PROTOCOLE USED FOR CONSTRUCTION OF THE *LACTOBACILLUS SAKEI* 23K SHOTGUN LIBRARY

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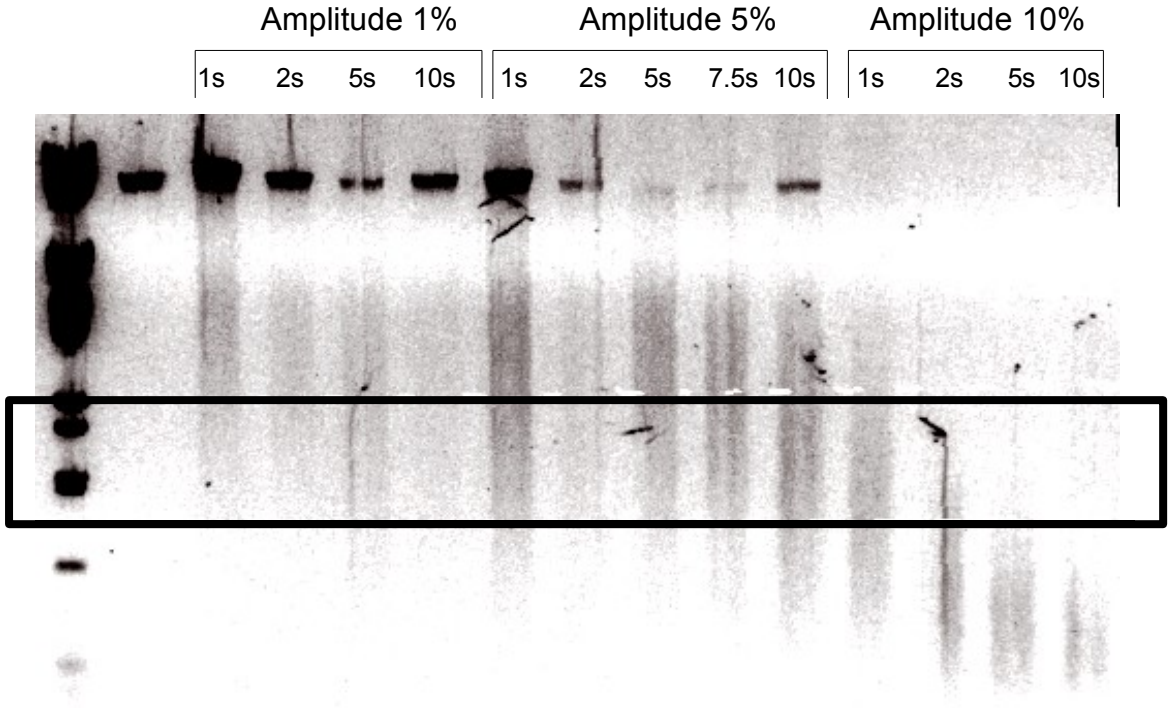
DNA PREPARATION

Chromosomal DNA was prepared from cells at stationary phase of growth and according to the protocol described by et Anderson *al.*, 1983. AEM 46(3): 549-552. Two additional cleaning steps were conducted to unsure a better quality of the DNA:

- Heat DNA at 72°C for 10 min (the DNA slightly denature and liberate mRNAs still attached to chromosomal DNA)
- Add 5 µl of RNase 10mg/ml and incubate 20 min at 37°C
- Add 20 µl of proteinaseK 20 mg/ml and incubate 20 min at 60°C
- Phenol/chloroform extraction and ethanol precipitation.

SONICATION

This procedure allows a mechanical shearing of the DNA, a process already initiated by the phenol/chloroform extraction (producing an slight shearing to 100/50 kb fragments). The shearing of the DNA by sonication is considered as a random process. However, Chromosomal DNA are known to contain regions with deviant GC% content and variations in supercoiled structures. Therefore at a given sonication condition, some DNA regions will be sheared more efficiently to small pieces than other DNA regions. If your plan is to carry out a size selection for cloning (you want inserts size to be from 1 kb to 2 kb), this means that you'll bias your library and miss much of 'fragile' regions and 'resistant' regions. To avoid such a bias, the best is to try different sonication conditions and to pool them after size selection as shown below:



The best sonication condition is found in a narrow range of amplitude % and time pulse, between A=5% Tp=7.5s and A=10%, Tp=1s using a 3 mm Ø probe. Aside this range, chromosomal DNA is either not efficiently sheared (bold band around 15 kb) but a fraction of it

is indeed sheared (fragile zones), or is reduced to small bands of < 100 bp but a fraction of it shows higher size (resistant zones). Choosing 4 conditions on the range and pooling them should give less biased sonicated DNA.

NB: DNA concentration seems to have no effect on sonication results.

- Perform 4 sonications in 170µl of TE containing 20 µg of chromosomal DNA.
- Take an aliquot of 10 µl and verify on gel the sonication efficiency and check it does correspond to your sonication set up.

END REPAIRING

The most critical step. The cloning efficiency will depend on it !

