Protocol BLaTM Assay

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This protocol is a detailed version of the method originally described in:

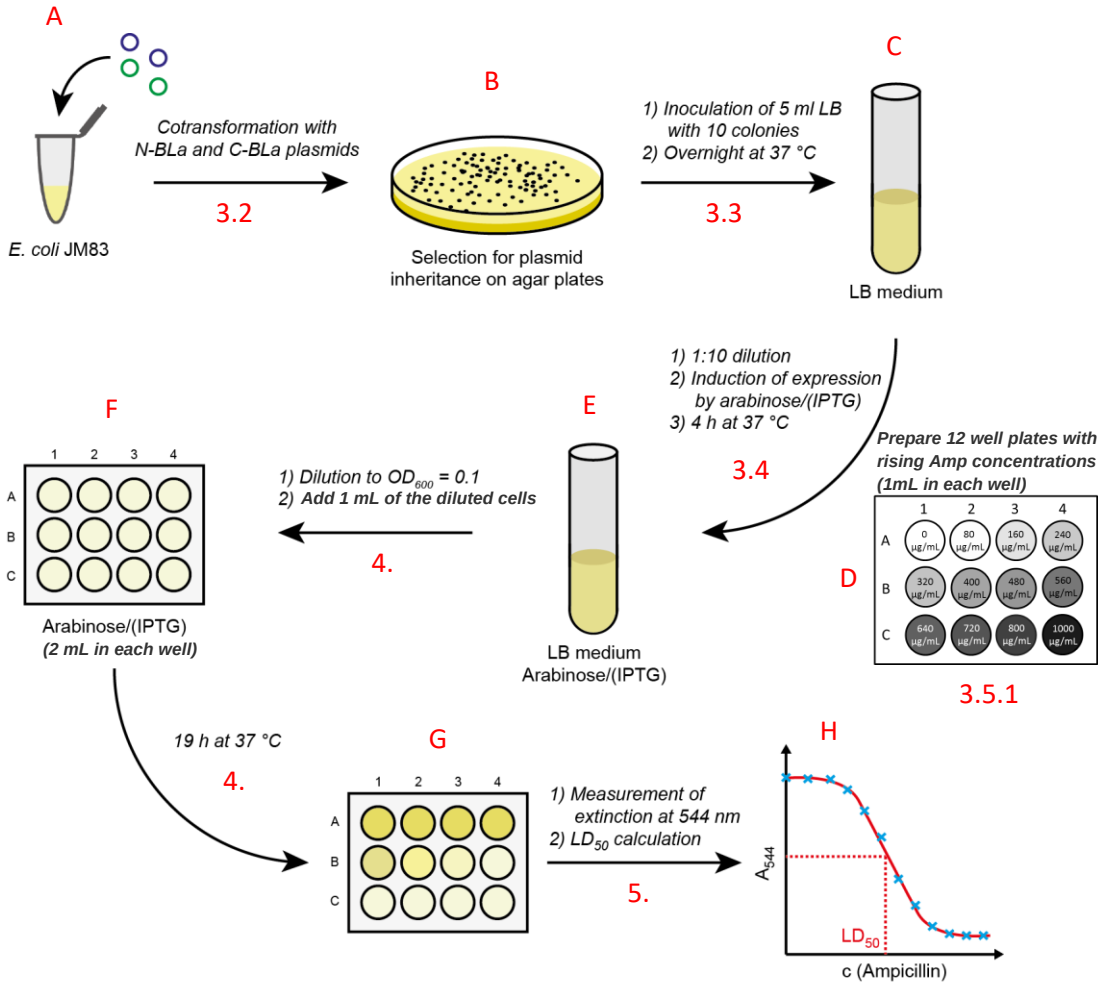
Schanzenbach, C., Schmidt, F.C., Breckner, P., Teese, M.G., and Langosch, D. (2017). Identifying ionic interactions within a membrane using BLaTM, a genetic tool to measure homo- and heterotypic transmembrane helix-helix interactions. Scientific Reports 7, 43476.

