



Promega

Technical Manual

Erase-a-Base[®] System

INSTRUCTIONS FOR USE OF PRODUCT E5750.



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Part# TM006

Erase-a-Base[®] System

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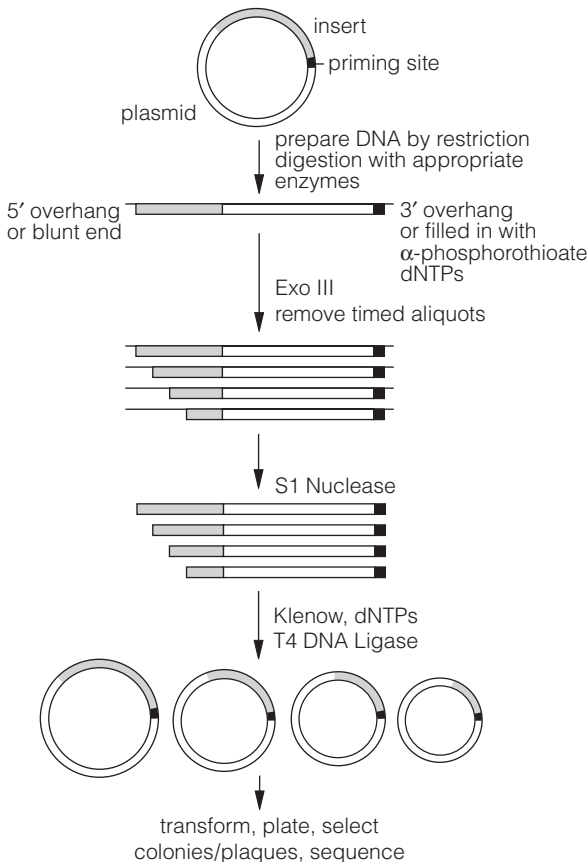
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I. Description

The Erase-a-Base[®] System^(a) is designed for the rapid construction of plasmid or M13 subclones containing progressive unidirectional deletions of any inserted DNA. The system is based on the procedure developed by Henikoff (1), in which exonuclease III (Exo III) is used to specifically digest insert DNA from a 5' protruding or blunt-end restriction site. The adjacent sequencing primer binding site is protected from digestion by a 4-base 3' overhang restriction site or by an α -phosphorothioate-filled end (2).

DNA fragments cloned into plasmid or M13 vectors are frequently greater than 500 bases in length and thus may be too long to sequence conveniently from a single primer binding site on the vector. An efficient way to sequence such large DNA inserts is to generate a nested set of deletions in the target DNA, effectively moving the priming site closer to the sequence of interest. The uniform rate of digestion of Exonuclease III allows deletions of predetermined lengths to be made simply by removing aliquots from the reaction at timed intervals. Starting with appropriately treated plasmid or M13 DNA, a collection of unidirectional deletions spanning several kilobases (kb) can be easily constructed in several hours.

A schematic diagram of the steps involved in the method is shown in Figure 1. The DNA fragment of interest is first cloned into the multiple cloning site of an appropriate vector so that at least two unique restriction sites lie between the end of the insert DNA and the sequencing primer binding site. The enzyme that cuts closest to the priming site must leave a 4-base 3' overhang, which is resistant to Exo III digestion. The enzyme that cuts closest to (or within) the insert DNA must leave a blunt end or 5' overhang. When this configuration of sites is not convenient, an enzyme producing a 5' overhang can be used closest to the priming site, and the recessed 3' ends can be filled in using α -phosphorothioate deoxynucleotides. This treated end is also resistant to Exo III digestion (2).



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Figure 1. Schematic diagram of the Erase-a-Base® System protocol.

