Aalto University
School of Chemical Engineering

Laboratory course in Biosystems and biomaterials

Part II - Assembly of genetic constructs using BioBricks and recombinant protein expression.

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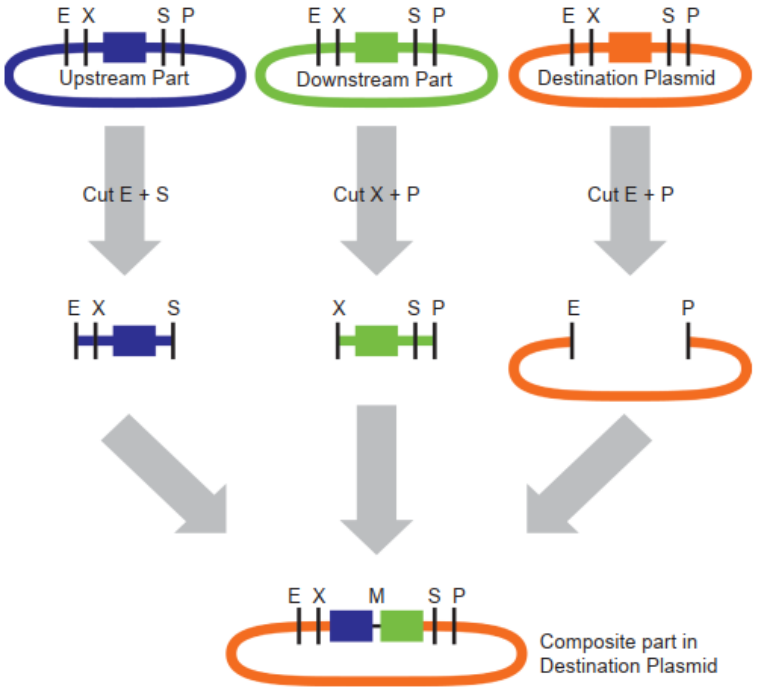
1. BioBricks for assembling genetic constructs.

Cloning is a crucial aspect of molecular biological research and is one of the most widespread techniques used in labs around the world. However, most labs work with completely different cloning methods, utilizing different insert strategies, using different vector backbones etc. This often requires a lot of time and energy to construct a suitable cloning strategy.

As mentioned before, the iGEM Biobricks are an attempt to reduce the complexity of molecular cloning strategies by introducing standardization. By using a BioBrick prefix before a gene of interest and a Biobrick suffix after a gene of interest, which is always identical (see Figure 1, the genes can always be combined and recombined in the same way. This allows people without a strong background in molecular biology to easily pick up cloning and use their own area of expertise to create a bigger diversity of parts. Besides offering a standardized cloning strategy, the iGEM repository (Registry of Standard Biological Parts, http://parts.igem.org/Main_Page) contains vast selection (>20,000 documented parts) of gene and gene regulatory elements that can be used as a basis for new ideas. These parts are grouped according to their function, promoters, ribosomal binding sites, coding regions, terminators etc. Furthermore, to ease the search for respective candidates for one's research, the registry has curated 'collections' pertaining to a research field. For instance, by selecting the Reporter protein tab (under catalog>collection in http://parts.igem.org/Main_Page), one, if searching for an IP-free fluorescent protein, can obtain a list (along with the sequence information) of suitable candidates.

There are four assembly standards, defining the genetic assembly's construction, reported in http://parts.igem.org [RFC10: original assembly standard; RFC21: Berkeley Standard; RFC23: developed by Pamela Silver (Harvard Medical School) and RFC25: developed by 2007 Freiburg iGEM team]. Among these, RFC10 is currently the most commonly used assembly standard.



	EcoRI	XbaI
BioBrick prefix:	GAATTCGCGGCCGCTTCTAGAG	
	SpeI	PstI
BioBrick suffix:	TACTAGTAGCGGCCGCTGCAG	

The scar between two joint BioBrick parts:

EcoRI	PstI
GAATTCGCGC...CGCTTACTAGTAGC...CCGCTGCAG	

Fig. 1. Basic principle of the iGEM Biobrick assembly method (RFC 10, https://static.igem.org/mediawiki/2013/1/1c/Uppsala2013_BioBrick_Assembly_Manual.pdf). A vector and insert are cut with two restriction enzymes (of which one overlaps) and after ligation, the Biobricks are recombined with a scar sequence between the bricks. Restriction enzymes: E, EcoRI; X, XbaI; S, SpeI and P, PstI.

