

Blue light system

The composite blue light repressible promoter was constructed using Restriction free cloning techniques and the inserted into pB1A2 backbone using a 2-A assembly. This promoter was built by stitching together 5 parts- A constitutive promoter, the YF1-FixJ construct, Double terminator, pFixK2 promoter and mrfp gene- in a specific order. For more information, please look at our **results**.

For the characterization of this promoter, cultures were grown in LB under 3 different conditions- Dark(No light), white light and blue light. The growth in blue light was carried out using a “blue light box” developed by Sayantan. Please see **hardware**.

The assay was conducted in DH5alpha cells. 2 incubators were used:- one for dark condition and the other for white light and blue light condition. It involved:

- Inoculating the positive colony (as confirmed from a colony PCR) into 5 mL LB broth. 3 inoculations were made from the same colony- one for all 3 light conditions.

A dark environment was created by wrapping the falcons with aluminium foil and taping it to ensure it doesn't have any opening for light to pass through.

- We took cells that constitutively express rfp, which was the cells containing the plasmid of constitutive promoter as our positive control and made 3 inoculations of them. The cultures were allowed to grow for 12 hours straight in the given conditions.
- After 12 hours, 1 mL of the culture was pelleted and resuspended in 1x PBS.
- Cells were pelleted and resuspended in 1x PBS.
- Now all the 3 for a given condition (negative control, test and positive control) were OD normalized.

This resuspensions and OD normalization had to be done quickly as we could not maintain the 3 conditions outside the incubator while resuspension and OD normalizations.

- The resuspensions after OD normalization were loaded into a 96 well plate and fluorescence was measured for rfp.

For characterization results, please have a look at our **results**.

Feedback loop system

The feedback loop system consists of the pLambda promoter, rfp gene and CI repressor in that order. To characterize this negative feedback loop, the major player of which being the CI repressor. RecA is a recombinase encoding gene in the bacterial genome that degrades the CI repressor [1,2,3]. So, upon inducing recA using Hydroxy-urea, more degradation of CI repressor would cause more rfp expression. To characterize this, two assays were done:-

1. Interval Fluorometry:-

- Secondary inoculations were made with 6 biological replicates in 96-well flat black and clear bottom plate.
- OD600 and Fluorescence intensity (Excitation wavelength= 584nm and Emission wavelength= 607nm) was measured for zeroth timepoint.
- The plate was incubated @37°C, 150rpm.
- After every hour, OD600 and Fluorescence intensity measurements were taken.
- This was done after 1, 2, 3, 4 and 10 hours after incubation.

2. recA mediated interference:-

- The secondary inoculation (1.5mL) was set by 1:1 dilution in NB agar (with Ampicillin).
- The cells were grown for 1 hour in that media. After an hour, one of the cultures (Test) was inoculated with 50mM of Hydroxy-Urea (HU)
- The culture was incubated for another 1 hour
- The cells were pelleted @8000rpm for 5mins.
- The pellet was washed with 1x PBS buffer twice by resuspending and pelleting down again @13000rpm for 3mins.
- After washes, solutions were resuspended in 1mL of 1x PBS. The OD600 measurements were taken and solutions were accordingly diluted to OD value - 1.0
- Take 100ul of solution in each well. Three technical replicates were set.
- Fluorescence intensity was measured using Excitation wavelength= 584nm and Emission wavelength= 607nm.

Mutation rate assay

The composite mutator plasmid was constructed using Restriction free cloning techniques and the inserted into pB1A2 backbone using a 2-A assembly.

For the characterization of this part “rifampicin-resistance assay” is performed to calculate the mutation rate .In this assay, the mutation rate per bp is calculated by $\mu_{bp} = f/[R * \ln(N/N_0)]$ where “f” is the frequency of mutants, N is the final population size and N_0 is the population size at which resistance is first observed.”R”is the number of unique sites yielding rifampin resistance (77 previously identified sites).

