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LDM protocol for estimating plasmid conjugation rates V.2

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ABSTRACT

This is a general protocol to implement the LDM approach for estimating plasmid conjugation rates. This version of the protocol was originally submitted with the accompanying manuscript. The recommended version of the LDM protocol will be linked in the corresponding GitHub repository. To get a conceptual overview of the approach, please read the accompanying manuscript:

Olivia Kosterlitz, Adamaris Muñiz Tirado, Claire Wate, Clint Elg, Ivana Bozic, Eva M. Top, and Benjamin Kerr. Estimating the rate of plasmid transfer with an adapted Luria–Delbrück fluctuation analysis. bioRxiv. https://doi.org/10.1101/2021.01.06.425583

The protocol is intentionally designed to facilitate proper implementation by explicitly verifying that the modeling assumptions are met. We provide 5 streamlined phases, where the last phase is executing the LDM conjugation assay. The initial 4 phases are to gather the necessary information for the proper execution of the LDM conjugation assay (i.e., phase 5). Here, we provide a brief description and goals for the initial 4 phases.

Phase 1: Minimum inhibitory concentration assay with the transconjugant-selecting medium. The goal is to find a concentration of transconjugant-selecting medium that inhibits donors and recipients while simultaneously permitting the growth of transconjugants.

Phase 2: Extinction probability assays for selective liquid and agar medium. The goal is to check the assumption that the focal cell type will successfully establish a lineage under the appropriate selective conditions.

Phase 3: Growth rate assay. The goal is to determine the donor and recipient-specific incubation times that will ensure the cultures enter exponential growth before initiating the conjugation assay.

Phase 4: Finding an incubation time and initial densities for executing the LDM conjugation assay. The goal is to find a combination of incubation time and initial densities such that the probability of a conjugation event occurring is between 0 and 1 (i.e., where there will be a mixture of turbid and non-turbid co-cultures after incubation with transconjugant-selecting medium).

Phase 5: LDM conjugation assay. The goal is to gather experimental estimates for the variables in the LDM equation $\gamma_D = f\left(\frac{1}{\tilde{t}}\left[-ln\hat{p}_0(\tilde{t})\right]^{\frac{lnD_{\tilde{t}}R_{\tilde{t}}-lnD_0R_0}{D_{\tilde{t}}R_{\tilde{t}}-D_0R_0}}\right)$ to calculate an estimate of the conjugation rate.

PROTOCOL CITATION

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Phase 1: Minimum inhibitory concentration assay with the transconjugant-selecting medium. medium. 4d

1 Prepare bacterial cultures. 2d

1. Start cultures from freezer stocks of the donors, recipients, and transconjugants.

Supplement the donor and transconjugant growth culture medium with the appropriate selection to maintain the plasmid. If using a microtiter plate format, surround the culture wells with the sterile growth medium to serve as contamination controls. Incubate **Overnight** .

2. Transfer the turbid overnight cultures from the previous step by diluting them 100-fold into growth medium initiating a second overnight culture (in order to acclimate the previously frozen strains to laboratory conditions). Prepare the growth medium and contamination controls as described in the previous step. Incubate **Overnight** .

2 Create a 2-fold gradient of the transconjugant-selecting medium. 15m

1. Add **500 µL** of growth medium to rows 2 through 8 (skip the first row) of a deep 96-well microtiter plate.
2. Add **1 mL** of growth medium supplemented with transconjugant-selecting antibiotics to row 1.
3. Create the 2-fold gradient by transferring **500 µL** serially down the rows of the deep-well plate mixing thoroughly by pipetting up and down.

The transconjugant-selecting medium is a dual-antibiotic medium to select for the conjugative plasmid and the recipient host cell. Here, we describe a one-way gradient with two antibiotics where the ratio of the two antibiotic concentrations is kept constant. A user could vary the ratio of the two antibiotics by running a two-way gradient.

3 Innoculate the transconjugant-selecting medium gradient. 1d 0h 15m

1. Dilute the donor, recipient, and transconjugant cultures 100-fold into growth medium.
2. Add **500 µL** of the diluted cultures to each well in the appropriate columns in the deep-well plate prepared in the last step.
3. Incubate **Overnight** .

4 Identify a viable transconjugant-selecting medium concentration. 1d

1. Record the turbidity from the MIC plate.
2. Analyze the turbidity data to determine the strain-specific MIC for the transconjugant-selecting medium. Identify a concentration(s) above the donor and recipient MIC, but below the transconjugant MIC.

