An Introductory Laboratory Course in Synthetic Biology

Autumn 2013

Table of Content

1. Introduction to Synthetic Biology, iGEM and Colisweeper

Synthetic biology uses engineering principles to create functional systems based on the molecular machines and regulatory circuits from living organisms. However, it also includes going beyond these natural circuits and develop new systems by adding synthetically engineered parts. This innovative nature of synthetic biology stems from the fact that the field represents a merger of molecular biology, genetic engineering and computer science. By following the engineering approach that has led to excellent results in other disciplines such as electrical engineering or computer sciences, synthetic biology requires decoupling of system design from system fabrication as well as standardization of components and conditions. Thus, synthetic biology aims at recruiting the knowledge base of engineering for biology and might thus enhance the speed and scope of the ongoing biological revolution.

The **International Genetically Engineered Machine (iGEM)** Foundation, addressing challenges mentioned above, fosters scientific research and education through organizing and operating the iGEM competition, the premier student synthetic biology competition. By these means, the organization promotes engagement of student researchers into synthetic biology projects, on the basis of an open sharing of DNA constructs. A visible result of the iGEM community efforts to introduce the engineering principles of abstraction and standarization into synthetic biology is the Registry of Standard Biological Parts (http://partsregistry.org/Main_Page). This repository is a collection of well-characterized biological components to be used as "parts" or "devices" in the design of novel systems. For the synthetic biology community in general, and particularly for the iGEM summer competition, it serves as a central reference point for the availability of parts.

Colisweeper is the project of the ETH Zurich iGEM team 2013. Colisweeper is an interactive, biological version of the computer game Minesweeper. The goal is to clear an agar "minefield" without detonating the mines. Genetically engineered Escherichia coli colonies are used as mines and non-mines. Mines secrete the signaling molecule AHL whereas non-mines process the signal. To distinguish different AHL-levels, a library of P_{LuxR} promoters with various AHL sensitivities was created through site-saturation mutagenesis. High-pass filters were constructed to control the expression of different orthogonal hydrolases in non-mines, depending on the number of surrounding mines.

2. Time Schedule for a one-day lab course

Time	Activity
08.00	Welcome & introduction
08.30	Lecture section “Biological Lab & Safety”
09.00	Colony plating
09.30	Plasmid test digestion
10.00	Lecture section “Synthetic Biology”
10.30	
11.30	Agarose gel for plasmid analysis
12.00	Lab cleaning
12.30	Lunch break
13.30	Analysis of gel
14.30	Lecture section “Mathematical Modeling”
16.00	Colisweeper tournament
until 17.30	Discussion & lab cleaning

3. The concepts behind Colisweeper

3.1. Quorum Sensing

Our Colisweeper system is based on a sender-receiver module. We use the well-known quorum sensing mechanism of the lux signaling system in our bio-game (Figure 1). In *Vibrio fischeri*, the *luxI* gene encodes for the enzyme that catalyses the production of the signaling molecule N-acyl-homoserine lactone, AHL. The AHL molecule is able to diffuse in and out of cells. Meanwhile, the LuxR protein is produced, driven by promoter P_{LuxL}. When unbound, this protein is in the inactive state. The binding of AHL to LuxR protein converts inactive LuxR to an active LuxR-AHL complex. The complex binds to the P_{LuxR} promoter and drives the gene expression upstream of the operon that leads to more production of AHL. Thereby, the available LuxR is bound with AHL which downregulates the P_{LuxL} promoter.

In our system the sender cells (mines) express LuxI protein, that catalyzes the production of the signalling molecule AHL (Figure 2). This molecule diffuses through the agar plate and reaches the receiver cells called non-mine colonies.

