iGEM 2018 Vibri*gens* InterLab Study Protocol

Before You Begin

Vibrio natriegens dies after about two weeks on plate. If you do not plan on starting the experiment in the two weeks after you receive the plate, take care to replate it every two weeks until you do (or make a glycerol stock).

Please read through this entire protocol sheet carefully before you start your experiment and prepare any materials you may need. In order to improve reproducibility, **we are requiring all participating teams to use plate readers to take measurements of fluorescence and absorbance**. If you have further questions, please contact our team at **igem2018@staff.uni-marburg.de**.

Plasmids work differently in different organisms. Don't expect the same measurement results for this experiment as you had in the iGEM InterLab.

Before beginning your experiments, it will be helpful to gather the following information about your plate reader, as you will be asked to provide this information when submitting your data to us:

- Instrument: brand and model

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- Is this the same instrument used in the iGEM InterLab study?

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- Can your instrument measure both absorbance and fluorescence?

  _____

- Does your instrument have pathlength correction, and if yes can it be disabled?

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- Does your instrument have variable temperature settings, and if yes can this be set to room temperature?

  _____

- What filters does your instrument have for measuring GFP? You will need information about the bandpass width (530 nm / 30 nm bandpass, 25-30nm width is recommended), excitation (485 nm is recommended) and emission (520-530 nm is recommended) of this filter.

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- Does your instrument use top or bottom optics (e.g. does your plate reader read samples from the top of the plate or the bottom)?

  _____

Materials:

You will need all of the following supplies and reagents to complete this entire protocol. Please take a moment to check that you have all of these supplies and reagents before you begin:

Our box contains:

❑1 Plate with *Vibrio natriegens* wild type

❑20 aliquots of recovery media (BHI + v2 + 680 mM Sucrose)

❑3 Falcon tubes with 50mL 10x v2 Salt (204mM NaCl, 4.2mM KCl, 23.14mM $MgCl_2$)

❑4-Falcon tubes with 2.5x Electroporation buffer (680 mM Sucrose , 7nM K_2HPO_4 , pH = 7)

❑1 10 mL-Falcon tubes with LB + v2 for the overnight culture for the competent cells

❑ 8 1.5 mL-Eppendorf tubes with the following plasmids from the InterLab study

- Interlab_Study_Test_Device_1 ❑
- Interlab_Study_Test_Device_2 ❑
- Interlab_Study_Test_Device_3 ❑
- Interlab_Study_Test_Device_4 ❑
- Interlab_Study_Test_Device_5 ❑
- Interlab_Study_Test_Device_6 ❑
- Interlab_Study_Pos_control ❑
- Interlab_Study_Neg_control ❑

What you also need:

❑ddH_2O (ultrapure filtered or double distilled water)
❑Competent cells (*Vibrio natriegens* WT, see protocol for transforming cells)
- ❑ LB (Luria Bertani)
- ❑ v2 salts (supplied in Vibri*gens* InterLab Distribution Kit)
- ❑ Sucrose & K_2HPO_4 (for electroporation buffer and recovery medium)

❑Chloramphenicol – Cm (stock concentration **2mg/mL** diluted in EtOH)
❑50 ml Falcon tube (or equivalent, preferably amber or covered in foil to block light)
❑Incubator at 37°C
❑1.5 ml Eppendorf tubes
❑Container with ice
❑Micropipettes (capable of pipetting a range of volumes between 1 µL and 1000 µL)
❑Micropipette tips
❑96 well plates, black with clear flat bottom preferred, at least 3 plates
❑Electroporator
❑Electroporation cuvettes

You have to do yourself:

❑ 500 mL LB + v2 salt media
- weigh in LB for 500 mL media and dissolve it in 450 ml H_2O
- add 50 mL from the v2 salt from our distribution box

