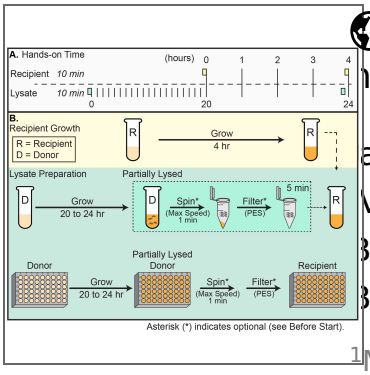

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Transformation of Bacillus subtilis with DNA obtained from natural donor cell lysis

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Protocol status: Working
We use this protocol and it's working as described.

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ABSTRACT

Natural transformation is a mechanism many bacteria use to acquire free DNA from the environment. *Bacillus subtilis* (*B. subtilis*) can perform natural transformation by entering the physiological state of competence, wherein *B. subtilis* produces the necessary proteins to acquire DNA and subsequently integrate homologous DNA into its genome. This trait is advantageous to researchers, for competence can be induced and utilized for efficient construction of new bacterial strains. For this purpose, the primary source of donor DNA is typically purified DNA in the form of genomic DNA (gDNA) or plasmid DNA. The benefit of using purified DNA is that a new strain can be constructed efficiently without concern for contamination with the donor cells. This involves growing both a donor and recipient strain, inducing competence in the recipient, extracting donor gDNA, and subsequently mixing the gDNA and recipient cells for the transformation to occur. The downside to using purified DNA is the purification process itself. Bacterial gDNA extraction usually involves phenol and chloroform or a commercial nucleic acid extraction kit. The time and resources involved in preparation of purified donor DNA severely limits the number of donors that are possible to prepare and use in a single experiment.

We propose to exploit natural cell lysis as a new method for transforming *B. subtilis* to bypass the time-intensive gDNA purifications. Described herein, the donor strain is grown to the point of sufficient, natural cell lysis with minimal post-incubation benchwork to obtain a lysate containing the desired donor DNA. This lysate is used as the donor substrate for a subsequent transformation. The growth and handling of the recipient is the same regardless of the donor DNA source. There are a few major advantages of this method. With this protocol, a researcher can obtain sufficient DNA for a transformation with less than 10 minutes of benchwork, affording the researcher more flexibility in scheduling their day. The utility of the application is further realized with its scalability. The natural donor cell lysis can occur in 96-well plates, and the resource cost of this is minimal compared to performing an equivalent number of gDNA extractions. Moreover, previously frozen lysates can be used as the donor DNA to obtain successful transformants with minimal loss of efficiency. By using cell lysate as the donor DNA source instead of purified DNA, we developed a method that reduces the time at the bench and improves scalability.

GUIDELINES

- Sterile technique is important during the protocol.

MATERIALS

Materials:

Note: Items necessary for protocols with a filtration step are marked with an asterisk (*****).

1. *Bacillus subtilis* (*B. subtilis*) **Note**: *B. subtilis subsp. subtilis str. 168* or *B. subtilis str.* PY79 are typically used.

2. Sterile pipette tips
3. Sterile 18 mm glass tubes (Fisher Scientific, catalog number: S63300)
4. Sterile 13 mm glass tubes (Fisher Scientific, catalog number: 14-961-27)
5. Sterile stainless-steel closures (18 mm neck size) (VWR, catalog number: 71000-330)
6. Sterile stainless-steel closures (13 mm neck size) (VWR, catalog number: 71000-322)
7. Sterile, clear, flat-bottom 96-well plates (Fisher Scientific, catalog number: 07-200-89)
8. Sterile, square-well, deep-well, 96-well plates (VWR, catalog number: 89237-526)
9. Aeration seal (Thomas Scientific, catalog number: T796200)
10. *****Sterile 1.7 mL microfuge tubes (Axygen, catalog number: MCT-175-C)
11. *****3cc disposable syringes (BD, Luer-Lok™, catalog number: BD-309585)
12. *****Sterile 30 mm Polyethersulfone (PES) syringe filter (0.22 µm pore size) (Celltreat, catalog number: 229747)
13. *****Sterile Steritop Threaded Bottle Top Filter (PES, 0.22 µm pore size, 45 mm neck size) (Millipore, catalog number SCGPT02RE)
14. 100 x 15 mm petri dishes (Crystalgen, catalog number: S-3006)
15. Sterile glass beads (diameter 0.2-0.3 mm) (Sigma-Aldrich, catalog number: G1277)
16. Sterile streaking sticks (flat toothpicks) (VWR, catalog number: 500029-808)
17. Inoculating sticks
18. LB agar (BD, Difco™, catalog number: 244510)
19. ddH_2O
20. Glucose (Amresco® Inc., catalog number: 50-99-7)
21. Casein hydrolysate (EMD, catalog number: 1.02245.0500)
22. KH_2PO_4 (Fisher Scientific, catalog number: P285-500)
23. K_2HPO_4 (Dot Scientific Inc., catalog number: DSP41300-1000)
24. Sodium citrate dihydrate ($Na_3C_6O_7 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: S4641-500G)
25. Potassium glutamate monohydrate (Sigma-Aldrich, catalog number: G1501-100G)
26. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)
27. Ammonium iron citrate (J.T. Baker, catalog number: 1185-57-5)
28. Antibiotics (for selection)
29. 10x competence medium (MC) (see Recipes)
30. 1 M $MgSO_4$ (see Recipes)
31. 1,000x Ferric Ammonium Citrate (see Recipes)