Phase 2: Extinction probability assays for selective liquid and agar medium. 4d

5 Prepare bacterial cultures. 2d

for instructions

6 Prepare the selective liquid and agar mediums.

1h

1. Prepare two deep-well plates to be inoculated with transconjugants: one with an antibiotic-free medium and one with the transconjugant-selecting medium. Add **950 µL** of growth medium to each well in the deep-well plate. Supplement the transconjugant-selecting medium with the relevant antibiotics at the concentration identified in Phase 1.
2. Prepare two agar plates for each cell type (donor, recipient, and transconjugant): one with an antibiotic-free medium and one with the strain-specific selecting medium (donor-, recipient-, and transconjugant-selecting agar, respectively). Supplement the agar plates with the strain-specific selection at the relevant concentrations that will be used throughout the assay.

We note that the transconjugant extinction probability is only needed in the transconjugant-selecting *liquid* medium. Thus, estimating the transconjugant extinction probability in an *agar* medium is not necessary for running the LDM conjugation assay. However, we include it here as it may be useful for troubleshooting later phases of the assay.

7 Dilute and innoculate the bacterial cultures.

1d 0h 30m

1. Dilute the donor, recipient, and transconjugant cultures from step 5.
2. Innoculate both deep-well plates (antibiotic-free and antibiotic-containing) by adding **50 µL** of diluted transconjugant culture. Note: the transconjugant cultures should be diluted to add approximately 1 transconjugant cell per well.
3. Innoculate both agar plates (antibiotic-free and antibiotic-containing) for each cell type (donor, recipient, and transconjugant) with **100 µL** of the appropriate diluted culture. Note: each culture should be diluted to add approximately 30 to 300 cells per plate.
4. Incubate the deep-well plates and the agar plates **Overnight** .

8 Determine the extinction probabilities.

1d

1. Record the turbidity from both deep-well plates.
2. Calculate the extinction probability π_x of the transconjugants in the transconjugant-selecting medium:

$\pi_x = 1 - \frac{lnP_x}{lnP_0}$ where P_x is the fraction of non-turbid wells in the deep-well

plate with the transconjugant-selecting medium and P_0 is the fraction of non-turbid wells in the deep-well plate with antibiotic-free medium.

1. Record the colonies from both agar plates for each cell type.
2. Calculate the strain-specific extinction probabilities π_x for the strain-specific agar plate.

$\pi_x = 1 - \frac{C_x}{C_0}$ where C_x is the number of colonies on the antibiotic-infused plate and C_0 is the number of colonies on the antibiotic-free plate for the same diluted culture.

If the extinction probabilities are indistinguishable from zero in each selection condition needed, then the user can proceed with no adjustments necessary. However, a non-zero extinction probability is likely and can be a source of bias if not considered. In this case, there are two ways to proceed.

1. Find a selection condition where the extinction probabilities are indistinguishable from zero. Alexander and MacLean found that the antibiotic concentration being sufficiently below the MIC of the focal strain can lower the extinction probability to a point that is indistinguishable from zero. Thus, the user can re-run this phase using lower antibiotic concentrations. However, we recognize that this solution may not be possible for certain selective conditions. For instance, the donor and recipient MIC for the transconjugant-selecting condition may be too close to the transconjugant MIC, such that there are no antibiotic concentrations that yield a zero transconjugant extinction probability in the transconjugant-selecting liquid medium that still counterselect donors and recipients. In this case, the user would have to proceed with the second solution.

Alexander HK, MacLean RC (2020). Stochastic bacterial population dynamics restrict the establishment of antibiotic resistance from single cells.. Proceedings of the National Academy of Sciences of the United States of America.
https://doi.org/10.1073/pnas.1919672117

2. Corrections can be applied using the estimated extinction probabilities:
   - Liquid-specific correction for a non-zero extinction probability of the transconjugants in the transconjugant-selecting medium occurs at the end of Phase 5 when calculating

the LDM estimate.

- Agar-specific correction for the non-zero extinction probabilities of the donors and recipients on the donor- and recipient-selecting agar plates, respectively, should occur for the remainder of the phases. In other words, to obtain the "corrected" density estimates, the density estimates resulting from the colony counts on the agar plate should be divided by the agar-specific extinction probability. Thus, if the experimenter has non-zero extinction probabilities on the agar plates then the appropriate corrections need to be applied for the remainder of the protocol.

