Φ29 TRANSFORMATION OF DNA TARGETS INTO *BACILLUS SUBTILIS*

The technique described in this protocol is used to transform a target plasmid into *B. subtilis* strains. To do this, target DNA is amplified to generate a double-stranded multimeric molecule (dsmDNA) which improves transformation efficiencies in the bacterium. Transformation itself is done using a modified method established by Anagnostopoulos and Spizizen[1]. This protocol takes advantage of the natural competency mechanism found in *B. subtilis*, which is activated at high cell densities and in nutrient deficient conditions[2]. This protocol is divided into 2 sections, for a total of 3 days of work in the alb. This protocol only works if your gene is already cloned inside a plasmid.

(A) Generation of multimeric DNA through Rolling Circle Amplification (RCA)[3]

NOTE: RCA is done using NEB's Φ29 DNA polymerase. Any Φ29 DNA polymerase will work. If using a different polymerase, please look at the manufacturer's recommended protocol for RCA and reaction mixture preparation.

1. Prepare a 25 µL RCA reaction and a (-) control as follows:

Reagent	Stock concentration	Final concentration	Sample (µL)	(-) control (µL)
ddH_2O	-	-	15.5	16
Φ29 DNA polymerase reaction buffer	[10 X]	[1 X]	2.5	2.5
dNTPs mix	[10 mM]	[0.2 mM]	0.5	0.5
DNA template	[1 ng/µL]	[1 ng]	1	1
Random hexamers*	[50 µM]	[5 µM]	2.5	2.5
Recombinant albumin	[1 mg/mL] **	[0.1 mg/mL]	2.5	2.5
Final volume:			24.5	25

*Random hexamers can be purchased form any provider and need to be resistant to degradation. Resistant hexamers should have phosphorothioate bonds found at the 3' end (5' – NNNN*N*N – 3').
** Must be prepared beforehand from the provided 20 mg/mL stock.

2. Prime template DNA using the following thermal-cycling protocol:

Denaturation	95 °C	3 minutes
Template priming [a]	95 °C to 20 °C	30 minutes

[a] Temperature will go down by 2.5 °C per minute of incubation.

3. Add 0.5 µL of Φ29 DNA polymerase [10 U/µL] (final concentration 5 U) to sample tube and use the following thermal-cycling protocol for RCA of desired template:

Extension	30 °C	18 hours
Heat inactivation	65 °C	10 minutes
Hold	12 °C	∞

4. Verify successful RCA via gel electrophoresis.
5. Perform a PCR clean-up on the successful RCA samples and (-) control using the appropriate kit. Follow the manufacturer's protocol provided with the kit.

(B) Transformation in *B. subtilis*.

1. Inoculate a single colony of the desired B. subtilis strain into 10 mL of LB media
2. Grow overnight at 37 °C with 200-250 rpm shaking. During the incubation, prepare 300 mL of competence media as follows:

Reagent	Mass	Final concentration (g/L)
KH_2PO_4 (monobasic)	3.21 g	10.7
K_2HPO_4 (dibasic)	1.56 g	5.2
Glucose	6.00 g	20
Trisodium citrate dihydrate	0.26 g	0.88
Ferric ammonium citrate	6.6 mg	0.022
Potassium aspartate	0.75 g	2.5
L-Trp	12.0 mg	0.04
Yeast extract	0.15 g	0.05 % *w/v*

   a. Dissolve solids from the above table in 283 mL of ddH_2O, then prepare a 1 M solution of $MgSO_4$ and a 1 mM solution of $MnCl_2$. Sterilize solutions by autoclave.
   b. Add 3 mL of $MgSO_4$ and 45 mL of $MnCl_2$ solutions into the competence media under sterile conditions (final concentrations of $MgSO_4$ and $MnCl_2$: 10 mM and 150 nM respectively). This will complete the recipe of the media and can be stored at room temperature in a sealed bottle until needed.

3. The next day, measure OD_{600} of culture, then dilute overnight culture in 10 mL of competence media to an OD_{600} of 0.1.
4. Grow at 37 °C with 200-250 rpm shaking until an OD_{600} of 1.5 or higher.
5. Transfer a 120 uL aliquot of the culture into a sterile eppendorf tube and add 50-100 ng of the purified multimeric DNA.
6. Mix the tube by flicking then incubate tube at 37 °C with 200-250 rpm shaking for 2 hours.
7. Plate whole contents of tube onto an LB agar plate containing the appropriate antibiotic for clone selection.
8. Grow overnight in a at 37 °C incubator.

References

(1) Anagnostopoulos, C.; Spizizen, J. Requirements for Transformation in Bacillus Subtilis . *J. Bacteriol.* **1961**, *81* (5), 741–746. https://doi.org/10.1128/jb.81.5.741-746.1961.

(2) Dubnau, D.; Blokesch, M. Mechanisms of DNA Uptake by Naturally Competent Bacteria. *Annu. Rev. Genet.* **2019**, *53*, 217–237. https://doi.org/10.1146/annurev-genet-112618-043641.

(3) Dean, F. B.; Nelson, J. R.; Giesler, T. L.; Lasken, R. S. Rapid Amplification of Plasmid and Phage DNA Using Phi29 DNA Polymerase and Multiply-Primed Rolling Circle Amplification. *Genome Res.* **2001**, *11* (6), 1095–1099. https://doi.org/10.1101/gr.180501.