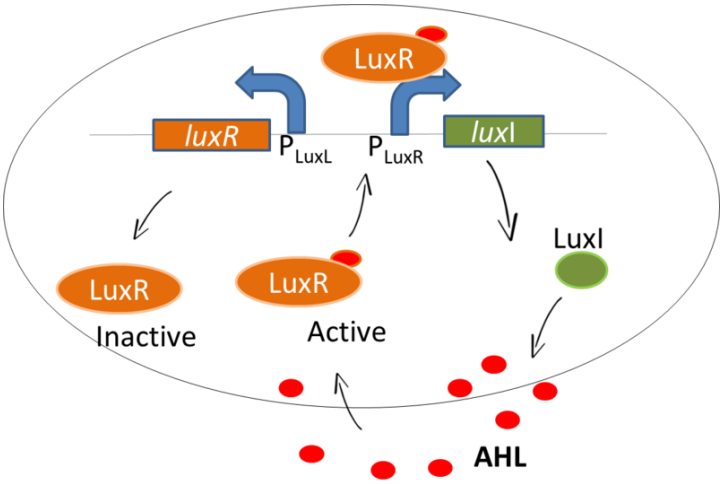

Figure 1 **The Vibrio fischeri quorum sensing system.**

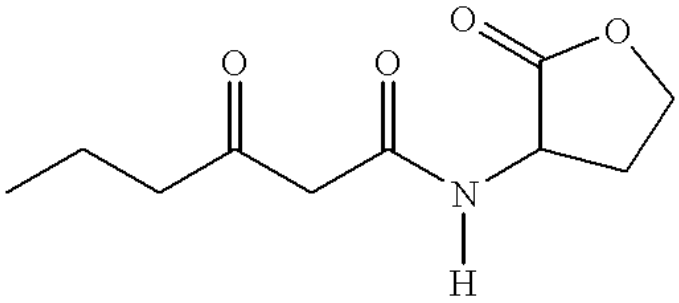

Figure 2 **The signalling molecule N-3-oxo-hexanoyl-L-homoserine lactone.**

3.2. Information Processing

The receiver (non mine) colonies in Colisweeper are designed to distinguish between different concentrations of AHL and translate this analog information into the expression of different sets of reporter enzymes. The continuous signal is digitized through a set of mutated P_{LuxR} promoters with different AHL sensitivities acting as highpass filters.

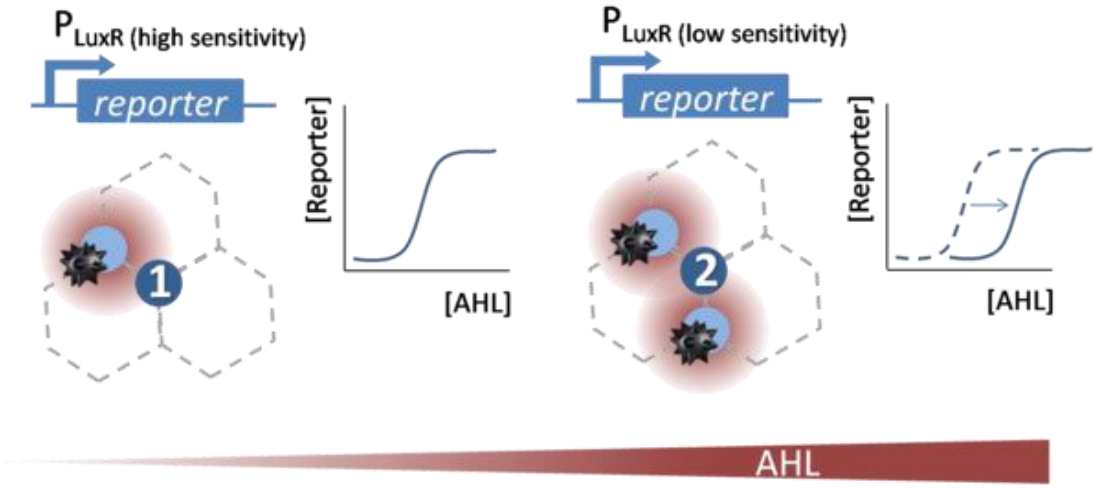

Figure 3 Theoretical sensivity shift of the PLuxR promoter to sense and distinguish different AHL concentrations.

The P_{LuxR} promoters have different sensitivities to AHL concentrations because of mutations within the activator binding site. In Figure 4 it can be seen that the LuxR/AHL activator complex interacts with the DNA at two sites. Inserting random mutations there leads to a shift in sensitivity like depicted in Figure 3.