Phase 3: Growth rate assay. 4d

9 Prepare bacterial cultures

2d

for instructions

10 Dilute and innoculate bacterial cultures.

30m

1. Dilute the donor and recipient cultures 10,000-fold into growth medium.
2. Add **250 µL** of the diluted cultures to each well in the appropriate columns in a standard microtiter plate prepared in the last step. Innoculate one row per time point.

We recommend tracking the cultural density at 1-hour intervals for at least 6 hours, which would correspond to 7 rows in the microtiter plate (i.e., T = 0, 1, 2, 3, 4, 5, 6). This may need to be adjusted depending on the specific experimental system.

11 Dilute and plate bacterial cultures at 1-hour intervals.

1d

1. Dilute and plate the donor and recipient cultures from the wells in the corresponding row. Incubate the agar plates **Overnight** .
2. Incubate the microtiter plate for **01:00:00** .
3. Repeat this step for each time point.

12 Determine an appropriate incubation time for the donors and recipients to isolate the cultures in exponential growth by calculating the growth rate over time.

1d

1. Record the colonies from the agar plates.
2. Calculate the growth rates ψ using the density information from neighboring time points.

$ln(N_{t_b} / N_{t_a})$

$\psi = \frac{\ln(N_b / N_a)}{t_b - t_a}$ where N is the population density at an earlier t_a and later t_b time points

Using the growth rate calculated over time, we recommend choosing a pre-assy incubation time that is at or close to the maximum growth rate for each strain to ensure bacterial cultures enter exponential growth before the start of the conjugation assay. We note that the only requirement of the LDM protocol is to maintain a constant growth rate during the assay period.

Phase 4: Finding an incubation time and initial densities for executing the LDM conjugation assay.
5d

13 Prepare bacterial cultures
2d

for instructions

14 Isolate bacterial cultures in exponential growth
4h

1. Dilute the donor and recipient cultures 10,000-fold into growth medium.
2. Incubate for strain-specific times determined in Phase 3 to ensure the cultures enter exponential growth.

15 Dilute bacterial cultures for a full factorial treatment of initial densities and incubation times.
1h

For ease of the reader, we illustrate a concrete example in this portion of the protocol using two-deep well plate maps: an experimental plate and a control plate. A visual of these plate maps is available in SI Figure 9 in the accompanying manuscript.

For the experimental plate, four columns are used for each initial density (10^4, 10^5, and 10^6-fold dilution) where 2 rows are used for each incubation time (0, 1, 2, and 3 hours) resulting in 8 wells per density-time treatment.

For the control plate, the same factorial layout is used for the experimental plate, but the 8 wells in each density-time treatment are not all co-cultures. Donor, recipient, and transconjugant monocultures serve as controls. One of the eight wells is empty to serve as a co-culture control and at each time point an extra donor and recipient monocultures are mixed into the empty well to verify that diluting with the transconjugant-selecting medium effectively prevents conjugation. In addition, two of

the eight wells are inoculated with co-culture and are sampled at each time point to uncover densities and determine whether donors and recipients maintain constant growth.

1. Dilute the exponentially growing donor and recipient cultures into pre-warmed growth medium to the relevant initial densities (e.g., 10^4, 10^5, and 10^6-fold dilution).
2. Mix the diluted donor and recipient cultures at equal volumes to create the co-cultures.
3. Dispense **100 µL** of diluted co-culture into each appropriate well in the corresponding columns in the experimental plate and the control plate according to the plate maps.
4. Dispense **100 µL** of diluted donor, recipient, and transconjugant monocultures into each appropriate well in the corresponding columns in the control plate according to the plate map.

16 Add transconjugant-selecting medium, plate from the mating co-cultures for estimating densities, and create the relevant controls at 1-hour intervals.

2d

1. Dilute and plate from the appropriate co-culture wells in the control plate onto donor- and recipient-selecting agar plates to uncover densities and determine whether donors and recipients maintain constant growth. Incubate the agar plates **Overnight** .
2. Mix **50 µL** from the appropriate donor and recipient monocultures into the corresponding empty well in the control plate to create a co-culture control and verify that new transconjugant cells do not form via conjugation after transconjugant-selecting medium is added.
3. Add **900 µL** of transconjugant-selecting medium into each appropriate well in the corresponding two rows in the experimental plate and the control plate according to the plate maps.
4. Incubate the deep-well plates for **01:00:00** .
5. Repeat this step for each time point. Upon completion of the last time point, incubate the deep-well plates until the turbidity is stable which will most likely occur between **48:00:00** to **96:00:00** (this depends on the experimental system).

