Electroporation of Bacillus Firmus 2020.02.15

Competent Cell Preparation

1. 5 ml of LB Medium where inoculated with *B. Firmus* and *B. Subtillis* respectively
2. The cells where incubated over night at 37°C and 200 rpm

3. 3.125 ml of the ON cultures where transferred into 50 ml of growth medium (diluted 16-Fold)
4. The culture was incubated at 37°C and 200 rpm until the OD_{600} reached 1.0
   OD Measurement *B. Firmus*: 0.404 – 0.456 – 0.848 – 1.286 (Avarage of 5 values)
   OD Measurement *B. Subtillis*:0.440 – 0.490 – 0.816 – 1.310
5. The culture was chilled by transferring the tube on ice and keeping it there for 10 minutes
6. The culture was centrifuged at 3202 rcf for 30 minutes at 4°C
7. The pellet was suspended in 15 ml of electroporation (washing-) buffer precooled to 4°C
8. Centrifugation and suspension was repeated 3 times
9. The pellet of the last centrifugation was suspended in 1.25 ml electroporation buffer
   OD Measurement *B. Firmus*: 40.8
   OD Measurement *B. Subtillis*: 36.34
10. The OD was set to 100 by centrifugation (5000 rcf / 5 min) and resuspending in the calculated volume of electroporation buffer supernatant to achieve an OD of 100
11. The cells where stored at -80°C to perform electroporation the next day

Electroporation

1. 60 µl of thawed (on ice) cells where transferred to precooled electroporation cuvettes with 1 mm gap
2. 100 ng precooled DNA was added to the electroporation cuvette
3. The mixture inside the gap was stirred with the pipette and gently knocking of the cuvette on the surface of the bench covered with tissue
4. The mixture was incubated for 15 minutes on ice
5. The mixture was shocked with a single pulse (3.5 – 5 ms)
6. The cells where diluted with 1 ml of recovery medium immediately after the shock
7. The recovering cells where incubated for 3 hours at 37°C and 200 rpm in 15 ml falcon tubes positioned in an angle to increase the surface of the mixture to the surrounding oxygen
8. The cells where spread on selective medium and non-selective medium

Samples

Organism	Plasmid	Antibiotic
1. Bacillus Subtillis	pAD43-25	Chloramphenicol
2. Bacillus Subtillis	pHT01	Chloramphenicol
3. Bacillus Subtillis	pBSMul1	Kanamycine
4. Bacillus Subtillis	Negative Control	Chloramphenicol
5. Bacillus Firmus	pAD43-25	Chloramphenicol
6. Bacillus Firmus	pHT01	Chloramphenicol
7. Bacillus Firmus	pBSMul1	Kanamycine
8. Bacillus Firmus	Negative Control	Kanamycine

Troubleshooting and later changed parameters

- Ratio of cell concentration (OD 100) to the mass of plasmid (100 ng)?
- Centrifugation of recovered cells and resuspending in volume-reduced supernatant after 3 h incubation to increase the chance of having transformed cells on one regular sized selective LB agarplate. The total volume of the recovered mixture is 60 µl cells (+ 2-5 µl DNA) + 1 ml recovery medium = 1.062 – 1.065 ml. The protocol advises to use a maximum of 200 µl per agarplate, requiring 5 agarplates per sample.
- Calculations correct ? (see Page 5)
- Applied Voltage, booth 1800 KV and 2100 KV where tried with electroporation cuvettes with 1 mm gap with a time constant of at least 5 ms
- Time of Incubation after electroporation: 3h and 4h
- Cells where concentrated before electroporation to OD 120 in a later performed electroporation
- Weight of used Plasmid: 200 ng of plasmid DNA instead of 100 ng was mixed with the electrocompetent cells
- Presence of organism of interest *B.firmus* on the currently used plates and the positive controls after electroporation was verified via colony PCR
- The protocol worked for *E.coli:* Cells where able to grow on ampicillin selective medium where cells without plasmid where not growing. Gfp expression was verified by fluorescence microscopy and plasmid DNA was harvested via plasmid preparation after preparation of an over Night culture of transformed *E.coli* cells in ampicillin selective liquid medium

Media

Growth Medium (55 ml + Blank)

LB:	1% Tryptone, 0,5% yeast extract, 0,5% NaCl
Sorbitol:	0,5 mol / L
Glycine Betaine:	7,5 % (W/V)

Electroporation Buffer (0,5 ml)

Sorbitol:	0,5 mol / L
Mannitol:	0,5 mol / L
Glycine Betaine:	7,5 % (W/V)
Glycerol:	10 % (W/V)

Electroporation (Washing-) Buffer (150 ml)

Sorbitol:	0,5 mol / L
Mannitol:	0,5 mol / L
Glycerol:	10 % (W/V)

Recovery Medium (1 ml)

LB:	1% Tryptone, 0,5% yeast extract, 0,5% NaCl
Sorbitol:	0,5 mol / L
Mannitol:	0,38 mol / L

