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Description :

Deletion Kit for Kilo-Sequencing (5 reactions) Cat. #6030

This kit is designed to aid sequence analysis of long DNA fragments that are inserted into the multiple cloning sites of M13 phage vectors (such as mp18/19) or pUC-related plasmid or phagemid vectors (such as pUC18/19 or pUC118/119). The kit is used to create nested unidirectional deletions in the target DNA, thereby progressively moving the primer binding site on the vector closer to the sequence of interest. The kit includes optimized reagent cocktails to ensure efficient self-circularization of the deletion subclones.

Kit Components :

Exo III buffer	500 μl
Exo III (180 units/μl)	10 μl
Mung Bean nuclease buffer	500 μl
Mung Bean nuclease (25 units/μl)	20 μl
Klenow buffer	250 μl
Klenow fragment (2 units/μl)	10 μl
Ligation solution A*	500 μl
Ligation solution B*	60 μl

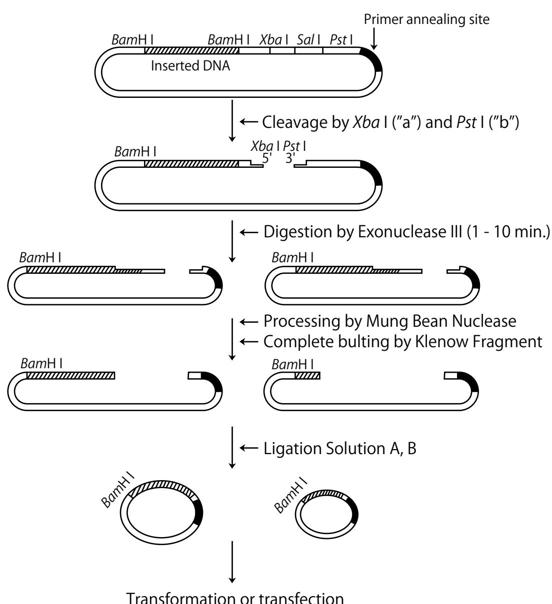
* : Ligation solutions A and B are also available as the DNA Ligation Kit (Cat. #6021)

Storage : − 20℃

References :

1. Henikoff, S. (1984) *Gene*, **28**, 351-359.
2. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, **33**, 103-119.
3. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning*, A Laboratory Manual, Cold Spring Harbor Laboratory Press.
4. Ozkaynak, E. and Putney, S. D. (1987) *BioTechniques*, **5**, 770-773.

Principle :



This kit is based on the procedure developed by Henikoff[1] and Yanisch-Perron *et al*[2], in which serial unidirectional deletions are created in a DNA insert by the 3' → 5' exonuclease activity of Exonuclease III. Exonuclease III progressively removes nucleotides from the 3' termini of 5'-protruding or blunt ends, but is generally less active against 3'-protruding ends. Basically, the inserted target DNA is digested progressively, while the sequencing primer annealing site is protected from digestion by an adjacent 4-base 3'-overhang restriction site (however, see CAUTION in section I, page 3 for exceptions). Aliquots removed at timed intervals from the Exonuclease III reaction are treated with the Mung Bean nuclease/buffer cocktail which stops the progression of the exonuclease and simultaneously removes the remaining 5'-overhangs on the complementary strand. The ends of the fragments are then made blunt using the Klenow fragment of DNA

polymerase, and are circularized by ligation using the Ligation cocktails. The DNA is then used to transform competent bacterial cells.

Protocol :

I. Preparation of plasmid clones and the choice of enzymes

The DNA to be sequenced is inserted into the multiple cloning site of an M13 vector or a pUC-related vector, and the DNA is prepared in double-stranded circular form. It is important to minimize the proportion of nicked (open circular) and linear molecules in the starting material, as Exonuclease III (*Exo* III) also digests these molecules and will create random deletions. Supercoiled DNA can be obtained by conventional equilibrium sedimentation in CsCl-ethidium bromide gradients [3)], or by acid-phenol extraction as described (Zasloff *et al*. 1978 *Nucl. Acids Res*. **5**: 1139). In order to create progressive unidirectional deletions, it is necessary to cut the DNA between the "primer annealing site" and the inserted DNA with two different restriction enzymes (see previous figure). The "a" enzyme will have to cut nearer to the insert DNA, and the "b" enzyme nearer to the primer annealing site. The "a" enzyme should leave either a 5' protruding end or a blunt end after digestion (such as *Eco*R I, *Sma* I, *Xma* I, *Bam*H I, *Xba* I, *Sal* I, *Acc* I, *Hin*c II, *Hin*d III), and the "b" enzyme should leave a 3'-protruding end (such as *Sac* I, *Kpn* I, *Pst* I, *Sph* I, *Sse*8387 I. *Sse*8387 I is especially useful as it is an 8-base cutter and its recognition site should occur rarely in DNA fragments). Recognition sites for "a" and "b" must not be present on the insert DNA. In case when a suitable "b" enzyme is not found, an alternative strategy is to fill in 3' recessed ends with α-phosphorothioate dNTPs. This allows 5' protrusions to be used as protecting sites against exonuclease as *Exo* III is resistant to α-phosphorothioate filled ends.[4)]

(Example)

If the DNA fragment is cloned into the *Bam*H I site of M13 mp18, *Xba* I, *Sal* I, *Acc* I, or *Hin*c II can be used as the "a" enzyme. Either *Sse*8387 I, *Pst* I or *Sph* I can be chosen as the "b" enzyme.