In Part II of this lab course, you will get acquainted with the BioBrick assembly method. In the lab, you will work to recombine standard Biobricks to create plasmids for constitutive expression of fluorescent proteins.

Learning outcomes

1. To understand and be able to use the basic steps involved in molecular *E. coli* cloning.
2. To understand how synthetic biology and "atomization" in biological research could be applied, but also what its limitations are.
3. To create GFP expressing *E. coli* strains.

BioBrick parts to be used in the assembly:

- GFP gene: BBa_I13504, a GFP reporter, consists of a ribosomal binding site (RBS), green fluorescent protein (GFP) coding gene, and a double terminator = only the GFP gene with no promoter
- Constitutive promoter: BBa_K823004, promoter without RBS = the promoter & vector backbone is used.

Database: http://parts.igem.org

Help pages: http://parts.igem.org/Help

2. Schedule –overview of procedures

Day 1. Plasmid Isolation, plasmid restriction & Gel Purification

Aim: Preparation of DNA fragments (the Biobricks) for assembly of the desired expression construct.

1. Use the Plasmid Isolation Kit from Macherey Nagel to **isolate plasmid DNA** from an overnight *E. coli* culture following the protocol 3.1. (Frozen cell pellet prepared from an overnight culture is provided)
2. **Measure the plasmid DNA concentration** using the Nanodrop Lite spectrophotometer.
    - Write down the DNA concentrations:
    - Calculate how much volume of the plasmid solution you need to get 2 µg of plasmid DNA.

plasmid name	DNA concentration ng/µl	Volume needed for 2 µg of DNA

3. Set-up the restriction digestions according to the example in protocol 3.2. Digest 2 µg of plasmid DNA per digestion. Incubate reactions at 37 °C for 30 mins.
4. During the digestion, cast an agarose gel according to the protocol 3.3.

! Work cautiously, SybrSafe/SybrGreen is a carcinogenic material!

5. After the digestion is completed and the gel has solidified, load the samples on the gel, and run at 80-120 V (depending on the gel size) for 40-60 minutes.
6. Use the Gel-dock to image the gel. Analyze the results and identify the bands that need to be excised from the gel.
7. While wearing the protective gloves and helmet, excise bands (= agarose gel pieces) from the gel with a scalpel on the transilluminator while minimizing the amount of time the gel is exposed to UV light (although UV light visualizes the bands, it also denatures the DNA over time).
8. If time allows, we continue with step 2 (& 3) from Day 2. If not, the agarose gel pieces are stored at -20°C.

Day 2. Preparation of Competent Cells & Transformation

Aim: Generation of the expression plasmid from the BioBricks fragments and its transformtion into *E. coli* cells.

1. Start with the **preparation of competent *E. coli* cells** according to protocol 3.6. You will start with step 2 of the protocol.

While the cells are growing, continue with the steps 2 to 5, then return to preparation of competent cells.

2. Use the Gel Purification Kit from Macherey Nagel to isolate the DNA from the gel pieces from Day 1. Follow the protocol 3.4. In the **last step elute DNA in 15 µl**.
3. Measure the concentration of your DNA after gel purification.
   Write down the DNA concentrations:

fragment	concentration
vector	
insert	

4. Use the purified fragments from the agarose gel to set up the ligation reaction according to the ligation protocol 3.5, while using a 3:1 molar ratio of insert to vector and 100 ng of vector DNA in the ligation.

   Formula for calculating the mass of insert needed:
   *required mass insert (ng) = desired insert/vector molar ratio **x** mass of vector (ng) **x** ratio of insert to vector lengths (in kb)*

   Or use the on-line calculator: NEBioCalculator

5. Prepare a control reaction only containing the vector, but no insert.

fragment	ligation	ligation without insert
vector, volume needed (µl) to get 100 ng		
insert, volume needed (µl) to get X ng		0 µl
H_20 needed		
10x T4 DNA ligase buffer	2 µl	2 µl
T4 DNA ligase	1 µl	1 µl

6. Continue with the protocol for preparing competent cells. Make sure the cells (including tubes and all washing solutions that are added) **are kept on ice**, since an increase in temperature will greatly reduce the competence of the *E. coli* cells.

