS **– MerA –** RBS **– MerB –** RBS **– GFP - Double Terminator**

Control:
**Wild type *Escherichia coli* DH5alpha**

****Note:*** The transport system is subject to change according to results of experiment 1 of characterization of the transport system.

***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.

The working of each part is given in detail above.

The circuit assembled using Gibson Assembly.

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

**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 control on LB agar overnight (1+1+ (duplicates if needed)).
- Then transfer the same to LB broth using a sterile loop. (5 + 5 + (duplicates if necessary) (of each circuit)) and incubate for 12 hours.
- Transfer into micro-centrifuge tubes and centrifuge the cells at 4000 rpm for 10 mins to avoid lysis of the cell, discard the supernatant and resuspend in metal medium containing LB broth and 2µM, 5µM, 10µM, 15µM and 20µM of $CH_3HgCl_2$ (respectively to the four different circuits).
- According to the results of preliminary experiment, let it grow for the required time.

- Then transfer 1 mL of the mixtures into the respective set of microfuge tubes and centrifuge again at 4000 rpm for 10 minutes to avoid cell lysis.
- Collect the supernatant in another set of microfuge tubes and centrifuge again at 13000 rpm for 10 minutes to remove the remaining cells. Collect the cells.
- Sonicate the samples.
- Measure the amount of mercury in each of the following using gas chromatography:

| | |
|---|---|
| *Supernatant set 1.1/ conc** | Represents the mercury present inside the cell containing the first circuit set. |
| *Supernatant set 1.2/ conc** | Represents the mercury remaining outside of the cell containing the first circuit set. |
| *Supernatant set 2.1/ conc** | Represents the mercury present inside the cell containing the second circuit set. |
| *Supernatant set 2.2/ conc** | Represents the mercury remaining outside of the cell containing the second circuit set. |

*Different concentrations used.

***Note:*** Steps 5-9 to be repeated 3-4 times hourly.

- Plot a graph of concentration vs time for each set and analyse.

**Expected observation:**

- Compare the graphs as given:

| | |
|---|---|
| Plot of set 1.1 vs set 2.1 | Set 1.1 has lower mercury concentration with time as compared to the control as it has the MerA and MerB mechanisms. |
| Plot of set 1.2 vs set 2.2 | Set 1.2 has lower concentration of mercury with time compared to the control as it has the transport system. |

- Since we know the initial concentration of mercury used, we will be able to determine the amount that has been converted to Hg(0).

**Expected result:**

- Plot of set 1.1 vs set 2.1 shows that the Hg(II) inside the cell having the BioBrick has been converted to Hg(0) and helps calculate its efficiency.
- Plot 1.2 vs set 2.2 shows that the mercury has been transported inside the cell at a much faster rate and helps calculate its efficiency.
- The Composite BioBrick 1 is thus very efficient in converting methylmercury to elemental mercury as compared to natural systems.

# 5. Glossary

| Term | Definition | Ref. |
|---|---|---|
| **2x assay buffer** | The 2X Reaction Buffer is optimized for caspase activity assay reactions | 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 |
| **Alkyl-mercury lyase** | Alkylmercury *lyase*, catalyzes the break of binding between organic radicals and *mercury*, releasing *Hg*(II). | Link |
| **Amplification** | PCR amplification is the selective amplification of DNA or RNA targets using the polymerase chain reaction | Link |
| **Anaerobic bacteria** | Anaerobic bacteria are bacteria that do not live or grow when oxygen is present. In humans, these bacteria are most commonly found in the gastrointestinal tract. | Link |
| **Annealing** | When the temperature is lowered to enable the DNA primers to attach to the template DNA. | 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 | Link |
| **Aseptic** | Free from contamination caused by harmful bacteria, viruses, or other microorganisms; surgically sterile or sterilized. | Link |
| **Atomic-absorption** | Atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES) is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. | Link |
| **Autoclave** | An autoclave is a machine that uses steam under pressure to kill harmful bacteria, viruses, fungi, and spores on items that are placed inside a pressure vessel | Link |
| **BioBrick** | BioBrick parts are DNA sequences that follow a specific restriction-enzyme assembly standard. | Link |

| | | |
|---|---|---|
| **Bromophenol Blue** | *Bromophenol Blue* is a pH indicator, and a dye appearing as a strong *blue* color. It is often used as a tracking dye during agarose or polyacrylamide gel electrophoresis. | 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 |
| **Kanamycin** | *Kanamycin* is a semisynthetic, broad-spectrum *antibiotic* derived from Streptomyces venequelae with primarily bacteriostatic activity. | Link |
| **Competent cells** | *Cell competence* refers to a *cell's* ability to take up foreign (extracellular) DNA from its surrounding environment. | 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 |
| **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, | Link |
| **dNTP mix** | The dNTPs are the artificial nucleotides used in the PCR to synthesize new DNA strands much like DNA replication | Link |
| **EDTA** | EDTA is a chemical that binds and holds on to (chelates) minerals and metals such as chromium, iron, lead, mercury, copper, aluminum, nickel, zinc, calcium, cobalt, manganese, and magnesium. | Link |
| **Enzyme** | Enzymes are biological molecules (typically proteins) that significantly speed up the rate of virtually all of the chemical reactions that take place within cells. | Link |
| ***Escherichia coli* DH5alpha** | DH5-Alpha Cells are *E.coli* cells engineered to maximize transformation efficiency | Link |