Julius, A., Laur, L., Schanzenbach, C., and Langosch, D. (2017). BLaTM 2.0, a Genetic Tool Revealing Preferred Antiparallel Interaction of Transmembrane Helix 4 of the Dual-Topology Protein EmrE. J Mol Biol 11, 1630-1637.

Strict adherence to the work-flow of ampicillin LD50 determination and GFP fusion protein expression control guarantees optimal performance of the BLaTM assay.

Note that this is a version were the concentration of the inducer arabinose is corrected (Nov. 29, 2019). In the original publications listed above the arabinose concentrations given are 10-fold lower than those that were actually used by us. Corrigenda were sent to both Journals and can be found at the end of the protocol.

1. Overview



2. What you need

2.1 For the BLaTM assay

- Chemically competent JM83 *E.coli* cells (reference: Chung CT, Niemela SL, Miller RH. One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. Proceedings of the National Academy of Sciences of the United States of America. 1989;86:2172-5.)
- C-BLa and N-BLa plasmids with the desired embedded transmembrane domains
- Test tubes
- Greiner Bio-one 12-well plates (Kremsmünster, Austria)
- Moisturized, sealed containers
- 37 °C shaking incubator
- Turning wheel/ Culture wheel/ culture rotator in an 37 °C environment (in our case New Brunswick Scientific, Tissue culture roller TC-7)
- Microplate reader (Absorption in 12 well plate, λ=550-600 nm)

- Python software with ECCpy installed (https://github.com/teese/eccpy)

2.2 For the GFP measurement

- Aliquot of 200 µL of the 4 h culture from the BLaTM assay
- Phosphate-buffered saline
- Black 96-well plate (e.g. Nunc)
- Microplate reader (λ_{em}=485 nm, λ_{ex}=520 nm)

3. BLaTM assay

3.1 Considerations before the start

- Instructions are written in a regular font.
- TIPS are written in *italics.*
- For best results it is recommended to run constructs, whose LD50 values are to be compared later, in parallel.
- Further, at least 2 biological and at least 2 technical replicates each (2*2=4) are recommended (for each TMD combination).
- For a total of 4 technical replicates, it is best to be able to work 4 consecutive days at least 6 hours a day; moreover, cells have to be transformed and the overnight cultures have to be prepared the days before. At the 5th day, only a measurement has to be carried out.
- ALWAYS use GpA wt_{+1} and GpA $G83I_{+1}$ (negative control) for measurements with the BLaTM 1.2 assay and EmrE TMD4_3 (positive control) and EmrE TMD4_3_G90V/G97V for measurements with the BLaTM 2.0 assay (find sequences in Fig.2 a) of Julius et al, 2017, http://doi.org/10.1016/j.jmb.2017.04.003) to be able to (i) validate assay performance and (ii) normalize your data.
- Overnight culture means 14 h to 18 h of incubation.
  !Try to do overnight incubations for the same length of time for all replicates!
- Agar plates should be sealed with Parafilm (Bemis, Oshkosh, WI, US) and stored at 4 °C.
- Plates can be used for inoculation for up to one week.
- Ampicillin stock solutions (20 mg/mL) have to be prepared freshly every day!

3.2 Co-Transformation of *E.coli* JM83 cells (A)

TIP: *For a perfect time management, transform the cells with the respective C-BLa and N-BLa plasmids on a Thursday, or Friday afternoon. If you transform them on Friday, keep in mind that the plates should be taken out of the incubator on Saturday morning.*

Chemically competent cells (Chung et al, 1989) are transformed by heat shock. A 100 µL cell aliquot is thawed on ice for 10 min and 50-100 ng of both plasmids (coding for the C-BLa or the N-BLa constructs, respectively) are added. After 30 min incubation on ice, the cells are incubated in a water bath at 42 °C for 1 min followed by incubation on ice for 2 min. Subsequently, 900 µL LB medium are added and cells are incubated for 1 h to 1.5 h in a turning wheel at 37 °C. Cells are then centrifuged for 1 min at 5,500 x g. About 900 µL of the supernatant is discarded and the pellet is resuspended in the remaining supernatant. Afterwards, the cells are plated on LB-agar plates (LB medium with 1.5% agar) containing chloramphenicol (34 µg/mL; Cm) and kanamycin (35 µg/mL; Kan) and incubated overnight at 37 °C. Afterwards, plates are sealed with Parafilm and stored at 4 °C for up to one week.