7. When the competent cells are ready and the ligation reactions is completed, transform the *E. coli* cells with the ligation mixture following protocol 3.7.

Think of the required controls that are needed in the transformation (no DNA, intact plasmid, no insert etc.) and what could be concluded from including them.

Day 3. Screening of colonies by colony PCR.

Aim: Identification of *E. coli* colonies that carry the desired plasmid.

1. Follow the colony PCR protocol 3.8. You will test 3 to 5 colonies.
2. Importantly, **restreak** the tested colonies on LB plates containing the correct antibiotic. Plates are incubated overnight at 37°C.
3. Prepare a gel for gel electrophoresis (Protocol 3.3) and find out which band size you expect to see.
4. Load the PCR reactions and run the agarose gel.

Day 4. Preparations for GFP measurement using the Cytation plate reader.

Aim: Adapt cells to growth in liquid media.

1. Culture one of the positive clones per plate in 2-4 ml LB medium (with the appropriate antibiotic) overnight at 37°C in the shaker incubator, 230 rpm.

Homework: Find out how the Cytation 3 (BioTek Cytation 3 Cell Imaging Multi-Mode Reader) works and what settings need to be used for the experiment on day 5. Things to find out include, What should we measure and how (wavelength, excitation, emission).

Day 5. Measuring growth and expression of GFP: “Cytation 3 experiment”

Aim: Grow cells under continuous monitoring of cell growth and GFP expression.

1. Discuss protocol 3.9. with teachers and decide whether some modifications should be done. All clones will be grown on a single microtiter plate. For each strain grow replicate cultures on the microtiter plate. Mark who uses which wells.

2. Set-up Cytation 3 for measurement and start measurements.

3. Protocols

3.1. Plasmid purification from *E. coli*

Before starting the preparation:

- Check if Wash Buffer A4 was prepared according to section 3.

1 Cultivate and harvest bacterial cells

Use **1–5 mL** of a saturated ***E.coli* LB culture**, pellet cells in a standard benchtop microcentrifuge for **30 s** at **11,000 x *g***. Discard the supernatant and remove as much of the liquid as possible.

Note: For isolation of low-copy plasmids refer to section 5.2.

11,000 x *g*, 30 s

2 Cell lysis

Add **250 µL Buffer A1**. Resuspend the cell pellet completely by vortexing or pipetting up and down. Make sure no cell clumps remain before addition of Buffer A2!

+ 250 µL A1

Resuspend

Attention: Check Buffer A2 for precipitated SDS prior to use. If a white precipitate is visible, warm the buffer for several minutes at 30–40 °C until precipitate is dissolved completely. Mix thoroughly and cool buffer down to room temperature (18–25 °C).

+ 250 µL A2

Mix

RT, 5 min

Add **250 µL Buffer A2**. Mix gently by inverting the tube **6–8 times**. Do not vortex to avoid shearing of genomic DNA. Incubate at **room temperature** for up to **5 min** or until lysate appears clear.

Add **300 µL Buffer A3**. Mix thoroughly by inverting the tube **6–8 times** until blue samples turn colorless completely! Do not vortex to avoid shearing of genomic DNA!

+ 300 µL A3

Mix

Make sure to neutralize completely to precipitate all protein and chromosomal DNA. LyseControl should turn completely colorless without any traces of blue.

3 Clarification of lysate

Centrifuge for **5 min** at **11,000 x *g*** at room temperature.

Repeat this step in case the supernatant is not clear!

11,000 x *g*, 5–10 min

4 Bind DNA

Place a NucleoSpin® Plasmid/Plasmid (NoLid) Column in a Collection Tube (2 mL) and decant the supernatant from step 3 or pipette a maximum of 750 µL of the supernatant onto the column. Centrifuge for **1 min** at **11,000 x *g***. Discard flow-through and place the NucleoSpin® Plasmid/Plasmid (NoLid) Column back into the collection tube.

Load supernatant

11,000 x *g*, 1 min

Repeat this step to load the remaining lysate.

