PCR CLONING UNKNOWN SAGE-TAGS

by Kornelia Polyak, November, 2000

STAGE I. Binding biotinylated oligo ODT-AscI to magnetic beads

Thoroughly resuspend Stretavidin magnetic beads (Dynal), transfer 100µl to an RNase-free, siliconized 1.5 ml tube (Ambion, #12450) and place on magnet. After ~30 sec remove supernatent.
Resuspend beads in 200 µl 2xB+W, remove this just before adding oligo.

Resuspend ~20 ug oligo in 100 ul 2xB+W buffer, bind to prewashed SA-beads. Incubate RT for 15 min with constant agitation (by hand). Wash beads with 2x200 ul lysis/binding buffer.

STAGE II. cDNA synthesis

Add total RNA (50-100 ug) to 1 ml of lysis/binding buffer, immediately reduce viscosity by pressing it through a 23 G needle using a 1ml syringe. Mix lysate with prewashed Dynabeads and incubate at RT for 3-5 min with constant agitation (by hand).
Place the tube on magnet for 2 min, then remove supernatant (the supernatant can be saved and used for DNA prep.).
Wash 2x 1 ml of **washing buffer with LiDS** + 20 µg/ml glycogen (add 1 µl of 20 mg/ml stock for 1 ml of buffer), 1x 1 ml of **washing buffer** + 20 µg/ml glycogen, then 4x 100 µl of 1x 1st strand buffer (dilute 5x 1st strand buffer from cDNA synthesis kit (Life Tech #18090-019) with DEPC treated water).

Resuspend beads in 1st strand synthesis mix:

DEPC water	54 µl
5x1st strand buffer	18 µl
0.1 M DTT	9 µl
10 mM dNTP	4.5 µl

Place the tube at 37°C for 2 min, then add 3 µl of SuperScript RT. Incubate at 37°C for 1 h, mix beads every 10 min by hand "vortexing". After incubation place tube on ice to terminate the reaction.

On ice add the components of the 2nd strand synthesis, in the order shown, to the first strand reaction:

water (prechilled)	227 µl
5x2nd strand buffer	150 µl
10 mM dNTP	15 µl
E coli DNA ligase	3 µl
E coli DNA pol I	12 µl
E coli Rnase H	3 µl

Incubate at 16 °C for 2 hours, mix beads every 10 min.

After incubation place tubes on ice and terminate reaction by adding 100 µl of 0.2 M EDTA.

Wash beads with 4x 200 µl of 1xBW +BSA, 2x200 µl of 1xbuffer 4 +BSA (transfer to new tubes after first wash). Take 2.5 ul of the last wash for checking the integrity of cDNA by RT-PCR. I use 2x amount of BSA in all the washing buffers, more BSA seems to reduce stickiness and improves the efficiency of the washes and the quality

of the library. After the SDS washing/heating step the beads are more sticky until the first BSA wash, but then they are OK.

STAGE III. Cleavage of cDNA with anchoring enzyme (Nla III)

Resuspend beads in following mix and incubate at 37°C for 1 hour:

LOTE	171 µl
BSA (100x, NEB)	4 µl
10xbuffer 4 (NEB)	20 µl
NlaIII (NEB, #125S)	5 µl

STAGE IV. Ligating linkers to cDNA

After incubation wash beads with 4x200 µl 1xB+W+BSA, then 2x200 µl 1xligase buffer. At final rinse transfer 100 µl of each sample into 2 new tubes (siliconized).

Remove last wash and resuspend beads as follows:

	Tube 1
LOTE	20 µl
5xligase buffer	8 µl
Linker 1A,B	10µl

Heat tubes at 50 °C for 2 min. then let sit at RT for 15 min. Add 2 µl of T4 ligase (High conc., Life Tech #15224-041) to each tube and incubate at 16 °C for 2 hours. Mix beads intermittently.

STAGE V. Releasing cDNA from beads

After ligation wash beads 4x200 µl 1xB+W+BSA, and 2x200 µl of 1xbuffer 4 +BSA then resuspend in

85 ul LOTE
10 ul 10x4
5 ul Asc I

and incubate at 37°C for 1 hour. After digestion cf off beads (14K 1 min), carefully take supernatant and transfer to a new tube 9not siliconized!). Wash beads once more with 100ul LOTE, combine sups. PC-8 extract and high concentration ethanol precipitate with 3 ul glycogen as carrier. 2x70% wash, resuspend pellet in 100 ul LOTE.

STAGE VI. Test "library"

Perform PCR using 1 ul of "library" as template and P1 and M13F as primers in a 25 ul PCR "usual" hot-start reaction. You should get a smear peaking ~ 500 bp. If this looks OK, then the "library" can be used to perfom PCR amplification of the unknown gene using tag specific and M13F primers. Usually touch-down PCR works best, but may have to try few different conditions. Use one known gene specific primers as control to make sure PCR is working.