Equipment:

1. Pipettes
2. Autoclave
3. Roller drum with disc for 18 mm tubes (New Brunswick, TC-7 style motor with disc for 18 mm tubes)
4. Vortexer (VWR, catalog number: 58816-121)
5. 30°C incubator
6. 37°C incubator

7. Microcentrifuge capable of 21,100 x g

Recipes:

- **10x Medium for Competence (MC, to make 100 mL)**
  10.7 g K_2HPO_4
  5.2 g KH_2PO_4
  20 g Dextrose anhydrous (glucose)
  0.88 g $Na_3C_6O_7 \cdot 2H_2O$ (sodium citrate dihydrate)
  1 mL 1,000x Ferric Ammonium Citrate (2.2% stock) (see Recipes)
  1 g Casein Hydrolysate EDM
  2.2 g Potassium Glutamate monohydrate
  Add ddH_2O to 100 mL
  Mix thoroughly; filter sterilize using 0.22 µm Threaded Bottle Top Filter
  Store at -30°C

- **1 M $MgSO_4$ (to make 100 mL):**
  12 g $MgSO_4$ (Magnesium Sulfate Anhydrous)
  Add ddH_2O to 100 mL
  Sterilize by autoclaving
  Store at room temperature

- **1,000x Ferric Ammonium Citrate (to make 100 mL):**
  2.2 g Ammonium iron citrate
  Add ddH_2O to 100 mL
  Filter sterilize using 0.22 µm Threaded Bottle Top Filter
  Wrap in aluminum foil. Note: This solution is light sensitive.
  Store at room temperature

- **Agar Plates**
  40 g LB Agar
  1 L House Distilled H_2O (diH_2O)
  Mix thoroughly with stir bar and stir plate
  Sterilize by autoclaving on a liquid cycle at 121.1°C for 40 or 45 minutes
  After sterilization and if necessary, add sterile antibiotic to the following final concentrations:
  100 µg/mL of spectinomycin
  10 µg/mL of kanamycin
  10 µg/mL of tetracycline
  5 µg/mL of chloramphenicol

Additional Notes:

- This protocol was exclusively tested with *Bacillus subtilis* strain PY79 but will likely work with other domesticated and undomesticated strains.
- If working with auxotrophic strains, then ensure appropriate amino acid supplementation in the MC medium. E.g., *B. subtilis str.* 168 is a tryptophan auxotroph, so tryptophan must be supplemented to enable propagation of *B. subtilis str.* 168.

SAFETY WARNINGS

- Follow the safety guidelines on each of the individual ingredients when preparing the materials and reagents.
- Follow appropriate local biosafety guidelines for handling microorganisms and recombinant samples.

BEFORE START INSTRUCTIONS

- Check the donor and recipient resistance markers to make sure they are compatible.
- If selection against donor cells is not possible, then it is necessary to filter the donor DNA crude cell lysate.
- It is not necessary to filter the crude lysate from the donor if selection can be used to remove donor bacteria.
- If filtration is necessary and many donor samples are to be used, such as in a 96-well plate format, then this will increase the cost of the protocol. Microfuge-based filtration of individual samples was validated here. 96-well plate filtration was not conducted here but also should be compatible with this transformation procedure.

Two days before the transformation: Preparing donor and rec... 10m

1 Streak out the **donor** strain onto a LB agar plate with appropriate antibiotics and incubate at 37 °C for approximately 18 hours to obtain fresh, single colonies. 5m

2 Streak out the **recipient** strain onto a LB agar plate and incubate at 30 °C for, at most, 42 hours to obtain fresh, single colonies in two days. 5m

One day before the transformation: Preparing the donor cultu...

3 If the **recipient** was not streaked out yesterday, then streak out the **recipient** onto a LB agar plate

with appropriate antibiotics and incubate at 37 °C for fresh, single colonies about 18 hours later.

4 In 18mm sterile, glass test tubes, prepare 1 mL 1x MC medium for each **donor**. 5m

4.1 Alternatively, standard 96-well plates may be used with 200 µL of 1x MC medium per well or square deep-well, 96-well plates may be used with 400 µL of 1x MC medium per well.

Note: ensure there is enough **donor** culture for the number of unique transformations.