❑ 500 mL LB + v2 salt media with Chloramphenicol
- weigh in LB for 500 mL media and dissolve it in 450 ml H_2O
- add 50 mL from the v2 salt from our distribution box
- add Chloramphenicol to an end concentration of 2 µg/mL
- store the media in a cool place until you use it

❑ 500 mL electroporation buffer
- take a sterile 500 mL bottle and add the 4 Falcon tubes of 2.5x electroporation buffer (200 mL)
- add 300 mL milliQ or other sterile water

❑ 500 mL LB + v2 – agarose
- weigh in LB-agarose (1% agarose) for 500 mL media and dissolve it in 450 ml H_2O
- autoclave the media and add the 50 mL v2 salt, later, from the second Falcon tube
- If the agar became solid, melt it in the microwave (without a cap screwed on) and add the v2 salts afterwards
- divide the agar in 100 mL for plates for the control and 400 mL for plates with antibiotic for the selection after the transformation
- pour the 100 mL LB + v2 agar in petri plates, you need at least one plate as control
- add to the 400 mL LB + v2 agar Chloramphenicol to an end concentration of 2 µg/mL
- pour the 400 mL LB + v2 agar with the antibiotic in petri plates, you need at least 8 plates

Preparation of electrocompetent cells

The information below are derived and modified from the publication of Weinstock et al (2016)

Take from the box or prepare before:

- LB media + v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14mM $MgCl_2$)
- Electroporation buffer (680 mM sucrose, 7 mM K_2HPO_4, pH = 7) (sterile filtrated)
- Overnight culture of *Vibrio natriegens* inoculated from plates or cryostock in LB + v2 salts (e.g. 5mL, 37 °C; at 200 r.p.m) (10mL Falcon from the box)

Preparing culture:

- Pre-chill the electroporation buffer (approx. 500 mL) and the centrifugation tubes you want to use (on ice)
- On the following day, 500 mL of the LB + v2 is inoculated with the overnight culture with a final OD of 0.05
- The culture is grown at 37 °C in a baffled flask with shaking at 200 r.p.m. until an OD600 of 0.5

Washing:

From here on try to always keep your culture on ice. Ensure you have chilled buffer ready

- Divide the culture into two (or more) pre-chilled centrifugation containments
- Put the culture on ice for 15 min
- The cells are pelleted at 3000x g. for 20 min at 4 °C to avoid cell damage
- The supernatant is carefully decanted and the cell pellets of both containers are gently resuspended in 10 mL of chilled electroporation buffer
- The suspensions are transferred to two chilled 50 mL Falcons
- The tubes are each filled to top with additional chilled electroporation buffer (max. 50 mL in total or fill to maximum volume in accordance with manufactures instruction)
- The Falcon tubes are inverted several times
- The cells are centrifuged down at 3000x g for 15 min at 4 °C
- The washing step (resuspend, fill-up, invert, centrifuge, discard supernatant) is repeated two times for a total of three washes

Aliquotation:

- After the final wash, the supernatant is carefully decanted and the cells are gently resuspended in residual electroporation buffer (equals approximately 500 µL)
- Measure the OD in a 1:20 dilution against electroporation buffer (e.g. 950 µL buffer & 50 µL sample)
- The volume is adjusted with additional electroporation buffer to bring the final OD600 to 16
- Cells are aliquoted (80 µL) into chilled tubes, frozen in liquid Nitrogen and stored at −80 °C until use

Electroporation protocol

prepare the competent cells using the protocol above

Take from the box or prepare before:

- recovery media 10 aliquots (a' 500µL)(BHI + v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14mM $MgCl_2$), and 680 mM sucrose)
- preheat the aliquots with the recovery medium to 45 °C (pipetting mixing with cold cells and resuspending in chilled cuvettes will cool down the media)
- Agar plates (LB + v2 + 1% agarose + 2 µg/ml chloramphenicol (Cm))