- The positive colony is inoculated into 3 ml of primary culture which is incubated overnight.
- 3 microliter of primary culture is inoculated into the secondary culture.
- Culture is induced with arabinose after 150 min.
- After 18-24 hr of incubation at 37 deg culture plated on the rifampicin plate to calculate the number of mutants.
- Also to calculate the total number of cells culture is diluted 1000000 times and then plated.

Standardization Measurements

Standard iGEM protocols for calibration of Absorbance 600 and green fluorescence measurements taken in a plate reader were performed using the iGEM 2019 measurement kit. The following graphs were obtained:

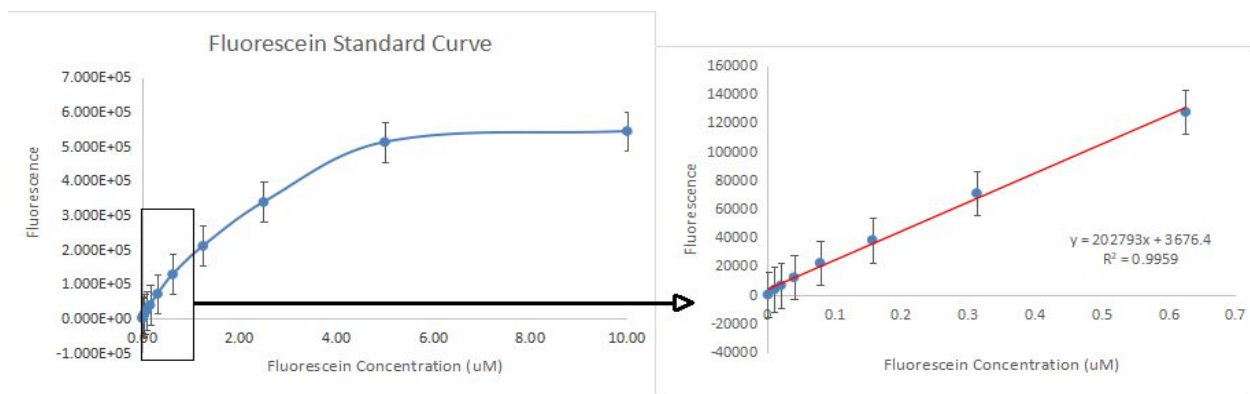


Fig: Graph showing calibration curve between Fluorescence readout (in AU) and number of fluorescence molecules.