| | | |
|---|---|---|
| **Fluorescence emission spectrum** | A fluorescence emission spectrum is when the excitation wavelength is fixed and the emission wavelength is scanned to get a plot of intensity vs. emission wavelength | Link |
| **Formazan dissolution** | Formazan dyes are artificial chromogenic products of the reduction of tetrazolium salts by dehydrogenases and reductases | Link |
| **Gas chromatography** | Gas chromatography (GC) is an analytical technique used to separate the chemical components of a sample mixture and then detect them to determine their presence or absence and/or how much is present. | Link |
| **Gel Loading Buffer** | Loading dye is mixed with DNA samples for use in agarose gel electrophoresis. It generally contains a dye to assess how "fast" your gel is running and a reagent to render your samples denser than the running buffer | Link |
| **Genetic burden** | The genetic debt due to harmful mutation but as yet undischarged. | 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 |
| **Homodimer** | A protein composed of two polypeptide chains that are identical in the order, number, and kind of their amino acid residues | Link |
| **Hydrophobicity** | Physical property of a molecule that is seemingly repelled from a mass of water | Link |
| **IL-10** | Interleukin 10 (***IL-10***), also known as human cytokine synthesis inhibitory factor ( CSIF), is an anti-inflammatory cytokine | Link |
| **Inflammation** | When inflammation happens, chemicals from your body's white blood cells enter your blood or tissues to protect your body from invaders | Link |
| **Laminar flow chamber** | 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 | Link |
| **LB Agar** | *LB agar* is LB broth that contains agar and is typically prepared in petri dishes. | Link |
| **LB broth** | *LB Broth*, also known as, *LB medium*, Lysogeny *broth*, *Luria broth*, or *Luria*-Bertani *medium*, is a commonly used nutritionally rich *medium* for culturing bacteria. | Link |

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|---|---|---|
| **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 |
| **Lipopolysacchari--de** | 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 |
| **Methylmercury** | Methylmercury (sometimes methyl mercury) is an organometallic cation with the formula $[CH_3Hg]^+$. Its derivatives are the major source of organic mercury for humans. It is a bio-accumulative environmental toxicant. | Link |
| **Microbiota** | Microbiota are "ecological communities of commensal, symbiotic and pathogenic microorganisms" found in and on all multicellular organisms studied to date from plants to animals. Microbiota includes bacteria, archaea, protists, fungi and viruses. | 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. Cloning refers to making numerous copies of a DNA fragment of interest, such as a gene | Link |
| **MTT Assay** | The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. | Link |
| **NanoDrop spectrophotomet-er** | The NanoDrop Spectrophotometer from NanoDrop Technologies is designed for measuring nucleic acid concentrations in sample volumes of one microliter. A single measurement cycle takes only 10 sec | Link |
| **OD** | Optical density, sometimes written as OD, is a measurement of a refractive medium or optical component's ability to slow or delay the transmission of light. It measures the speed of light through a substance, affected primarily by the wavelength of a given light wave. | Link |
| **pH** | pH is a measure of how acidic/basic water is. The range goes from 0 to 14, with 7 being neutral. pHs of less than 7 indicate acidity, whereas a pH of greater than 7 indicates a base. | Link |

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|---|---|---|
| **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 Chain Reaction** | Polymerase chain reaction (PCR) 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 |
| **Primer** | A primer is a short, single-stranded DNA sequence used in the polymerase chain reaction (PCR) technique | Link |
| **Promoter** | A promoter is a sequence of DNA needed to turn a gene on or off. The process of transcription is initiated at the promoter | Link |
| **Restriction digestion** | Restriction Digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction Endonucleases (sometimes just called Restriction Enzymes or RE's) | Link |
| **Repressor** | In molecular genetics, a repressor is a DNA- or RNA-binding protein that inhibits the expression of one or more genes by binding to the operator or associated silencers | Link |
| **Restriction endonucleases** | Restriction enzyme, also called restriction endonuclease, is a protein produced by bacteria that cleaves DNA at specific sites along the molecule | Link |
| **Ribosomal binding site** | This site, located at the 5' untranslated region (UTR) of the mRNA transcript, regulates protein synthesis based on its sequence and structure. | Link |
| **Serva DNA stain** | *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 | Link |
| **Standard solution** | *Standard solution* is a *solution* containing a precisely known concentration of an element or a substance | 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 (inoculum) from a culture of microorganisms, e.g. for streaking on a culture plate. | Link |

| | | |
|---|---|---|
| **Stock solution** | Stock solution is a concentrated solution that will be diluted to some lower concentration for actual use. | Link |
| **Supernatant** | Denoting the liquid lying above a solid residue after crystallization, precipitation, centrifugation, or other process. | Link |
| **Thermocycling** | *Thermocycling* of selected samples is done to artificially age some specimens by creating a temperature difference. | Link |
| **Template DNA** | 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 |
| **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 |
| **Tris-buffer saline** | Tris, or tris(hydroxymethyl) aminomethane, is a common biological buffer, used throughout the DNA extraction process | Link |
| **Tris-HCl** | Tris HCl is an organic compound often used in buffer solutions such as TAE or TBE for electrophoresis gels | Link |
| **Viable cells** | A viable cell count allows one to identify the number of actively growing/dividing cells in a sample. | Link |
| **Vortex** | A vortex mixer, or vortexer, is a simple device used commonly in laboratories to mix small vials of liquid. | Link |
| **Wild type** | Wild type (WT) refers to the phenotype of the typical form of a species as it occurs in nature | Link |

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