Here we are recommending that the user dilute the mating cultures ten-fold with transconjugant-selecting medium. After overnight incubation, if the turbidity of the transconjugant control well is too similar to the mating control wells making it difficult to distinguish turbidity coming from transconjugant growth and turbidity coming from inhibited/dead donor and recipient cells, then this may be indicative that the final densities of the donors and recipients are too high relative to the dilution factor. One option for mitigating this issue is to dilute the mating cultures more than ten-fold. This would require a larger deep-well format or a smaller mating culture volume.

17 **Find an incubation time and initial density where there is a mixture of turbid and non-turbid cultures and the bacterial cultures have a constant growth rate.** 1d

1. Record the colonies from the agar plates.
2. Calculate the growth rates ψ for each time point. **for the relevant equation.**
3. Record the turbidity from both deep-well plates.
4. Analyze the turbidity data (in conjunction with the growth rates) to determine an incubation time and initial density that satisfies the assay requirements.

The goal is to identify a density-time combination in the experimental deep-well plate where the p0 requirement is met and there are both transconjugant-containing and transconjugant-free co-cultures (i.e., both turbid and non-turbid co-cultures). Even though a density-time combination meets the p0 requirement, the controls need to be checked in the corresponding density-time wells in the control deep-well plate. For the donor, recipient, and transconjugant monocultures, the transconjugant-selecting medium should prohibit the growth of both donors and recipients (non-turbid wells) while permitting the growth of the transconjugants (turbid well). For the co-culture created at the specific time point, the transconjugant-selecting medium should effectively dilute and prevent conjugation (non-turbid well). For the co-cultures used for plating, the donor and recipient growth rates are calculated to verify that the growth rates were relatively constant throughout the assay period.

Phase 5: LDM conjugation assay. 6d

18 **Prepare bacterial cultures.**

and **for instructions**

19 **Dilute and innoculate bacterial cultures and plate from the mating cultures for estimating initial densities.**

1. Dilute the exponentially growing donor and recipient cultures into a pre-warmed growth medium to the relevant initial densities identified in Phase 4.
2. Mix the diluted donor and recipient cultures at equal volumes to create the co-cultures.
3. Dispense **100 µL** of diluted co-culture into each appropriate well in the corresponding columns according to the plate map. An example plate map is available Figure 5 in the accompanying manuscript.
4. Dilute and plate the diluted co-culture used in the last bullet point onto donor- and recipient-selecting agar plates for estimating the initial densities (D_0, R_0). Incubate the agar

plates **Overnight** .

5. Dispense **100 µL** of diluted donor, recipient, and transconjugant monocultures into each appropriate well in the corresponding columns according to the plate map.
6. Incubate the deep-well plate for the identified incubation time $\tilde{t}$ in Phase 4.

20 6d

At the chosen incubation time $\tilde{t}$, add transconjugant-selecting medium and plate from the mating cultures for estimating final densities.

1. Dilute and plate from the appropriate co-culture wells onto donor- and recipient-selecting agar plates for estimating the final densities ($D_{\tilde{t}}, R_{\tilde{t}}$). Incubate the agar plates **Overnight** .
2. Mix **50 µL** from the appropriate donor and recipient monocultures into the corresponding empty well(s) to create a co-culture control and verify that new transconjugant cells do not form via conjugation after the transconjugant-selecting medium is added.
3. Add **900 µL** of the transconjugant-selecting medium into each appropriate well according to the plate map.
4. Incubate the deep-well plate until the turbidity is stable which will most likely occur between **48:00:00** to **96:00:00** (this depends on the experimental system).

21 **Calculate the LDM estimate.**

1. Record the colonies from the agar plates.
2. Record the turbidity from both deep-well plates.
3. Calculate the LDM estimate for the conjugation rate.

Upon completion of this phase, the experimenter should have the following data:

- $\tilde{t}$: actual incubation time when the transconjugant-selecting medium was added.
- $D_0, D_{\tilde{t}}$: initial and final donor densities
- $R_0, R_{\tilde{t}}$: initial and final recipient densities
- $\hat{p}_0$: the proportion of non-turbid cultures from the deep-well plate
- f : the reciprocal of the co-culture volume in mL
- Confirmation from the controls. These are the same controls used in Phase 4. **for details.**

The equation to be applied will depend on the value of the extinction probability for the transconjugants (i.e., zero or non-zero) in the transconjugant-selecting medium determined in Phase 2. **for details.**

Step 21 includes a Step case.

Zero transconjugant extinction probability

Non-zero transconjugant extinction probability