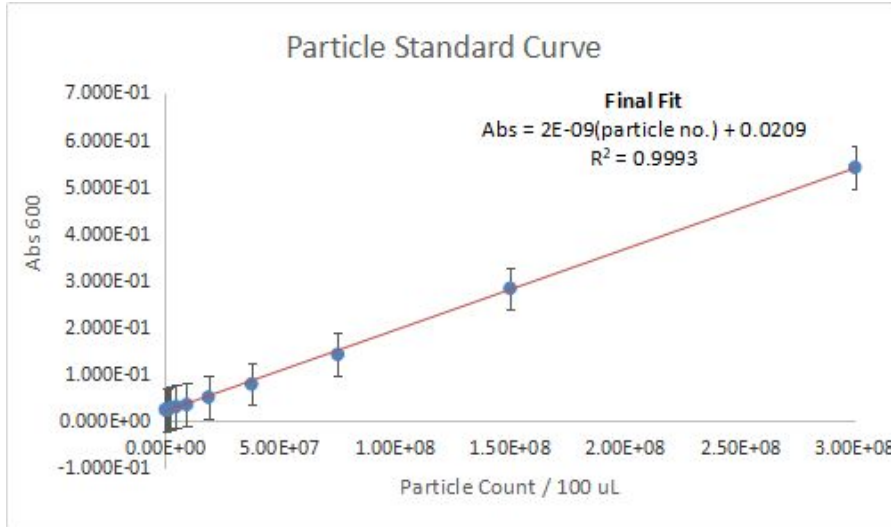
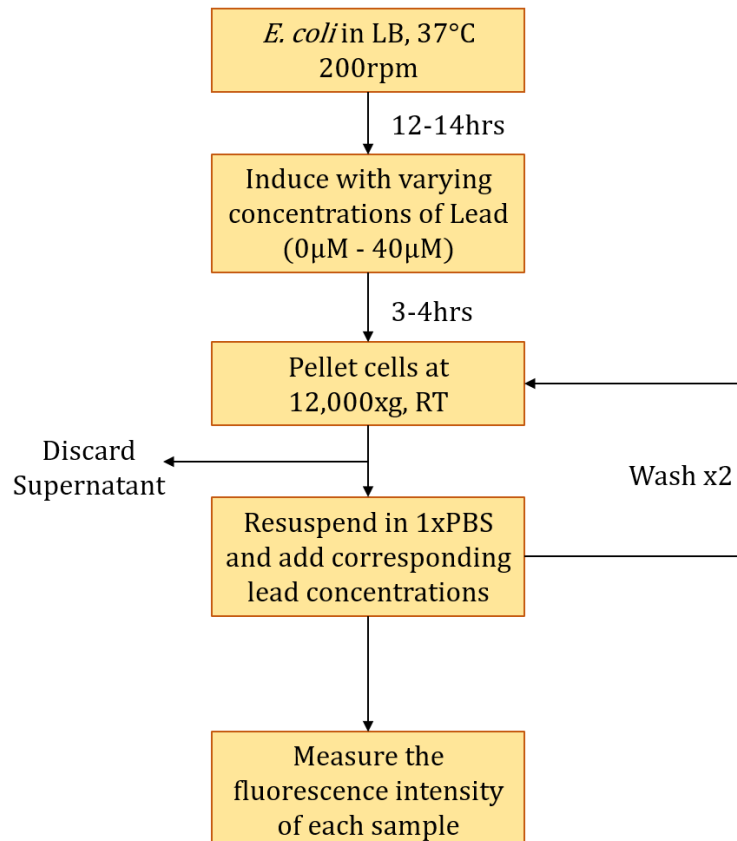


Fig: Graph showing calibration curve between Absorbance 600 and number of cells.

Biosensor measurement assay

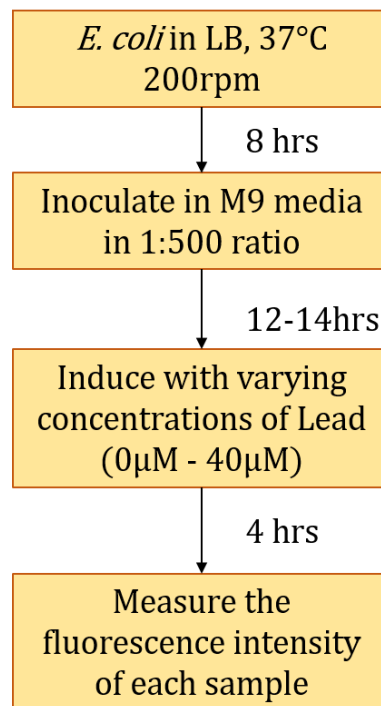
All experiment were performed in E coli DH5a cells. Cells were transformed with the lead biosensor construct (BBa_K1758333) taken from the repository. The characterization of the part was done in LB media in presence of different concentrations of lead nitrate (Pb^{2+}).

The protocol is described below:



The fluorescence intensity was measured using PerkinElmer's EnSight Plate Reader in a 96-well black plate. For negative control, *E. coli* transformed with TetR Repressible Promoter (BBa_R0040). For the positive control, cells transformed with constitutively expressed GFP (BBa_J364001) is used.

To determine the lead concentrations of unknown sample, M9 media was used as follows:



The *pbrD* genes was amplified using PCR with the Prefix and Suffix sequences as primers. The *pbrT* gene was synthesised as 2 parts and was stitched using overlap PCR.