3.3 Overnight cultures

TIP: *For a perfect time management, inoculate the first overnight culture for the first of the 2 technical replicates, on Sunday evening. Try to do overnight incubations for the same length of time for all replicates!*

10 colonies, picked from one agar plate (B) are combined in 5 mL LB medium in (Cm, Kan) in test tubes and incubated in a turning wheel overnight (14 h to 18 h) at 37 °C (C). *Always seal the plates after usage!*

3.4 Expression culture

TIP: *To enhance the stability of the expression medium, keep it in the fridge, but keep in mind to NEVER use it cold (→ expression medium has to be at room temperature prior to inoculation!).*

4.5 mL of LB expression medium (Cm, Kan, 1.33 mM Arabinose; Ara) with 0.3 mM IPTG (for measurements with the BLaTM 1.2 assay) or 0.6 mM IPTG (for BLaTM 2.0), are inoculated with 500 µL of the overnight culture (1:10 dilution) and incubated at 37 °C for 4 h for expression in a turning wheel. *Always prepare 2-3 uninduced samples, by inoculating of 4.5 mL of LB medium (containing 34 µg/mL Cm and 35 µg/mL Kan) with 500 µL of randomly choosen overnight cultures, as controls for the GFP measurement.*

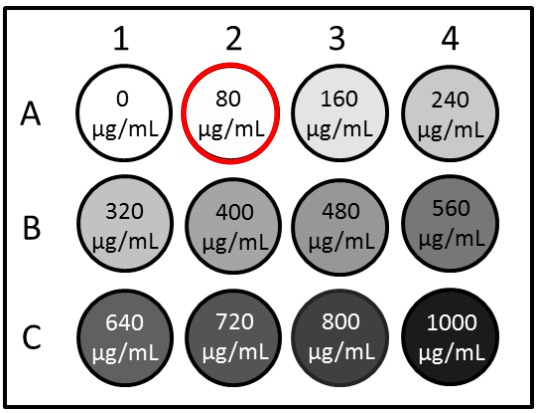
3.5 Prearrangements during the 4 h expression period

3.5.1 Preparation of ampicillin stocks and the 12-well plates

- Estimate the interaction strength of your TMDs of interest (do you assume a low/medium/high interaction strength?)
- Depending on your assumption, count the number of samples for each "affinity-group" (low-affinity, medium-affinity and high-affinity)

- *It might be that your estimation is not correct; that is, the cells die at lower ampicillin concentrations as expected or do not die at all. The first measurements must therefore be used to adjust the ampicillin concentration for achieving best survival curves. The first measurement with the estimated affinity of your TMDs then gives you a hint about how to proceed further.*
- After the estimation proceed as follows:
  - Group your samples and count the number of samples in each group. *Do not forget the controls! (Of course, GpA wt_{+1} is in the "high-dimerizing" and GpA $G83I_{+1}$ is in the "low-dimerizing group").*
  - Prepare a sufficient volume (by summing up the volume needed for the ampicillin solution for each well) of a 20 mg/mL ampicillin stock solution (*Weight the exact amount of Amp! Solubilize the ampicillin in expression medium*). *Always prepare at least 10% more than the calculated volume of the 20 µg/mL ampicillin stock solution to compensate possible pipetting errors.*
  - Calculate the ampicillin solutions for each well depending on the amount of samples you have. You need 1 mL for 1 sample → 12 mL (+ 5-10% more; = 12.5 – 13 mL) for 12 samples.
    Exemplary calculation for the 2. well for 12 samples from the "high-dimerization group":
    You need 12.5 mL of expression medium with a concentration of 80 µg/mL. Thus, you need 50 µL (80 [µg/mL] / 20 [mg/mL] * 12.5 mL) of the 20 mg/mL Amp stock solution.
    Proceed like this for the calculation of the volume of needed Amp stock solution for each of the wells with the following ampicillin concentrations:

For the "high-dimerization group":



Well number	concentration	final concentration (see F)
1. well	0 µg/mL	0 µg/mL
2. well	80 µg/mL	40 µg/mL
3. well	160 µg/mL	80 µg/mL
4. well	240 µg/mL	120 µg/mL
5. well	320 µg/mL	160 µg/mL
6. well	400 µg/mL	200 µg/mL
7. well	480 µg/mL	240 µg/mL
8. well	560 µg/mL	280 µg/mL
9. well	640 µg/mL	320 µg/mL
10. well	720 µg/mL	360 µg/mL
11. well	800 µg/mL	400 µg/mL
12. well	1000 µg/mL	500 µg/mL

For the “low-dimerization group”:

Well number	concentration	final concentration (see F)
1. well	0 µg/mL	0 µg/mL
2. well	40 µg/mL	20 µg/mL
3. well	80 µg/mL	40 µg/mL
4. well	120 µg/mL	60 µg/mL
5. well	160 µg/mL	80 µg/mL
6. well	200 µg/mL	100 µg/mL
7. well	240 µg/mL	120 µg/mL
8. well	280 µg/mL	140 µg/mL
9. well	320 µg/mL	160 µg/mL
10. well	360 µg/mL	180 µg/mL
11. well	400 µg/mL	200 µg/mL
12. well	500 µg/mL	250 µg/mL

Note that the final concentration results after adding a volume of bacterial culture equivalent (1 mL : 1 mL) to that of the ampicillin solution.

The ampicillin concentrations should be adjusted such that the cells of a given sample die somewhere in between the 5^{th} to the 9^{th} well. For samples with medium, very high, or very low affinity, the ampicillin concentrations should be adjusted for most accurate results. A very high ampicillin concentration in the well 12 prevents bacterial growth there.

Try to run as many samples as possible (up two 18 plates at one time are easy to handle) in parallel to improve the accuracy of your results!

- After the calculations, you can start preparing the ampicillin dilutions in Falcon tubes. Therefore, you fill the determined amount of expression medium (e.g. 12.5 mL for 12 samples) into labelled Falcon tubes with an appropriate capacity (15 mL or 50 mL).
- In the next step, you pipette out the calculated volumes (e.g. 50 µL for the 2. well) and refill the tubes by the same volume of the 20 mg/mL ampicillin stock solution.
- After the ampicillin solutions for all wells are prepared you can transfer them into the labeled 12-well plates with increasing concentrations. (Starting with no Amp in the 1. well, the lowest concentration in the 2. well, and so on (as drawn above and in D).
- The 12-well plates are then placed in a moisturized, sealed container and pre-warmed in a 37 °C incubator until their use at the end of the 4 h expression period.

3.5.2 Further preparations

- Prepare Falcon tubes for the dilution of the expression culture (OD_{600} = 0.2) for each sample. Since you need 1 mL of the OD_{600} = 0.2 cell suspension for each well of the 12-well plate, you add 12 mL of expression medium to each Falcon tube (to end up at a final volume of 13 mL after adding the expression culture → 1 mL is in addition). *(In the example you have an overall of 12 samples → you will need 12 Falcon tubes (labeled with your sample names) filled with 12 mL of expression culture).*
- As a last step, prepare cuvettes for measuring the OD_{600}. *Samples normally grow to an OD_{600} of 2.8 to 3.5. Thus, samples should be diluted (1:10 = 100 µL of cell suspension : 900 µL of LB medium) for OD_{600} measurement to be in the linear range.*

4. Determining ampicillin resistance

- After EXACTLY 4 h remove the glass-tubes containing your cultures (E) from the turning wheel.
- *All following steps have to be performed as quickly as possible (to avoid cooling of cells and media)!*
- Measure the OD_{600}. *(Always blank with the LB medium you used for diluting the samples).*
- Calculate the volume of cell suspension you need for your induced cell samples.