5 Wash silica membrane

*Recommended: If plasmid DNA is prepared from host strains containing high levels of nucleases (e.g., HB101 or strains of the JM series), **it is strongly recommended** performing an additional washing step with **500 µL Buffer AW, optionally preheated to 50 °C**, and centrifuge for **1 min** at **11,000 x g** before proceeding with Buffer A4. Additional washing with Buffer AW will also increase the reading length of DNA sequencing reactions and improve the performance of critical enzymatic reactions.*

Optional: + 500 µL AW

11,000 x *g*, 1 min

Add **600 µL Buffer A4** (supplemented with ethanol, see section 3). Centrifuge for **1 min** at **11,000 x *g***. Discard flow-through and place the NucleoSpin® Plasmid/Plasmid (NoLid) Column back into the **empty** collection tube.

+ 600 µL A4

11,000 x *g*, 1 min

6 Dry silica membrane

Centrifuge for **2 min** at **11,000 x *g*** and discard the collection tube.

Note: Residual ethanolic wash buffer might inhibit enzymatic reactions.

11,000 x *g*, 2 min

7 Elute DNA

Place the NucleoSpin® Plasmid/Plasmid (NoLid) Column in a 1.5 mL microcentrifuge tube (not provided) and add **50 µL Buffer AE**. Incubate for **1 min** at **room temperature**. Centrifuge for 1 min at **11,000 x *g***.

+ 50 µL AE

RT, 1 min

Note: For more efficient elution procedures and alternative elution buffer (e.g., TE buffer or water) see section 2.5.

11,000 x *g*, 1 min

3.2. FastDigest restriction enzyme protocol

1. Prepare the reaction mixture at room temperature according to this table.

Water, nuclease-free	fill-up to 30 µl
10x FastDigest buffer	3 µl
DNA	2 µg
FastDigest enzyme I	1.5 µl
FastDigest enzyme II	1.5 µl
Total Volume	30 µl

2. Mix gently and spin down.
3. Incubate at 37 °C in a heat block.
4. Optional: Heat inactivate enzyme(s)

3.3. Agarose gel electrophoresis

Preparing the agarose gel

For other percentages and tray sizes, adjust amounts and volumes accordingly! This is only one example.

1. Measure 1.25 g (or 0.6 g) agarose powder and add it to a 500 ml (or 250 ml) flask.
2. Add 125 ml (or 60 ml) TAE Buffer to the flask. (the total gel volume will vary depending on the size of the casting tray)
3. Melt the agarose in a microwave or hot water bath until the solution becomes clear. (if using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).
4. Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
5. Add the correct amount of SYBR Green (1:10'000) to the solution.
6. Seal the ends of the casting tray with two layers of tape or with the bigger tray, use the gel caster with rubber stoppers.
7. Place the combs in the gel casting tray.
8. Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
9. Carefully pull out the combs and remove the tape or the stoppers.
10. Place the gel in the electrophoresis chamber.
11. Add enough 1 x TAE Buffer so that there is about 2-3 mm of buffer over the gel.
12. Note – gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

1. Add 5 µl of 6X Sample Loading Buffer to each 25 µl PCR reaction (just an example)
2. Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.
3. Carefully pipette desired amount (5-10 µl for check-up PCR, up to 100 µl for fragment cutting) of each sample / loading buffer mixture into separate wells in the gel.
4. Pipette 5 µl (check the amount from teacher) of the DNA ladder standard into at least one well of each row on the gel.

Note – if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel.

Running the gel

1. Place the lid on the gel box, connecting the electrodes.
2. Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected. (Remember – "Run to Red")
3. Turn on the power supply to about 80 volts with a small gel and about 100 volts with a big gel. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – it should not exceed 5 volts/ cm between electrodes!
4. Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.
5. Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
6. Let the power run until the blue dye approaches the end of the gel.
7. Turn off the power.
8. Disconnect the wires from the power supply.
9. Remove the lid of the electrophoresis chamber.
10. Using gloves, carefully remove the tray and gel.

3.4. Purification of DNA from agarose gels

Before starting the preparation:

- Check if Wash Buffer NT3 was prepared according to section 3.