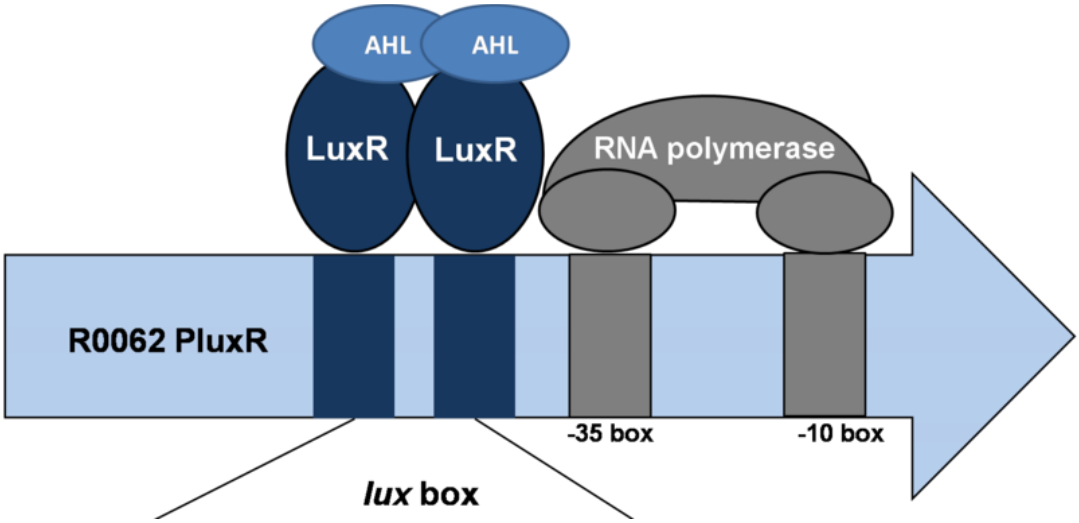

Figure 4 The AHL/LuxR inducible P_{LuxR} promoter.

3.3. Hydrolase Enzyme Reporters

Colisweeper depends on the processing of molecular signals and generation of visible and distinguishable color outputs. By using the wild type P_{LuxR} promoter and mutant promoters with varying AHL/LuxR affinities, gene expression can be induced for different concentrations of AHL. We coupled this system with reporter enzymes, called hydrolases, which catalyze hydrolysis of their colorless substrate into a colored product. Therefore, the player triggers the colored response upon addition of a substrate.

The set of hydrolases we use include the *Citrobacter* **Alkaline phospohatase**, the *Bacillus subtilis* **β-Glucuronidase** and the *Escherichia coli* **Acetyl esterase**, **β-N-Acetylglucosaminidase** and **β-Galactosidase**. These enzymes have features that make them attractive as reporters. They are known to be relatively stable, exhibit activity under different conditions (e.g. pH and temperature), and catalyze the cleavage of various colorimetric and fluorescent substrates, which is ideal for visual screening. These hydrolases are all native to Escherichia coli and orthogonal to each other, which means that each substrate can only be processed by the corresponding specific enzyme. In order to prevent background expression of these native enzymes, Colisweeper uses a triple knockout strain that has three hydrolase genes knocked out: uidA (β-glucuronidase), aes (acetyl esterase) and nagZ (β-N-acetylglucosaminidase).

Addition of a multi-substrate mix by the player leads to an enzyme-substrate reaction which specifically cleaves the chromogenic substrates, thereby producing a visible color output. Chromogenic substrates incorporate a chromophore whose absorbance properties change after the enzyme reaction, and the color signal produced then is directly related to the enzyme-catalyzed reaction.

Figure 5 Colorimetric output. This image shows liquid cultures of our mutant Escherichia coli strain expressing the enzymes PhoA, NagZ, LacZ, Aes or GusA to convert specific chromogenic substrates into colored outputs.

Indican is a chromogenic glycoside hydrolase substrate which belongs to a family of natural glycosides found in plants. Cleavage of the glycosidic bond forms an unstable hydroxyindole intermediate, which dimerizes by oxidation to form indigo as a blue precipitate (Figure 6). Numerous enzyme substrates have been designed following this natural product example, giving rise to many colored phenols that are used to detect enzyme activities.

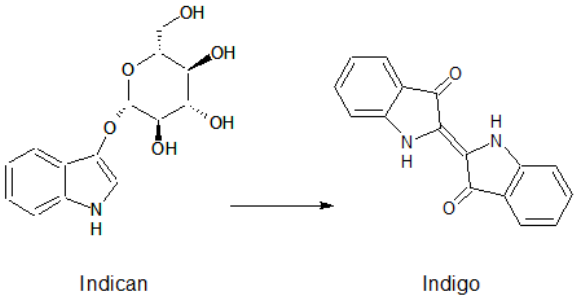

Figure 6 Colorimetric conversion of Indican to Indigo.

4. Protocols

4.1. Transformation of the Mines and Non Mines

Transformation is used to introduce plasmid DNA into bacterial cells. For Colisweeper we need to insert different plasmids into the mine and non mine cells.