  Exemplary calculation:
  Measured OD_{600} in the 1:10 dilution = 0.28 (→ OD_{600} of the sample = 2.8)

  *→ 0.2 (= desired OD_{600})/2.8 (= measured OD_{600})*13 mL (final volume)*

  = <u>0.929 mL</u>

- Afterwards, adjust the OD_{600} to 0.2 for all your samples. Therefore, you need the prepared Falcon tubes with the 12 mL expression medium (3.5.2). Taken the exemplary calculation above, you then add 0.929 mL of your cell suspension and 0.071 mL of expression medium to get a final volume of 13 mL. (*In cases of OD_{600} values below 2.6 you have to remove the*

superfluous as you have to add more than 1 mL of cell suspension). Proceed in that way for all the samples.

Pipette and calculate as exact as possible – survival in ampicillin does depend on the initial cell density in a very sensitive way!

- After the 13 mL of cell suspensions at OD_{600} = 0.2 are prepared, the remaining volumes of the expression cultures are stored in the fridge for GFP measurement.
- Then, the containers with the 12- well plates are taken out of the 37 °C incubator.
  Do not take out more than 4 plates at one time – to avoid cooling of the medium!
- Add 1 mL of the OD_{600} = 0.2 cell suspension to each well of the 12-well plate. After pipetting 4 plates, place them into the moisturized containers, make sure that they are sealed properly, and incubate them for 19 h at 140 rpm in the 37 °C shaking incubator.
  (Now, the 19 h incubation time starts! Thus, make sure to note the time! Moreover, note the order of the plates in the incubator.)

5. Determination of the LD50

- After 19 h, cell density of each well of each plate is measured at λ = 550-600 nm *(depending on the filters available)* with a microplate reader. *For similar periods of incubation, measure the cells in the order you placed them in the incubator the day before.*
- The read out is a survival curve for each sample (G).
  - The LD_{50} values are then determined by the Python software with the ECCpy script installed (https://github.com/teese/eccpy) (H). The LD50 values are taken as TMD-TMD dimerization affinities, relative to the positive control (e.g. parallel GpA TMD-TMD homodimerization or antiparallel EmrE TMD4-TMD4 homodimerization).

6. Preparations for the next day

- Prepare overnight cultures of your samples for the BLaTM assay at the next day (to reproduce the same experiment for the second time).
- On Mondays *(if you perform the assay as recommended)* do another (2^{nd}) transformation if you want to do another round of LD_{50} determinations.

7. GFP fluorescence measurement

- Take the 4 h expression cultures that have been stored in the fridge while preparing the 12-well plates for the BLaTM assay.

- Take an aliquot of 200 µL of the 4 h expression culture and centrifuge it at 19,000 x g for 1 min at room temperature.
- Carefully discard the supernatant, resuspend the pellet in 200 µL phosphate-buffered saline (PBS) and transfer it into a black Nunc 96-well plate.
- Then, the GFP fluorescence is measured using a microplate reader at λ_{Ex} = 485 nm and λ_{Em} = 520 nm.
- From each of the 2-3 uninduced samples, 200 µL culture are measured in parallel and represent background fluorescence of the cells.
- For the calculation, the mean GFP fluorescence of uninduced cells is subtracted from the measured data and the fluorescence signal per single cell is calculated, assuming that an OD_{600} = 1.0 is equivalent to $8 * 10^8$ cells/mL.

BLaTM 2.0, a genetic tool revealing preferred antiparallel interaction of transmembrane helix 4 of the dual-topology protein EmrE

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J. Mol. Biol. 11, 1630-1637

Corrigendum:

We note that all concentrations of arabinose that are given in this paper (page1635) have to be multiplied by a factor of 10. Thus, use 1.33 mM arabinose instead of 133 µM and 13.3 mM arabinose instead of 1.33 mM. We regret any inconvenience.

Identifying ionic interactions within a membrane using BLaTM, a genetic tool to measure homo- and heterotypic transmembrane helix-helix interactions

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Scientific Reports, 7, 43476

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