Calculations

Growth Medium 100 ml

Sobitol:
V = 100 ml = 0,1 l
c = 0,5 mol / l
M = 182,2 g / mol

n = c * V = 0,5 mol / l * 0,1 l = 0,05 mol
m = M * n = 182,2 g / mol * 0,05mol = 9,11 g

Glycine Betaine: (7,5 % (W/V)):
m1 / V1 = m2 / V2
m2 = m1 * V2 / V1 = 7,5g * 100 l / 100 l = 7,5 g

Electroporationbuffer 10 ml

Sobitol:
V = 10 ml = 0.01 l
c = 0,5 mol / l
M = 182,2 g / mol

n = c * V = 0,5 mol / l * 0,01 l = 0,005 mol
m = M * n = 182,2 g / mol * 0,005 mol = 0.91 g

Mannitol:
V = 10 ml = 0.01 l
c = 0,5 mol / l
M = 182,2 g / mol

n = c * V = 0,5 mol / l * 0,01 l = 0,005 mol
m = M * n = 182,2 g / mol * 0,005 mol = 0.91 g

Glycine Betaine: (7,5 % (W/V)):

m1 / V1 = m2 / V2
m2 = m1 * V2 / V1 = 7,5g * 10 ml / 100 = 0.75 g

Glycerol: 10 % (W/V):

c1 * V1 = c2 * V2
10 % * 10 ml = 87 % * x ml
x ml = 10 % * 10 ml / 87 % = 1.15 ml
10 ml = 1.15 ml (Glycerol) + 8.85 ml (ddH_2O)

Electroporation (Washing-) Buffer 150 ml

Sobitol:
V = 150 ml = 0.15 l
c = 0,5 mol / l
M = 182,2 g / mol

n = c * V = 0,5 mol / l * 0,15 l = 0,075 mol
m = M * n = 182,2 g / mol * 0,075 mol = 13.66 g

Mannitol:
V = 150 ml = 0.15 l
c = 0,5 mol / l
M = 182,2 g / mol

n = c * V = 0,5 mol / l * 0,15 l = 0,075 mol
m = M * n = 182,2 g / mol * 0,075 mol = 13.66 g

Glycerol: 10 % (W/V):

c1 * V1 = c2 * V2
10 % * 150 ml = 87 % * x ml
x ml = 10 % * 150 ml / 87 % = 17.24 ml
150 ml = 17.24 ml (Glycerol) + 132.76 ml (ddH_2O)

Recovery Medium 50 ml

Sobitol:
V = 50 ml = 0,05 l
c = 0,5 mol / l
M = 182,2 g / mol

n = c * V = 0,5 mol / l * 0,05 l = 0,025 mol
m = M * n = 182,2 g / mol * 0,025mol = 4,555 g

Mannitol:
V = 50 ml = 0,05 l
c = 0,38 mol / l
M = 182,2 g / mol

n = c * V = 0,38 mol / l * 0,05 l = 0,019 mol
m = M * n = 182,2 g / mol * 0,025mol = 3,4618 g

Antibiotics

Chloramphenicol

Stock: 25 mg / ml
Work: 10 µg / ml
V = 500 mL (= 20 Plates)

m_{Work}: m1 / V1 = m2 / V2
<-> 10 µg / 1 ml = X / 500 ml
<-> X = 10 µg * 500 ml / 1 ml = 5 mg (*CM required to have 500 ml of a 10 µg / ml solution*)

V_{Stock}: m1 / V1 = m2 / V2
<-> 25 mg / 1 ml = 5 mg / X
<-> X = 5 mg * 1 ml / 25 mg = 0,2 ml (*of 25 mg / ml CM stock solution is required for 500 ml of a 10 µg / ml work solution*)

Kanamycine

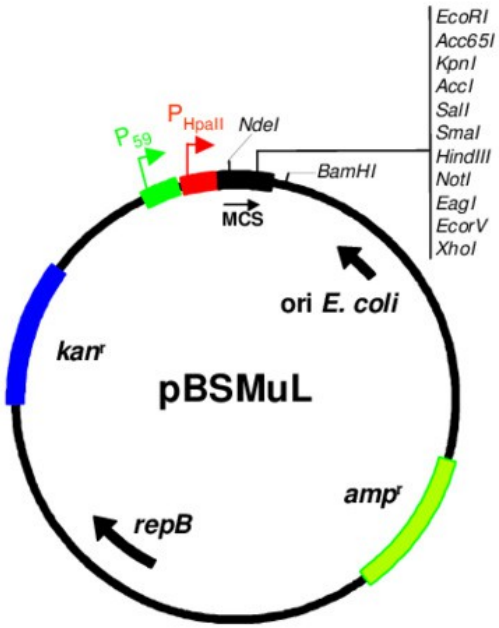
Stock: 50 mg / ml
Work: 50 µg / ml
V = 500 mL (= 20 Plates)

m_{Work}: m1 / V1 = m2 / V2
<-> 50 µg / 1 ml = X / 500 ml
<-> X = 50 µg * 500 ml / 1 ml = 25 mg (K*M required to have 500 ml of a 50 µg / ml solution*)