CAUTION

While most 3'-protruding ends are resistant against *Exo* III cleavage, some such as are created by *Apa* I, *Sac* II, or *Sfi* I are found to be susceptible. These restriction enzymes should not be chosen as the "b" enzyme as they will not protect the vector from exonuclease processing.

II. Production of deletions

Reagents to be supplied by the user :

- Restriction enzymes and appropriate buffers
- TE-saturated phenol
- Chloroform/isoamylalcohol
- 3M sodium acetate
- Ethanol (100% and 70%)
- Competent cells and SOC media

1) Prepare 5 - 10 μg of double-stranded supercoiled DNA (M13 RF DNA or pUC-type plasmids carrying the DNA fragment to be sequenced).
2) Digest the DNA with "a" and "b" enzymes.
3) Extract with an equal volume of TE-saturated phenol, centrifuge, and recover the aqueous (upper) layer into a fresh tube (TE buffer : 10 mM Tris-HCl, 1 mM EDTA, pH8.0). Extract once with an equal volume of chloroform/isoamylalcohol (24/1, v/v).

4) Centrifuge, and transfer the aqueous (upper) layer into a fresh tube, then add 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Store the tube at −70℃ for 10 minutes, and recover the DNA precipitate by centrifugation. Rinse with 70% ethanol, then dry under reduced pressure.
5) Dissolve the DNA in 100 μl of *Exo* III buffer.
6) Dispense 100 μl of Mung Bean nuclease buffer in a separate tube.
7) Add 1 μl (180 units) of *Exo* III to the DNA solution prepared in step 5). Mix by vortexing, and incubate at 37℃. Transfer 10 μl aliquots at one minute intervals to the 100 μl Mung Bean nuclease buffer prepared in step 6). Under the above conditions, approximately 300 bases will be removed every minute. If successive deletions varying in length by less than 300 bases are desired, the incubation should be done at 25℃, and the samples should be removed at 30-second intervals. The rate of *Exo* III digestion can be altered simply by changing the incubaton temperature.
   The combined mixture will sum up to a total volume of 200 μl when the periodical transfers from the *Exo* III reaction are completed.
8) Inactivate Exo III by incubation at 65℃ for 5 minutes, and then return the tube to 37℃.
9) Add 2 μl (50 units) of Mung Bean nuclease.
10) Incubate at 37℃ for 15 - 30 minutes.
11) Repeat steps 3) and 4).
12) Dissolve the DNA precipitate in 50 μl of Klenow buffer.
13) Add 1 μl (2 units) of Klenow fragment, and incubate at 37℃ for 15 minutes*1.
14) Add a 5 - 10 μl sample of the above DNA solution to 100 μl of Ligation solution A.
15) Add 12 μl of Ligation solution B, and mix by vortexing.
16) Incubate the mixture at 16℃ for one hour*2.
17) Add 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol, leave at -70℃ for 10 minutes, and then recover the precipitate by centrifugation. Rinse with 70% ethanol, then dry under reduced pressure.
18) Dissolve the DNA in 40 μl of appropriate buffer for restriction enzyme "a". Cut the DNA solution with a few units of restriction enzyme "a", and use this DNA solution to transform competent cells (use ≧ 200 μl competent cells)*3.

*1 : Although most of the DNA becomes blunt-ended by Mung Bean nuclease, the Klenow fragment completes this reaction and secures higher ligation and transformation efficiency.
*2 : Although most of the molecules will be ligated efficiently between 15 minutes to 2 hours, an overnight incubation sometimes secures more complete ligation. Ligation solutions A and B are also available as the TaKaRa Ligation Kit (Cat. #6021). See section IV for the rapid protocol.
*3 : Restriction enzyme "a" is used here to exclude DNA molecules which are left uncut at step 2). This procedure reduces the appearance of background transformation.

III. Screening of the deletion mutants

Usually, several tens to hundreds of colonies or plaques will be obtained on one plate if the recommended protocol in section II is used. Pick 50 - 100 clones, and inoculate each in a small volume of liquid culture media (2 ml). Purify the plasmid clones by the alkaline lysis[3)] method. Cut with an appropriate enzyme, and check the size of the deletion created in the DNA insert by agarose gel electrophoresis. Select clones which carry deletions of various sizes, prepare the templates of the chosen clones, and proceed to DNA sequence analysis.