1 Excise DNA fragment / solubilize gel slice

Note: Minimize UV exposure time to avoid damaging the DNA. Refer to section 2.5 for more tips on agarose gel extraction.

Take a clean scalpel to excise the DNA fragment from an agarose gel. Remove all excess agarose.

! Determine the weight of the gel slice and transfer it to a clean tube.

+ 200 µL NTI per 100 mg gel

For each **100 mg of agarose gel < 2 %** add **200 µL Buffer NTI.**

For gels containing > 2 % agarose, double the volume of Buffer NTI.

Incubate sample for **5–10 min** at **50 °C.** Vortex the sample briefly every 2–3 min until the gel slice is **completely** dissolved!

50 °C
5–10 min

2 Bind DNA

Load sample

Place a **NucleoSpin® Gel and PCR Clean-up Column** into a Collection Tube (2 mL) and load up to 700 µL sample.

11,000 x *g*
30 s

Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

Load remaining sample if necessary and repeat the centrifugation step.

3 Wash silica membrane

Add **700 µL Buffer NT3** to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

+ 700 µL NT3

11,000 x *g*
30 s

Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and low A_{260}/A_{230} (see section 2.7 for detailed information).

+ 700 µL NT3

11,000 x *g*
30 s

4 Dry silica membrane

Centrifuge for **1 min** at **11,000 x *g*** to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube.

11,000 x *g*
1 min

Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.

5 Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a **new** 1.5 mL microcentrifuge tube (not provided). Add **15–30 µL Buffer NE** and incubate at **room temperature** (18–25 °C) for **1 min**. Centrifuge for **1 min** at 11,000 x *g*.

+ 15–30 µL NE

RT
1 min

11,000 x *g*
1 min

Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min. See section 2.6 for detailed information.

3.5. Ligation

This protocol is for the DNA Insert Ligation (sticky-

Sticky-end Ligation

1. Prepare the following reaction mixture:

Linear vector DNA	20-100 ng
Insert DNA	1:1 to 5:1 molar ratio over vector
10x T4 DNA Ligase buffer	2 µL
Thermo Scientific T4 DNA Ligase (Cat #EL0016)	1 U
Water, nuclease-free	to 20 µL
Total volume	**20 µL**

2. Incubate 10 minutes at 22 °C.

3.6. Preparation of competent *E. coli* cells

!! All steps from harvesting the cells should be carried out at 4°C and the cells should then be kept on ice whenever possible! Make sure the $CaCl_2$, $CaCl_2$+25% glycerol and Eppendorf tubes are pre-chilled as well!

Materials:

- *E. coli* cells (from a plate or -80°C competent stock) – Top10, XL-blue, DH5α
- LB medium (sterile)
- 0.1 M $CaCl_2$, ice cold (sterile)
- 0.1 M $CaCl_2$+20% glycerol, ice cold (sterile)
- 1.5 mL Eppendorf tubes, ice cold (sterile)

1. (**DONE**) Inoculate strain of *E. coli* into 3-5 mL LB and grow O/N at 37°C + shaking.
2. Next morning, use 1 v/v % AND 2 v/v % of the O/N culture to make a 10 ml liquid culture of cells (LB, in a sterile plastic tube). Grow the cells at 37°C + shaking (~220 rpm) until the OD_{600} is between 0.3 – 0.4 (usually takes around 1,5 - 2 hours). Do NOT let the OD_{600} get > 0,5!!
3. Keep the tubes on ice for 5-10 min.
4. Centrifuge the cells for 3 min at ~3300 g with a centrifuge. Check that there is a visible cell pellet on the bottom of the tube.
5. Gently discard the supernatant. Place the tubes back on ice and gently resuspend the cells with 5 ml of cold 0,1M $CaCl_2$ by pipetting.
6. Incubate cells for 30 minutes on ice.
7. Centrifuge the cells again for 3 min at ~3300 g with a centrifuge. Check that there is a visible cell pellet on the bottom of the tube.
8. Gently discard supernatant and resuspend cell pellet in 0,25 ml of cold 0.1 M $CaCl_2$+20% glycerol.
9. **KEEP CELLS ON ICE UNTIL THEY ARE NEEDED**.
10. Optional step for storage of competent cells: Transfer cell suspension into a pre-chilled, (**&labelled**) sterile Eppendorf tube. Quick-freeze the aliquoted cells with liquid nitrogen and store at -80 ˚C.