V_{Stock}: m1 / V1 = m2 / V2
<-> 50 mg / 1 ml = 25 mg / X
<-> X = 25 mg * 1 ml / 50 mg = 0,5 ml (*of 50 mg / ml KM stock solution is required for 500 ml of a 50 µg / ml work solution*)

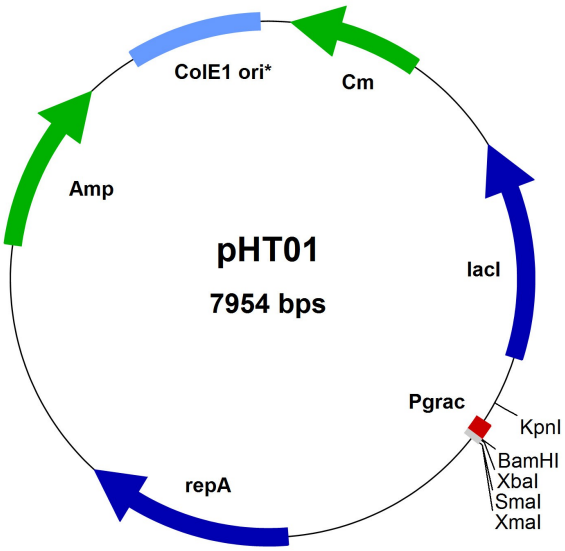
Plasmids:

PBSMul1



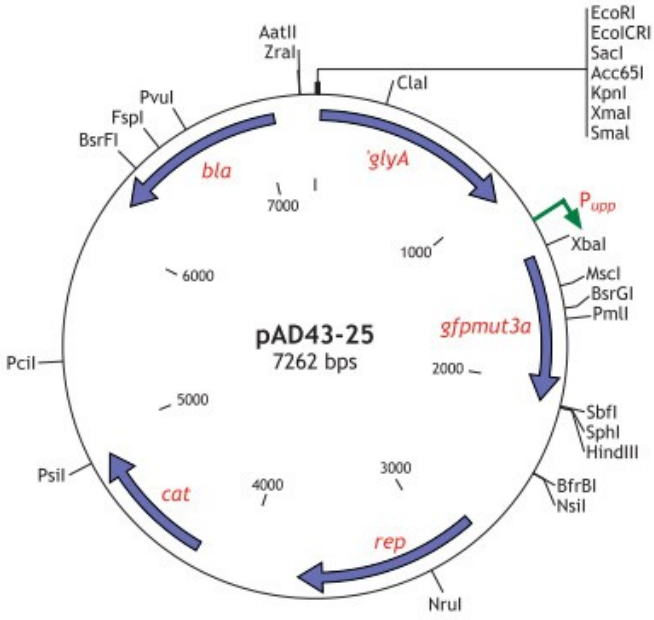
P HpaII , constitutive Gram-positive promoter;
P 59, second constitutive gram-positive promoter only present in pBSMuL2;
MCS, multiple cloning site;
ori E. coli, gene required for replication in E. coli;
amp r , β-lactamase gene conferring ampicillin resistance in E. coli;
repB, gene required for replication in B. subtilis;
kan r , kanamycin resistance gene

pHT01



The pHT01 is a high-level intracellular expression vector for recombinant protein production with *B. subtilis*. The vector is based on the strong σA-dependent promoter preceding the groES-groEL operon of *B. subtilis* which has been converted into an efficiently controllable (IPTG-inducible) promoter by addition of the lac operator. pHT01 is an *E. coli/ B. subtilis* shuttle vector, that provides ampicillin resistance to *E.coli* and chloramphenicol resistance to *B. subtilis*.

PAD43-25



Features:

*gfp*mut3a	promoter-less gene encoding a variant of Green Fluorescent Protein from plasmid pFPV25 Valdivia, R. H. and S. Falkow. 1997. Science **277**:2007-2011
rep	Replication initiation protein from cryptic rolling circle plasmid pTA1060 (GenBank U32380) from *Bacillus subtilis "natto"*
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
'glyA	last 1010 bp of the *Bacillus cereus* structural gene for glycine/serine hydroxymethyltransferase
P_{upp}	constitutive promoter from the *Bacillus cereus upp* (uracil phosphoribosyltransferase) gene

Description: pAD43-25 is a shuttle vector, replicating in *E. coli* from the pBR322 origin and in *Bacillus* from the pTA1060 origin. A chromosomal fragment from *Bacillus cereus* UW85, containing the first 21 bp of the *upp* gene and all of its upstream regulatory regions, allows for high-level constitutive expression of a Green Fluorescent Protein variant.

Construction: pAD43-25 was constructed by ligating chromosomal DNA *Sau3*A fragments from UW85 into the pAD123 BamHI site and screening for clones that express high levels of GFP in *Bacillus cereus* during vegetative growth.

Use: The placement of the *B. cereus* UW85 *upp* promoter upstream of *gfp*mut3a allows for constitutive expression of a mutant GFP that has been optimized for use in fluorescence-activated cell sorting, with an optimal excitation wavelength of 498 nm. This shuttle vector should replicate in a wide variety of Gram-positive organisms along with *E. coli*. Plasmid pAD43-25 should serve as a useful marker for quantifying or sorting cells in a wide variety of applications.