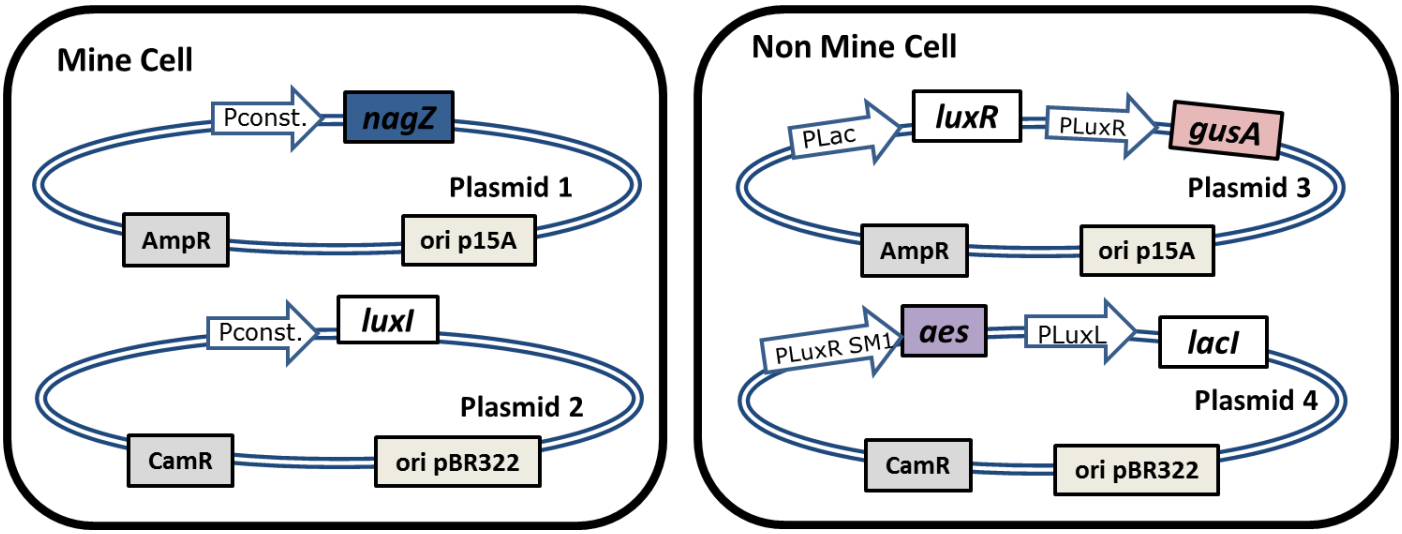

Figure 7 Mine and non mine cells containing their respective plasmids.

1. Thaw chemically competent "Colisweeper" cells on ice for 10 minutes
2. Mix 1 µl of resuspended plasmid for 50 µl competent cell suspension
3. Mine cells need plasmids 1&2, non mine cells need plasmids 3&4 (Figure 7)
4. Incubate 30 min on ice
5. 1 min heat shock at 42°C
6. Incubate 3 minutes on ice
7. Add 900 µl LB
8. 1h shaking at 37°C
9. Centrifuge at 12000 rcf
10. Remove 750 µl of the supernatant
11. Resuspend the pellet in the remaining 200 µL LB
12. Plate on pre-warmed plates containing Ampicillin (100 µg/ml) and Chloramphenicol (36 µg/ml) antibiotics

4.2. Colisweeper Grid Plating

To plate the Colisweeper plate you need mine and non mine liquid cultures in exponential growth phase, more specifically at OD_{600} = 0.5. The petri dish containing LB Agar with Ampicillin and Chloramphenicol antibiotics is placed onto the picture of the plate with the grid. For each “gamefield” 1.5 µl of liquid culture are placed on a dot. You can choose yourself where to plate mines and where to plate non mines. To ensure equal information processing the non mines are plated first. After 11 h of incubation at 37°C be sure exchange your plates with the ones from another group, otherwise you would be able to cheat during the Colisweeper tournament ☺.