4.2 Table for making 1 mL or 400 µL of 1x MC medium. 5m

	A	B
	900 µL sterile ddH2O	360 µL sterile ddH2O
	3 µL 1M MgSO4	1.2 µL 1M MgSO4
	100 µL 10x MC (stored at -30°C)	40 µL 10x MC (stored at -30°C)
	1 mL of 1x MC	400 µL of 1x MC

Column A is for **1 mL of 1x MC medium.**
Column B is for **400 µL of 1x MC medium.**

5 Inoculate 1 mL 1x MC medium with one **donor** colony. 1m

6 Vortex at setting 3 to 4 for 10 seconds and remove the inoculating stick. 30s

00:00:10 Vortex

6.1 Alternatively, if using 96-well plates, then use a pipette tip to inoculate each well with a colony.

7 Incubate **donor** culture at 37 °C with aeration. 2m

Note: 18 mm test tubes can aerate rolling in a roller drum.
Note: 96-well plates should have an aeration seal applied and aerate on a platform shaker at 350 rpm.

8 Incubate for 20-24 hours to obtain sufficient cell lysis. 1d

24:00:00 Donor Incubation

Day of the transformation: Mixing donor and recipient. 4h

9 Four hours before the end of the 20-24 hours incubation time for the donor, prepare 1 mL 1x MC medium for the **recipient** in sterile, 18mm glass test tubes.

9.1 Alternatively, standard 96-well plates may be used with 200 µL of 1x MC medium per well or square deep-well, 96-well plates may be used with 400 µL of 1x MC medium per well.

Note: ensure there is enough **recipient** culture for the number of unique transformations.

9.2 Table for making 1 mL or 400 µL of 1x MC medium.

	A	B
	900 µL sterile ddH2O	360 µL sterile ddH2O
	3 µL 1M MgSO4	1.2 µL 1M MgSO4
	100 µL 10x MC (stored at -30°C)	40 µL 10x MC (stored at -30°C)
	1 mL of 1x MC	400 µL of 1x MC

Column A is for **1 mL of 1x MC medium.**
Column B is for **400 µL of 1x MC medium.**

10 The **recipient** can be grown in either glass test tubes or 96-well plates:

Will the recipient be grown in Test Tubes or 96-well plates?
(Select from the first step case below and continue within that step case for the remainder of the protocol.)
Step 10 includes a Step case.

Test Tubes
96-well Plates

step case 3m

Test Tubes

This step case is to be used when the **recipient** is growing in a test tube.

11 Inoculate 1 mL 1x MC medium with one **recipient** colony using a sterile inoculation stick.

12 Incubate the **recipient** cultures at 37 °C for 4 hours with aeration. 4h
Use the same aeration methods as used for the donor cultures.

go to step #7 for aeration conditions

04:00:00 Recipient Incubation

13 Shortly before 4 hours have elapsed, prepare the **donor** for transformation:

Is it necessary to filter the donor? (see Before Start)
Step 13 includes a Step case.
Filtration
No Filtration

step case

Filtration

This step case should be used when the **donor** needs to be filtered and the **recipient** is growing in a test tube.

14 **Is the donor growing in test tube or 96-well plate format?**
Step 14 includes a Step case.
Test Tubes
96-well Plate

step case 2m

Test Tubes

This step case should be used with the **donor** is growing in a test tube and:

- when the **donor** needs to be filtered and
- when the **recipient** is growing in a test tube.

15 Aliquot 1 mL sample of the **donor** to a 1.7-microfuge tube.

16 Spin the sample at 21,000 x g (max speed) in a centrifuge for 1 minute. 1m

21000 x g, 00:01:00 , Spin the donor sample

17 Remove supernatant to a new microfuge tube. Take care not to disrupt cells and cell debris in pellet. 1m

18 Filter the supernatant through a 0.22 µm PES filter. 1m

This filtrate is the source of donor DNA.
Note: cellulose acetate and nylon filters are equally effective, but PES is usually most cost effective.

19 **Donor** DNA source may sit at room temperature while preparing the transformation tubes, but it can be stored at -30 °C.

20 Transfer 200 µL of the **recipient** to a sterile, 13mm glass tube (this is the transformation tube). 2m

21 Transfer 10 µL of **donor** filtrate to the transformation tube. 2m

22 Place transformations at 37 °C for 2 hours with aeration. 2h
Use the same aeration methods as used for the donor cultures.

go to step #7 for aeration conditions

Note: Use the large 18 mm tubes with small caps or 1 mL pipette tips inside to hold the small 13 mm glass tubes (See Figure 1 in Loyo & Burton 2018, link in *References Section*).

02:00:00 Transformation Growth

23 Dilute transformation cultures 1:10 and 1:100. 5m

24 Dispense 100 µL to 200 µL on appropriate selective plates. 10m

Note: ensure the agar plates are not so dry that they prevent even spreading.

25 Use 4-5 beads per petri plate to spread the cells evenly. 5m

Note: do not allow the liquid to sit on the agar plate for too long before spreading.

26 Incubate plates at 37 °C overnight, 30 °C for two days, or Room temperature for three days.

Note: **the resulting colonies should be the desired transformants.**