Electroporation

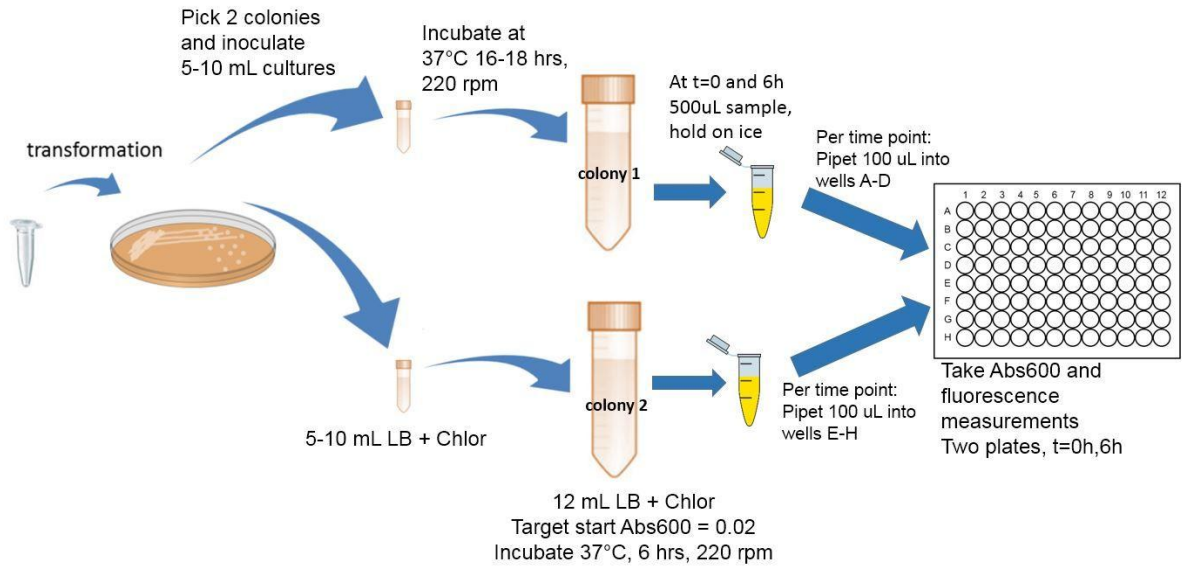
- Take nine aliquots of the prepared competent cells and place them on ice.
- 1µL of the Plasmid DNA (100 ng/µL) and the electrocompetent cells are combined and gently mixed (NO VORTEX, avoid foaming) in a chilled 1.5 mL microcentrifuge tube.
  (each aliquot gets one 1 µL of another plasmid from the box one aliquot is a control and will later not be plated out on a plate with antibiotics)
- The cell-DNA suspension is transferred to a chilled electroporation cuvette with a 0.1 cm gap size
- Cells are electroporated with the following settings: 900 V, 25 mF, 200 Ω (Eppendorf electroporator)
- Cells are immediately recovered in 500 µL (one aliquot) preheated (45°C) recovery medium and transferred to a 1.5 mL tube
- The cells are recovered by incubating at 37 °C for 1.5 h. (also put the agar plates for preheating in the incubator at 37 °C)
- The cells are centrifuged down at 3000x g, most of the supernatant is removed
- The pellet is resuspended in the leftover media (approx. 50 µL) and plated out on warm agar plates (37 °C) containing the appropriate antibiotic (2 µg/ml Chloramphenicol - Cm)
- The plates are incubated for several hours or overnight at 37 °C for colonies to appear. Check the rest of the InterLab Protocol to keep track of colony growth

Cell measurement protocol

For all measurements, you must use the same plates and volumes. Please set the gain against test device 5 at the 3h timepoint and use this gain when measuring the whole plate for timepoint 0h and 3h.

If you encounter problems at any stage of the *Vibri*gens InterLab, please contact us at igem2018@staff.uni-marburg.de.

