Protocol to generate half Lambda DNA for optical/magnetic tweezer

by Wei Cheng, Feb. 2006

Make Dig handle:

Rationale: PCR portion of Lambda DNA, during PCR, incorporate Dig-dUTP, after PCR, purify product, digest with XbaI and dephosphorylation

PCR primers: forward, TGA TTT CCA GTT GCT ACC GA (Tm=54˚C in 50 mM Na+); backward, CAG GTA TCG TTT GGA GGC AG (Tm=56˚C in 50 mM Na+)

The PCR product has the following sequence:

tgatttccag
24241 ttgctaccga ttttacatat tttttgcatg agagaatttg taccacctcc caccgaccat
24301 ctatgactgt acgccactgt ccctaggact gctatgtgcc ggagcggaca ttacaaacgt
24361 ccttctcggt gcatgccact gttgccaatg acctgcctag gaattggtta gcaagttact
24421 accggatttt gtaaaaacag ccctcctcat ataaaaagta ttcgttcact tccgataagc
24481 gtcgtaattt tctatctttc atcatattct agatccctct gaaaaaatct tccgagtttg
24541 ctaggcactg atacataact cttttccaat aattggggaa gtcattcaaa tctataatag
24601 gtttcagatt tgcttcaata aattctgact gtagctgctg aaacgttgcg gttgaactat
24661 atttccttat aacttttacg aaagagtttc tttgagtaat cacttcactc aagtgcttcc
24721 ctgcctccaa acgatacctg

510 bp total, XbaI site is at 278, after XbaI cut, should get two fragments: 278 and 232 bp

XbaI site:

T^CTAG A
A GATC^T

PCR protocol as follows (from Mike Stone):

Make the following dNTP cocktail (Dig-dUTP, Roche, 1 mM stock, cat# 11093088910; dNTP, 10 mM stock, usually dNTP comes as 100 mM stock, so dilution is necessary):

		1X	10X
Bio/Dig dUTP	.33mM	6.6 µl	66 µl
dTTP	.66 mM	1.4 µl	14 µl
dATP	1 mM	2 µl	20 µl
dCTP	1 mM	2 µl	20 µl
dGTP	1 mM	2 µl	20 µl
Sterilized H2O		6 µl	60 µl
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Total		20 µl	200 µl

The PCR reaction is prepared as follows.

10 ng DNA template	2.0 µl (1 µgλDNA, NEB cat#N3011S)
.5 µM Primer 1	2.4 µl (.5 µM final)
.5 µM Primer 2	3.1 µl (.5 µM final)
10x Taq Buffer	10 µl
Mg+2	4 µl (2mM final)
dNTP cocktail	20 µl (200 µM final each dNTP(G,C,T/U,A))
Taq Polymerase	1.5 µl (1.5 Units)
Sterilized H2O	57.0 µl

Total	100 µl

Note: for DNA template, which is 510 bp in Lambda DNA, 2.0 µl Lambda DNA (from NEB, 500 ng/µl) gives 10 ng of the target DNA sequence.

The PCR reaction conditions are as follows:

1. Initial Denaturation Step	95 C	6 min.
2. Annealling	54 C	45 sec.
3. Extension	72 C	1.5 min.
4. Denaturation	95 C	45 sec.
5. Repeat 2-4 29 times		
6. Terminal Extension	72 C	10 min.
7. Store at 4 C	4C	Forever

These reaction conditions work effectively. Usually 1-3 µg of product/ rxn is expected and run parallel reactions to scale up.

After the PCR is complete you may want to run 5 µl of a few of your reactions out on a diagnostic gel in order to determine that your reactions worked.

Purify the PCR product with Qiagen PCR purification kit, note the binding capacity of one Qiagen spin column is 10 µg, combine product if necessary to avoid dilution of the product. The product is eluted in buffer EB provided by Qiagen.

Set up XbaI digest:
32 µl Purified PCR product
4 µl 10×NE buffer 2
0.4 µl 100×BSA
4 µl XbaI (NEB)

Total 40 µl, incubate in 37˚C warm room for 12-16 hrs, then 65˚C for 20 min to inactivate the enzyme.
Add 4.7 µl 10× Antarctic phosphatase buffer, and 2 µl Antarctic phosphatase (NEB, cat# M0289S, 5 U/µl), incubate at 37 ˚C for 1 hrs, then inactivate enzyme at 65˚C for 5 min.