- Add 1µl of RNAseA 20 mg/ml per tube. Incubate 20 min at 37 °C.
- Extract with PCI (phenol/chloroform/isoamylic alcohol) and recover the upper water phase.
- Add 19 µl of sodium acetate 3M pH = 7.0 and precipitate with 2.5 volume of ethanol 100% (wait at least 2 hours at -20°C). Centrifuge 30 min at 15000 rpm and 4°C. Wash with ethanol 70%. Dry the pellet.
- Resuspend in 200 µl of 1x T4 DNA polymerase buffer. **Mix the 4 fractions** and keep half of it for further use if your DNA end-repairing trial is failing. Separate the second half into **10 fractions of 40 µl** (it is about 4 µg of DNA per fraction)
- Add - 6 µl of dNTPs 0.5 mM
  - 3 µl of a T4 DNA polymerase 6u (3u/µl) Klenow 10u (10u/µl) mixture.

- Vortex gently et incubate 30 min **precisely** at room temperature (~20°C).

- Inactivate the two End-Repairing enzymes by heating 15 min at 75 °C.
- Cool down on ice 10 min (very important !)
- Add - 3 µl of rATP 0.5 mM
  - 1 µl of T4 polynucleotide kinase (10u/µl)

- Vortex gently and incubate 30 min at 37°C.
- Pool all 10 fractions and extract with PCI. Recover the upper water phase. (which should be about 500 µl).
- Add 19 µl of sodium acetate 3M pH = 7.0 et precipitate with 2.5 volume of ethanol 100% (wait at least 2 hours at -20°C). Centrifuge 30 min at 15000 rpm and 4°C. Wash with ethanol 70%. Dry the pellet. Resuspend in 100 µl of TE (~40 µg of DNA).

SIZE SELECTION

Load 10 fractions of 10 µl on gel (TAE 1x buffer) for size selection. This represents 4 µg of DNA per slot. Cut the gel to recover the 1-2kb fraction (corresponding to less than 2 µg of DNA)- (minimize exposition to UVs). Purify by freeze-drying method (SpinX for instance) and resuspend the DNA pellet in 20 µl of TE for each purification. Only one or two fractions will be used for cloning. Keep the other fractions in case the ligation to adaptators is failing or you need more than one fraction to reach your number of clones expected. Each fraction contains now about 1 µg of DNA (there is a certain loss during purification).

LIGATION TO *Bst*XI ADAPTATORS

On end-repeaired DNA, perform a ligation with *BstXI* adaptators (*InVitrogen*). Considering that 1 μg of DNA with average size 1.5 kb is about 1.5 pmols of DNA, it makes 3 pmols of DNA ends in your fraction. *BstXI* adaptators are about 10 bp double-stranded and are at a concentration of 1μg/μl. Thus they are at 150 pmols/μl. This solution is diluted 10 times and is added to the ligation mixture with an 10 times excess to DNA ends.

Test
- DNA end-repaired 20 μl (1μg)
- Adaptors 1/10° 2 μl
- PEG 8000 20% 3 μl
- T4 ligase buffer 10x 3 μl
- rATP 10 mM 1.0 μl
-T4 ligase 10u/μl 1.0 μl

Control 1
- DNA sonicated (non-repaired) 20 μl (1μg)
- Adaptors 1/10° 2 μl
- PEG 8000 20% 3 μl
- T4 ligase buffer 10x 3 μl
- rATP 10 mM 1.0 μl
-T4 ligase 10u/μl 1.0 μl

Control 2
- Control insert blunt-end 5 μl (100 ng)
- Adaptors 1/10° 2 μl
- PEG 8000 20% 3 μl
- T4 ligase buffer 10x 3 μl
- rATP 10 mM 1.0 μl
-T4 ligase 10u/μl 1.0 μl

- Vortex gently and incubate ON at 16°C.
- Separate each ligation on gel to purify the DNA from adaptators doublets. Purify the DNA (Geneclean). For control 1 purify only the 1-2 kb fraction. Resuspend in 10 μl of TE. Each chromosomal DNA fraction – test and control contains about 500 ng → check the concentration on gel which should be around 50ng/μl).

CLONING IN pcDNA2.1

	1	2	3	4	5	6
pcDNA2.1 *BstXI*-digested (50 ng/μl)	1 μl	1 μl	1 μl	1 μl	1 μl	1 μl
Control insert blunt-end 0.5 kb (10 ng/μl)	1 μl					
Control 2 - 0.5 kb + adaptators (10 ng/μl)		1 μl				
Control 1 + adaptators (50 ng/μl)			3 μl			
DNA end-repaired + adaptators (50 ng/μl)				0.5 μl	1.5 μl	4 μl
PEG 8000 20%	2.5 μl	2.5 μl	2.5 μl	2.5 μl	2.5 μl	2.5 μl
T4 ligase buffer 10x	1 μl	1 μl	1 μl	1 μl	1 μl	1 μl
rATP 10 mM	0.5 μl	0.5 μl	0.5 μl	0.5 μl	0.5 μl	0.5 μl
T4 ligase 5u/μl	1 μl	1 μl	1 μl	1 μl	1 μl	1 μl
Sterile water	3 μl	3 μl	1 μl	4.5 μl	3.5 μl	-

- Vortex gently and incubate ON at 16°C.

The number of positive clones should increase almost proportionaly from ligation 4 to 6. Con–trol I should give almost no positive clones.

Use strain TOP10F' if possible (we noticed that XL2-blue Mrf' gave highbackgorund of false positive). Use chemically competent cells rather than electro-competent cells if possible. The Blue/white ratio should be about 1:1 and using TOP10F' competent cells with efficiency of 10^9 CFU/μg we could get as much as 8,500 clones per ligation mixture (6 transformations). Select 100 clones for PCR and check the insert size.