Workflow



Method

Day 1: Transformation of Plasmids

please transform *Vibrio natriegens* with these following plasmids (all in pSB1C3, follow the electroporation protocol for *Vibrio natriegens*):

Devices (from Distribution Kit, all in pSB1C3backbone):

Device	**Part number**
Negative control	BBa_R0040
Positive control	BBa_I20270
Test Device 1	BBa_J364000
Test Device 2	BBa_J364001
Test Device 3	BBa_J364002 B
Test Device 4	Ba_J364007
Test Device 5	BBa_J364008
Test Device 6	BBa_J364009

Vibri*gens* Challenge: How long until you can see your first colonies forming?

4 hours after plating, keep an eye on your plates and check every half an hour for small colonies. If you see small colonies forming, note the time and import data into Excel sheet provided (**first colonies on plates**).

Pick two colonies from each of the transformation plates and inoculate in 5 mL LB + v2 + 2 µg/mL Chloramphenicol. Grow the cells overnight (16-18 hours) at 37 °C and 220 r.p.m.

Day 2: Cell growth, sampling, and assay.

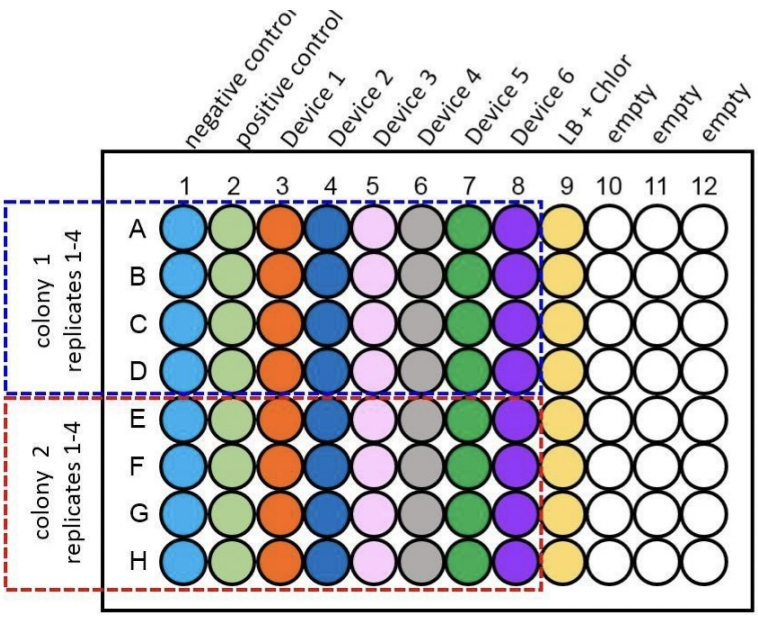
- ❏ Make a 1:20 dilution of each overnight culture in 0.25 mL of culture into 4.75 mL of LB + v2 + 2 µg/mL Chloramphenicol
- ❏ Measure Abs_{600} of 200 µL of these diluted cultures and with a blank in a 96-well plate (samples should be laid out according to the plate diagram below)
- ❏ Record the data in your notebook
- ❏ Dilute the cultures further to a target Abs_{600} of 0.02 in a final volume of **12 mL** LB + v2 + 2 µg/mL Chloramphenicol in 50 mL Falcon tube (amber, or covered with foil to block light)
- ❏ Take 500 µL samples of the diluted cultures at 0 hours into 1.5 mL Eppendorf tubes, prior to incubation. (At each time point 0 hours and 3 hours, you will take a sample from each of the 8 devices, two colonies per device, for a total of 16 Eppendorf tubes with 500 µL samples per time point, 32 samples total). Place the samples on ice
- ❏ Incubate the remainder of the cultures at 37 °C and 220 r.p.m for 3 hours
- ❏ Take 500 µL samples of the cultures at 3 hours of incubation into 1.5 ml Eppendorf tubes. Place the samples on ice
- ❏ At the end of sampling point you need to measure your samples (Abs_{600} and fluorescence measurement), see the below for details
- ❏ Record data in your notebook
- ❏ Import data onto Excel sheet provided (**fluorescence measurement tab**)

Measurement and Layout for Abs_{600} and Fluorescence measurement

Samples should be laid out according to the plate diagram below. Pipette 100 µL of each sample into each well. From 500 µL samples in a 1.5 ml Eppendorf tube, 4 replicate samples of colony #1 should be pipetted into wells in rows A, B, C and D. Replicate samples of colony #2 should be pipetted into wells in rows E, F, G and H. Be sure to include 8 control wells containing 100 µL each of only LB + v2 + 2 µg/mL Chloramphenicol on each plate in column 9, as shown in the diagram below.