Samples of the Exo III digestion are removed at timed intervals and added to tubes containing S1 Nuclease, which removes the remaining single-stranded tails. The low pH and the zinc cations in the S1 Nuclease buffer effectively inhibit further digestion by Exo III. S1 Nuclease, unlike mung bean nuclease, is active in the Exo III buffer, so it is not necessary to change buffers between the Exo III and S1 steps. After neutralization and heat inactivation of the S1 Nuclease, Klenow DNA Polymerase is added to fill in the ends, which are then ligated to circularize the deletion-containing vectors. The ligation mixtures are used directly to transform competent cells. Each successive time point yields a collection of subclones containing clustered deletions extending further into the original insert.

A number of subclones from each time point are screened to select appropriate intervals between deletions. Sequence analysis can be conveniently performed directly with double-stranded plasmid constructs using the SILVER SEQUENCE™ DNA Sequencing System or the *fnol*® DNA Cycle Sequencing System with SP6 or T7 promoter primers (for pGEM® Vectors) or other appropriate primers.

The Erase-a-Base® System can be used with any plasmid or M13 vector. However, the pGEM®-5Zf(+/-) and pGEM®-7Zf(+/-) Vectors (Cat.# P2241, P2251, P2351, P2371) are designed for use with the Erase-a-Base® System. The multiple cloning regions in these vectors contain blunt end and 5' overhang restriction sites flanked by 3' overhang restriction sites. Thus, either the SP6 or T7 primer binding site can be protected from Exo III digestion simply by cutting with an enzyme that generates a specific 3' overhang restriction site.

Citations using the Erase-a-Base® System

- Song S.I. and Miller W.A. (2004) *Cis* and *trans* requirements for rolling circle replication of a satellite RNA. *J. Virol.* **78**, 3072-82.

Notes: In this report, satellite RNA from the full-length RPV serotype (satRPV RNA) of Cereal yellow dwarf virus was transcribed *in vitro* using the RiboMAX™ Large Scale RNA Production System. Self-cleavage of the synthesized RNA was then induced by incubation with a cleavage buffer. Serial deletions were created in the wild-type satRPV cloned in pGEM®-3Zf(-) Vector using the Erase-a-Base® System. The mutants generated were tested to see how well they replicated with internal sections of satRPV sequence removed.

- Lee, H.Y., An, J.H., and Kim, Y.S. (2000) Identification and characterization of a novel transcriptional regulator, MatR, for malonate metabolism in *Rhizobium leguminosarum* bv. trifolii *Eur. J. Biochem.* **267**, 7224-9.

Notes: Total RNA was isolated from *Rhizobium leguminosarum* bv. trifolii using the SV Total RNA Isolation System. This RNA was used in a primer extension assay using AMV Reverse Transcriptase to determine the transcriptional start site of the *mat* operon. Nested deletions during the sequencing of the *mat* promoter were prepared using the Erase-a-Base® System.

For additional peer-reviewed articles that cite use of the Erase-a-Base® System, visit:

www.promega.com/citations

II. Product Components

Product	Cat.#
Erase-a-Base® System	E5750

Includes:

- 10,000u Exonuclease III
- 2,000u S1 Nuclease
- 150u DNA Polymerase I Large (Klenow) Fragment
- 100u T4 DNA Ligase
- 500µl Exonuclease III 10X Buffer
- 500µl S1 Nuclease 7.4X Buffer
- 1ml S1 Nuclease Stop Buffer
- 1ml Klenow 1X Buffer
- 500µl dNTP Mix (0.125mM each)
- 2ml Ligase 10X Buffer
- 250µl DTT, 100mM
- 2ml PEG, 50%
- 10µg Erase-a-Base® Control DNA
- 1 Protocol

Refer to Section VIII.C for information on Erase-a-Base® System components available separately.

Storage Conditions: Store at -20°C.