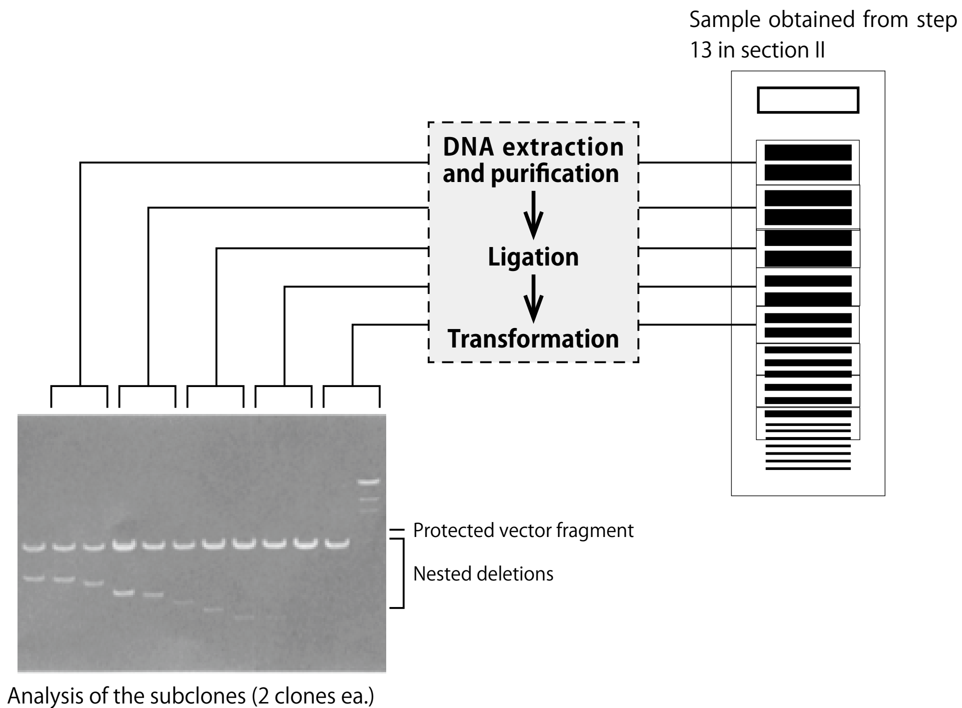
IV. Size selection

In some cases, it is preferred to obtain subclones that carry deletions within a relatively narrow size range. The following protocol is convenient for this purpose.

1) Separate the sample obtained in the above section II-13 (5 - 10 μg DNA) by agarose gel electrophoresis. Excise the gel slice from the region of the desired size range, and extract the DNA (Rapid extraction can be conveniently performed by SUPREC-01 cartridges Cat. #9040)

2) Dissolve the DNA in 10 μl TE buffer, add 80 μl of Ligation solution A and 15 μl of Ligation solution B. Incubate at 16℃ for 15 minutes. (Longer incubation is also possible, although usually, 15 minutes is sufficient if the DNA is in small amounts and purified.)

3) Use 10 μl of this mixture to transform 100 μl of competent cells. Incubate the DNA/competent cell mixture in ice for 15 minutes. Then heat at 42℃ for 30 seconds. Add 890 μl of SOC media and incubate for 15 minutes at 37℃. Plate on selective media.



Analysis of the subclones (2 clones ea.)

Vectors and primers for sequence analysis

M13 mp18 RF DNA		10 μg		Cat. #3118
pUC18 DNA		25 μg		Cat. #3218
pUC19 DNA		25 μg		Cat. #3219
pUC118 DNA		25 μg		Cat. #3318
pUC119 DNA		25 μg		Cat. #3319
pTV118N DNA	(pUC118 deriv.)	25 μg	*Nco* I site at initiation codon of *lacZ*'	Cat. #3328
pHSG298 DNA	(pUC18 deriv., kan^r)	25 μg		Cat. #3298
pHSG299 DNA	(pUC19 deriv., kan^r)	25 μg		Cat. #3299
pHSG398 DNA	(pUC18 deriv., cm^r)	25 μg		Cat. #3398
pSTV28 DNA	(pACYC184 deriv., cm^r)	25 μg	multiple cloning site of pUC118	Cat. #3331
pSTV29 DNA	(pACYC184 deriv., cm^r)	25 μg	multiple cloning site of pUC119	Cat. #3332
pTWV228 DNA	(pUC118 deriv.)	25 μg	replication origin of pBR322	Cat. #3333

M13 primer M1	(plus*)	150 pmol	Cat. #3810
M13 primer M2	(plus*)	170 pmol	Cat. #3820
M13 primer M3	(plus*)	130 pmol	Cat. #3831
M13 primer M4	(plus*)	150 pmol	Cat. #3832
M13 primer M13-20	(plus*)	120 pmol	Cat. #3881
M13 primer RV	(minus*)	130 pmol	Cat. #3830
M13 primer RV-P	(minus*)	120 pmol	Cat. #3882
M13 primer RV-M	(minus*)	120 pmol	Cat. #3880
M13 primer RV-N	(minus*)	150 pmol	Cat. #3833

* : M13 primers can be used to sequence DNA inserted in the multiple cloning sites of M13 and pUC-derived vectors. Primers are positioned in either plus or minus orientations with sequences complementary to the (+) and (-) strands of single-stranded phage and phagemid templates, respectively. With double-stranded DNA templates, the minus primers are used to read DNA sequences in the same orientation as the *lac*Z' transcript, whereas the plus primers are used to read in the opposite direction.

NOTE : This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

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