The same gain has to be used for all measurements. Please first measure the samples that were taken after incubation (3 h). If your plate reader has the function to automatically adjust the gain, set it to 90 % of the maximal gain using the wells of test device 5. If your plate reader does not have this function, start with a high gain and reduce it subsequently until the most intense samples (probably device 5) gives an analyzable result.

At the end of the experiment, you should have two plates to read. Each plate should be set up as shown below. You will have one plate for each time point: 0 and 3 hours. On each plate you will read both fluorescence and absorbance.



Help Debugging:

- If you have measurements that are off scale ("OVERFLOW"), that data will not be usable. You need to adjust your gain so that the data will be in range and re-run your calibration.

- If your Abs_{600} measurements for your colonies are very close to that of your blank (LB + v2 + 2 µg/mL Chloramphenicol), then your *V. natriegens* has probably not been transformed or grown successfully.

- If your negative and positive control values are very close to each other, that probably means something has gone wrong in your protocol or measurement.

Troubleshooting & Annotations – Competent cells

- Our LBI media (ROTH) has the following composition: Tryptone 10 g/L, Yeast 5 g/L, NaCl 10 g/L. The media is made with 25g per liter. Salt content is very important for *Vibrio natriegens,* so please double check if unsure and adjust the NaCl content accordingly.

- You can prepare your own cryostock from the plates we send you. We use a cryostock in LB + v2 salts media with 20% glycerol stored at -80°C

- Ensure to take an appropriate size for your baffled flask. For 500 mL of culture we recommend at least a 1.5 L flask. If not available you can divide the cultures equally in smaller flasks

- The large batch size of the overnight culture (500 mL) for competent cells is to ensure that you will have enough competent *Vibrio natriegens* to complete the study and account for potentially needed spares.

- If no large centrifuge containments are available, it is possible to divide the culture into several large Falcon tubes (e.q. 50 mL).

- If no liquid nitrogen is available to you, the cells may be frozen in a dry ice bath (ethanol or isopropanol + crushed dry ice) or placed directly in the freezer at -80°C (CAUTION: transformation efficiency might suffer from these alternative methods)

Troubleshooting & Annotations - Electroporation

- If no BHI (brain heart infusion) is available for the recovery medium use LB + v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14mM $MgCl_2$), and 680 mM sucrose (same media as for competent cells with extra sucrose). Media has to be filtrated sterile.

- You can simply add the 1% agarose to the prepared LB + v2 media to turn it into plate media by heating until you get homologous medium again (add the Cm after heating). We used standard petri dishes (92x16mm) for our cell cultures. If your cells overgrow and you cannot select a single colony you have the option to streak out the overgrown colony on a new plate.

- The high sucrose content advised to be avoided in some electroporation protocols has not been an issue with any of the electroporators we tested.

- We tried 0.2 cm gap cuvettes before for electroporation and it did not work. You can clean and reuse cuvettes after use. (e.g. flush them four times with distilled water followed by ethanol. Let them dry in the oven at up to 75 °C or clean them with diluted HCl).

- Remember to flush the cuvette several times to account for most of the cells. Try in advance which pipette tips fit in gap of your cuvette. If the tip/pipette is not long enough try combining two pipette tips of different volumes.

- Yes, the Cm concentration needed for *Vibrio natriegens* is much lower than in *E. coli* (2µg/ml chloramphenicol).