Notes & Hints:

- Chemically competent cells have lower transformation efficiencies than with electroporation, but are simpler to transform, less work to make and have the advantage that larger volumes of DNA containing buffers etc. (e.g. after Golden Gate cloning) can be transformed more easily.
- After making competent cells, the transformation efficiency should be checked with a known plasmid of known concentration (**won´t be done during this course**). e.*g.*: To calculate

transformation efficiency, transform 2 µl of the provided control pUC19 DNA (100 pg) into 50 µl of cells. Follow regular transformation protocol and dilute 10 µl of cells up to 1 ml in SOC. Plate 100 µl of this dilution. In this case, 150 colonies will yield a transformation efficiency of 1.5 x 10^9 cfu/µg (µg DNA=0.0001, dilution=10/1000 x 100/1000). For good transformation results, the transformation efficiency of the competent cells should be ~1 x 10^9 colony forming units (cfu) per µg. *(source: NEB Q5 SDM)*

3.7. Transformation of *E. coli*

Transforming chemically competent cells using heat shock method.

1. On ice, pipet 50 µL of competent cells and the ligation reaction into an Eppendorf tube, mix gently.
2. Incubate tubes on ice for 10-20 minutes.
3. Incubate tubes in 42°C for 45 sec - 1 minute (heat shock).
4. Incubate tubes on ice for 5 minutes.
5. Add 500 µL of LB medium.
6. Incubate cells at 37°C and 200 rpm for 30 min - 1 hour.
7. Collect cells by centrifugation (3 mins at 3300 g) and remove about 450 µL supernatant.
8. Resuspend cells in the remaining supernatant and plate onto an appropriate antibiotic LB-agar plate.
9. Incubate overnight at 37°C or over weekend in RT.

3.8. Colony PCR using Kapa2G mix

1. Create the following mixture for the PCR reactions, calculate the amount needed for X reactions. Hint: If you test 3 colonies, prepare the mixture for 3.5 reactions:

Reagent	Volume for one reaction	Volumes for X reactions
Kapa2G mix	5 µL	
Primer 1 (10 µM)	0.5 µL	
Primer 2 (10 µM)	0.5 µL	
Template DNA: *E. coli* colonies are added in step 4 of this protocol		
PCR-grade water	4 µL	
Total	10 µL	

IMPORTANT: When using the PCR machine in teaching laboratory make sure to use the specific PCR tubes (8-strips with domed caps, ask the teacher)

2. Pipette 10 µL into PCR tubes
3. Pick individual colonies from the transformation plate with a yellow tip.
4. Swirl the tip in the PCR tubes containing the ready colony PCR mixture.
5. At the end, streak the tip onto a LB agar plate containing the correct selection.
6. Put the samples in the thermocycler and run the following program:

1) Initial denaturation	95 C	5 min	
2) Denaturation	95 C	15 s	
3) Annealing	60 C	15 s	
4) Extension	72 C	60 s	35x back to 2
5) Final extension	72 C	2 min	
6) Cool samples	10 C	∞	

When the PCR is finished, adjust the samples with 6X DNA sample buffer and load the samples on the gel. Run at 80-100V until separated sufficiently.

3.9. Microtitre Plate Growth Experiment

1. Inoculate cells from a single colony (solid plate) into 2-4 ml of LB medium (containing the appropriate antibiotic) and incubate the cells at 37°C overnight.
2. Add 80 µl of this culture to 4 ml of fresh LB medium containing the appropriate antibiotic and incubate at 37°C until the OD_{600} reaches 0,6.
3. Add 10 µl of cells to 200 µl of medium in the experimental setup (which equals $4{,}8 \times 10^6$ cells to start with).
4. If fluorescence is measured, measure from the bottom (the top reads can reach unreadable high measurements).

Cytation 3 Settings

1. Set the Temperature to 37°C.
2. Start Kinetics. Experiments should last 6 hours minimal, but preferably 8-10 h.
3. Shake Orbital for 6 min.
4. Measure OD at 600 nm.
5. Measure Fluorescence (Bot).
6. Measure every 10 minutes.