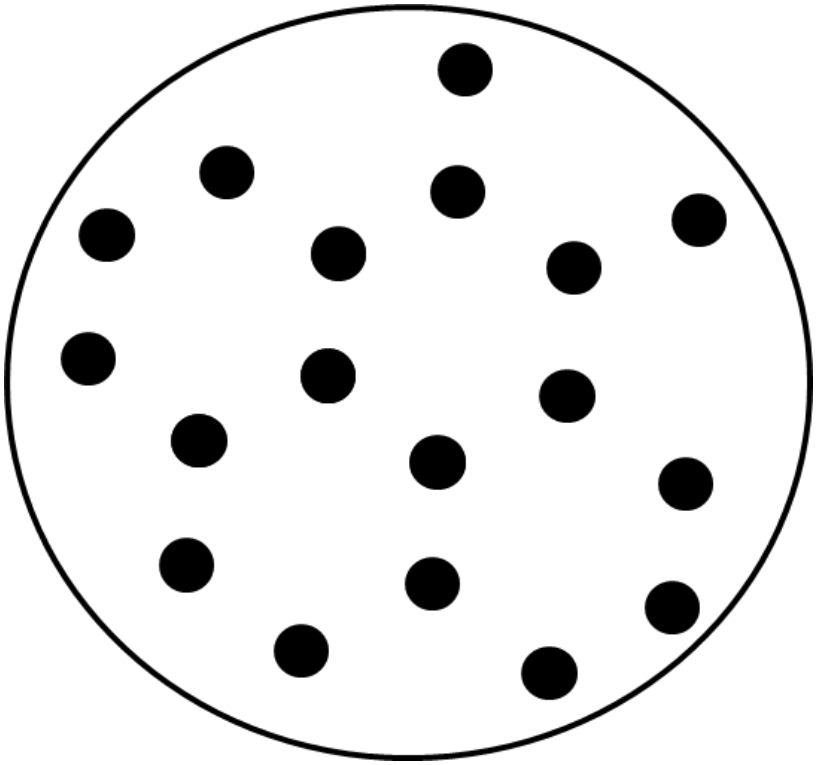

Figure 8 Colisweeper grid to help plating the Colisweeper plate.

4.3. Plasmid Test Digest/ Plasmid Mapping

To get an idea of how the four Colisweeper plasmids look you will set up a restriction digest with the restriction enzymes XbaI and PstI. The DNA will be analyzed later by agarose gel electrophoresis to determine the fragment sizes.

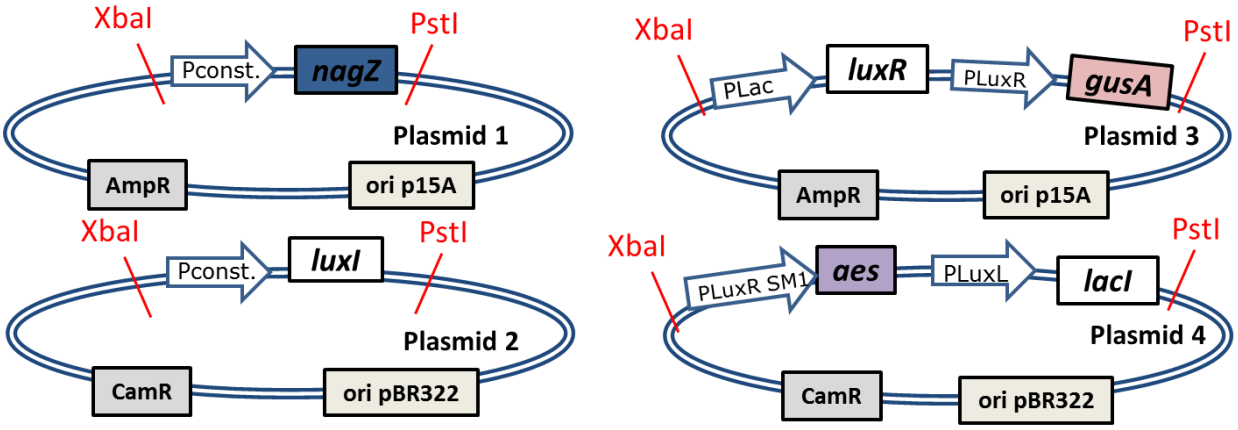

Figure 9 Plasmid maps. Your task ist o find out the size of each fragment.

1. Use 2 ug of plasmid DNA (max 45 µl for low concentration minipreps)
2. Add 5µl NEB CutSmart buffer
3. Fill up with H_2O to 50 µl
4. Add 0.5 µl of each restriction enzyme XbaI, PstI
5. Keep enzymes always on cooling block and don't take them out for too long.
6. Mix through pipetting
7. Incubate 1h at 37°C
8. Add 10 µl of loading buffer (6X)
9. Analysis on agarose gel

4.4. Agarose Gel Electrophoresis

1. Dissolve 1 g of agarose in 100 ml TAE buffer by heating the mixture in the microwave
2. Add 10 µl of GelRed staining solution
3. Pour the gel and let it solidify for 40 min
4. Add 5 µl of DNA loading dye to the restriction enzyme reaction
5. Load 5 µl of the 1 kb DNA ladder and the prepared DNA samples onto the agarose gel
6. Run the gel at 100 V for 1 h
7. Visualize the DNA under UV illumination and analyze the fragment sizes