Use Qiagen PCR purification kit to purify the DNA, and determine concentration of the purified DNA by ABS260nm, store the DNA at -20˚C.

Make ½ Lambda DNA (to generate ssDNA)

1. Biotin fill in by Klenow exo-:

It is important to use fresh order of Lambda DNA (NEB, cat#N3011S). Take 48 µl Lambda DNA (NEB, 500 ng/µl), heat at 65˚C for 5 min to melt the COS sites, then put on ice ***immediately*** to quench, COS sites now forms hairpin with itself, and ready to use Klenow exo- to fill in Biotin label.

48 µl Lambda DNA (quenched on ice)
4.95 µl Biotin-14-dATP (0.4 mM stock, Invitrogen, cat# 19524-016)
4.95 µl Biotin-dCTP (0.4 mM stock, Invitrogen, cat# 19518-018)
0.5 µl dGTP (diluted to 10 mM from 100 mM stock)
0.5 µl dTTP (diluted to 10 mM from 100 mM stock)
7 µl 10×NE buffer 2
2.5 µl Klenow exo- (NEB, cat# M0212S, 5U/µl)

Mix well by stirring gently with pipette tip, and incubate at 37˚C for 1 hr, stop by adding 1.2 µl 0.5 M EDTA, then heat at 70˚C for 5 min, pass through Bio-Rad spin P6 (cat# 732-6221) equilibrated in 1×NE buffer 2.

Instructions for using Bio-Rad P6 (MW. Cut off 6 kD) spin column: please follow the instruction that comes with the spin column. Briefly, you can follow my '2-1-1-1-4' rule for buffer exchange and sample loading: the spin column comes with either Tris buffer or SSC buffer, the buffer needs to be exchanged to your buffer of interest before you load your sample! First of all, snap the bottom plastic piece and remove the cap, place the column on a collection tube that comes with the column, let the column drain by gravity until the flow stops by itself, now centrifuge at 1000g for 2 min, discard the flow through from collection tube, now put 500 µl your buffer of interest (For example, in above case, 1×NE buffer 2) into the column, centrifuge at 1000g for 1 min, discard flow through again, repeat this step for two more times, now remove the collection tube, and place the column in a new 1.7 ml centrifuge tube, pipette your sample right at the center of the column, note for Micro spin column, the maximum loading volume is 75 µl, and centrifuge at 1000g for 4 min, the follow through contains the DNA in 1×NE buffer 2.

2. Set up RE digest by XbaI in NE buffer 2, with BSA

80 µl DNA post P6
1.0 µl 10×NE buffer 2
0.9 µl 100×BSA
8 µl XbaI (NEB)

37˚C for 1 hr, then add 1.5 µl 0.5 M EDTA, and then 65˚C for 20 min. Note: after this XbaI digest, the DNA should NOT be treated with phosphatase, the reason being if dephosphorylation, after ligation, it leaves a nick on the strand with Biotin label and

there is no way to generate ssDNA from the product molecule. In addition, if you want to selective only one half of the Lambda DNA for single molecule experiments, you can perform double digestion of XbaI + Acc65 I in NE buffer 2, and follow the treatment as above to heat inactivate the enzymes.

3. DNA from step 2 should be ready for ligation without further treatment. Determine the concentration of DNA by ABS260 nm. Ligate with Dig handle that has been treated with phosphatase, molar ratio handle: Lambda DNA=2:1

½ Lambda DNA, biotinylated, post XbaI digest: 1.5 µg (100 ng/µl, take 15 µl)
XbaI digested Dig handle, treated with phosphatase: 34 ng (170 ng/µl, take 0.2 µl)
2×Quick ligase buffer (from NEB quick ligation kit, cat# M2200S): 15 µl
T4 ligase (from NEB quick ligation kit, cat# M2200S): 1.5 µl

20 min @ RT, 65˚C for 10 min to inactivate the ligase, then store at 4˚C, or add glycerol to a final concentration of 25% v/v and store at -20˚C