In-vitro SELEX protocol
Troubleshooting guide

Library preparation

1. Let ssDNA pool denature by heating at 95°C for 5 min
2. Dissolve 20pmol naive ssDNA library in 500µl binding buffer (BB)
   - ➢ 20 µL of 1 µM library in 500 µL BB.
3. Cool on ice for 10 min → Formation of stable tertiary structure *Stop point*

Negative selection → don't do in the first SELEX round

4. Wash BSA coated cell culture plate 2x 10 min with 500 µl Wash buffer (WB) by using a shaker
5. Wash BSA coated cell culture plate 1x 10 min with 500 µl BB by using a shaker
6. Incubate ssDNA library with bacteria 10^7 CFU in 1mL BB for 30min on orbital shaker at 4ºC
   - ➢ Supernatant contains unbound DNA sequences

Positive selection

7. Spin down 0.4 mg beads from stock solution at 1200g for 10 min
   - ➢ stock 10 mg/ml
   - ➢ take 40µl and add 500µl MES buffer
8. Wash beads 2x with 500µl WB at 1200g for 10 min
9. Wash beads 1x with 500µl BB at 1200g for 10 min
10. Resuspend beads in 500 µL BB
11. Mix dissolved library (500 µL) together with resuspended beads → in total 1mL
12. Incubate for 1h on orbital shaker at 4°C
    → Place orbital shaker in the fridge
13. Centrifuge at 1200 g for 10 min
14. Wash 1x with 500µl WB at 1200 g for 10 min

→ in later rounds: increase number of washing steps by one more wash and one more minute → until you reached 5 minutes of washing time

Elution of bound aptamers

15. Resuspend in 500µL BB
16. Heat at 95°C for 15min
17. Cool on ice for 5 min
18. Centrifuge at 14,000 rpm for 2 min → Supernatant contains aptamers *Freeze point*

DNA precipitation

19. Add 0.1V x 3M Sodium acetate
    - ➢ 50µL 3M Sodium acetate for 500µL

20. Add 100µg/mL glycoblue to solution
    - ➢ 5µL glycoblue for 500µL
21. Add 2.5V 95 – 99% cold (- 20 °C) EtOH ⇒ 1.375mL EtOH for 500µL

    Alt. a. 1h at -20ºC

    Alt. b. O/N at -80°C
22. Centrifuge at 10 000 g for 30 min at 4°C
23. Remove supernatant
24. Wash in 50 µL 70%ethanol → just to cover the pellet
25. Take as much supernatant as possible and let dry
    - María: You shouldn't be able to smell EtOH anymore. Shouldn't look glossy. Don't let it dry too much either otherwise pellet resuspension becomes harder.
26. Resuspend in 30 µL of nuclease free water (Ambion, 100 mL bottle)
27. Measure with Nanodrop *Freeze Point*

Aptamer library amplification

A) <u>Library pre-amplification</u>

28. Prepare 1 × 50 µL PCR tube:

	Working conc. / amount
– 5 µL of each primer (10 µM)	1 µM / 50 pmol
– 5 µL of dNTP Mix (2.5 mM each)	0.25 mM each
– 1 µL DNA Template (~100 ng/µL)	100 ng
– 5 µL 10X polymerase buffer	1 X
– 0.25 µL of DNA polymerase (5 U/µl)	1.25 U
– 28.75 µL NF-H_2O to adjust total volume to 50 µL	

    ⚠️ *Keep tubes on ice.*
    ⚠️ *Add polymerase last.*

29. PCR for Library pre-amplification:

    - Initial denaturation - 95ªC 3'

    - Denaturation - 95ºC 30''
    - Annealing - 55ºC 30'' × 6 Cycles
    - Elongation - 72ºC 30''

    - Final elongation - 72ºC 3'

    - ➢ Now we have a new library!!!

B) <u>Library amplification</u>

30. Prepare 4 × 50 µL PCR tube:

	Working conc. / amount
– 5 µL of each primer (10 µM)	1 µM / 50 pmol
– 5 µL of dNTP Mix (2.5 mM each)	0.25 mM each
– 1 µL DNA Template (~100 ng/µL)	100 ng
– 5 µL 10X polymerase buffer	1 X

- 0.25 µL of DNA polymerase (5 U/µl) 1.25 U
- 28.75 µL NF-H_2O to adjust total volume to 50 µL

⚠️ *Keep tubes on ice.*
⚠️ *Add polymerase last.*

31. PCR for Library amplification:
    - Initial denaturation - 95ªC 3'

    - Denaturation - 95ºC 30''
    - Annealing - 57.5ºC 30'' × 35 Cycles
    - Elongation - 72ºC 30''

    - Final elongation - 72ºC 3'

* Take out one tube after: 25, 30 and 35 cycles
4 tubes = 3 for the cycle testing (above) + 1 NTC (Non Template Control) (0 µL template, 29.75 µL NF-H_2O)

32. Run 2µL of each PCR product on a 2% agarose, 120V for 15 min
    - 2% agarose gel: 1g agarose, 50mL 1% TBE, 5µL SybrSafe
    - Low range ladder: 2 µL Ladder (ready to use, dye not needed), 4 µL ddH2O
    - Samples: 2 µL sample, 1 µL 6X DNA dye (Ladder kit), 3 µL ddH2O

    ➢ Decide the optimal PCR cycle number based on laddering in the gel:

    6 cycles (from pre-amplification) + ??? cycles (from amplification)

33. Repeat PCR library amplification with the determined number of cycles

Separation of sense and antisense ssDNA

34. Add 50 µl of Dynabeads to bind antisense ssDNA → separation from sense ssDNA
35. Add the magnets and discard the supernatant
36. Wash the beads + bound sequences with 500 µL WB
37. Elute the ssDNA from the beads by melting in a 0.1M NaOH solution 5'
38. Add the magnets and recover supernatant (try to keep under 600µl)
39. Desalt by using GE healthcare Cytiva illustra NAP column NAP-5
    - ➢ Run the protocol as given in this kit to filter out the NaOH
    - ➢ Recover the flow through

Flow cytometry and yield evaluation

40. Repeat binding step (Positive selection)
41. Flow cytometry to assess library enrichment after the SELEX round

➢ SELEX cycle 1 finished !!!
➢ Repeat until flow cytometry shows plateau → send "plateau" library for sequencing

Buffers

Wash Buffer (WB)

- PBS (1X)

- BSA (1 mg/mL)
- 5mM MgCl2

Binding Buffer (BB)

- yeast tRNA (0.1mg/mL) in WB