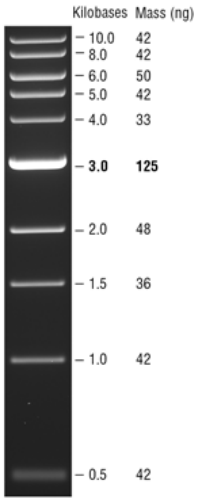

Figure 10 NEB 1 kb DNA ladder (https://www.neb.com/products/n3232-1-kb-dna-ladder)

4.5. Play Colisweeper

Playing Colisweeper is easy. All you need is a prepared Colisweeper plate (incubated for ~11h), playing substrate and flagging substrate. Pipette 2 µl of the playing substrate onto a colony of choice and wait for the color reaction to happen. As soon as you see the color you know about the identity of the colony and can plan the next move. If you are sure that a colony is a mine you can flag it with 2 µl of the flagging substrate. The first to finish a plate wins the tournament!

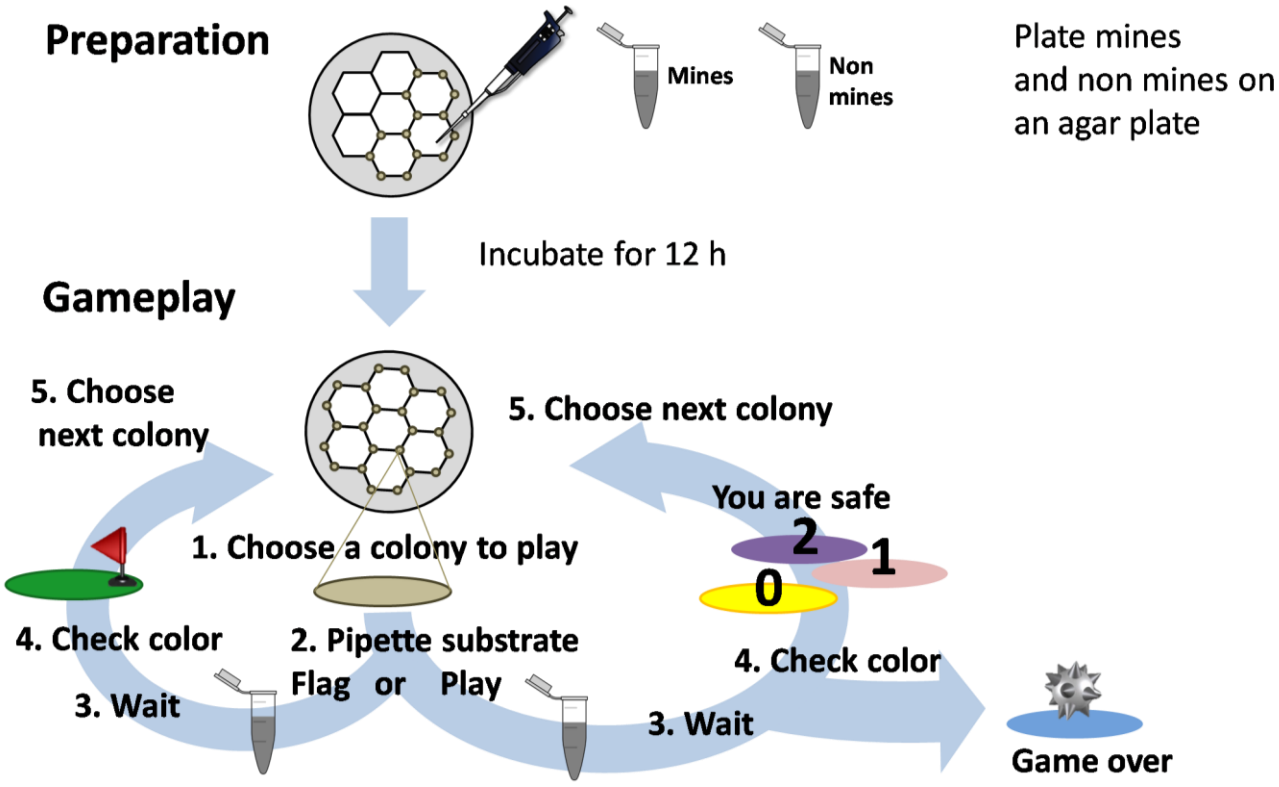

Figure 11 How to play Colisweeper?