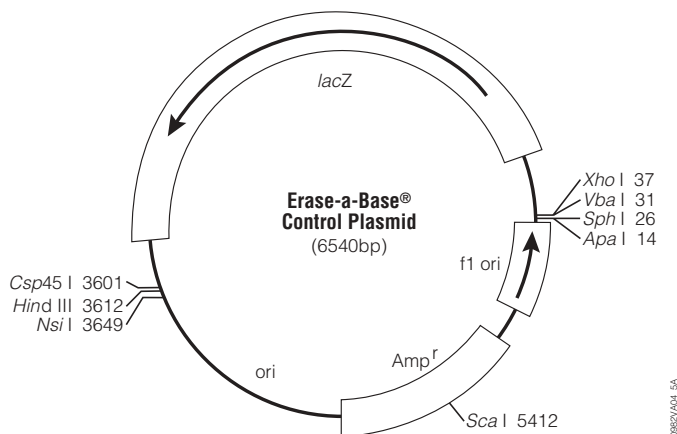


Figure 2. Erase-a-Base® Control plasmid. The linear Erase-a-Base® Control DNA supplied with the system is a *Sph* I/*Xho* I digest of this plasmid.

III. Preparation of Starting DNA

III.A. General Considerations

The generation of ordered sets of unidirectional deletions relies on the uniform digestion rate of Exonuclease III (Exo III) from appropriate DNA ends. However, this enzyme also digests from nicks in double-stranded DNA molecules (3), creating single-stranded gaps. The effect of random nicks in the starting DNA is, therefore, to randomize the deletions obtained. The greater the percentage of nicked molecules in the starting material, the more random the deletions become and the more difficult it becomes to screen for the desired (predicted) deletions among subclones.

Therefore, it is important to minimize the proportion ($\leq 20\%$) of nicked molecules in the starting DNA. This can be accomplished by 1) minimizing the amount of nicked (and linear) molecules in the plasmid preparation, and 2) minimizing the generation of single-stranded nicks during restriction enzyme digestion. Some restriction enzyme preparations contain nuclease contamination. For best results, use Promega restriction enzymes. Closed circular DNA can be purified by conventional equilibrium sedimentation in CsCl-ethidium bromide gradients (4). Alternatively, nicked and linear DNA can be selectively separated from supercoiled DNA by acid-phenol extraction as described by Zasloff *et al.* (5). The latter procedure is presented in Section VIII.A.

Erase-a-Base® Control DNA (Figure 2) is provided as a non-nicked, predigested control. The Erase-a-Base® Control Plasmid has been digested with *Xho* I (5' overhang) and *Sph* I (Exo III-resistant 3' overhang) and suspended in TE buffer at a concentration of 0.5 $\mu\text{g}/\mu\text{l}$. This construct is designed to check for deletions. The *lacZ* insert is not intended for use in a cloning strategy.

III.B. Restriction Digestion of Plasmid DNA

When possible, double-digest 10 μg of closed circular DNA with two different restriction enzymes: one that generates a 4-base 3' protrusion protecting the primer binding site and another that leaves a 5' protrusion or blunt end adjacent to the insert from which deletions are to proceed (see Tables 1 and 2). If salt conditions do not allow a double digestion, it is usually best to digest with the restriction enzyme that results in the 3' overhang first. However, some restriction enzymes do not cut well when their cleavage site is near the end of a DNA fragment (6). It is important to be sure that both restriction enzymes have cut the DNA to completion. Since Exo III is strongly inhibited by as little as 20mM NaCl (7), the plasmid DNA must be extracted with phenol:chloroform and precipitated with ethanol following restriction digestion.

Note: *Hha* I, *Pvu* I and *Sac* II generate 3' overhangs, but these are not resistant to Exo III digestion.

Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.B.)

- 3M sodium acetate (pH 5.2)
- restriction enzymes and appropriate buffers
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)

1. The following restriction digestion reaction is provided as an example:

recombinant plasmid DNA	10 μ g
appropriate restriction enzyme 10X buffer	10 μ l
1mg/ml acetylated BSA (optional)	10 μ l
appropriate restriction enzymes	20–40u each
nuclease-free water to final volume of	100 μ l

Incubate at the appropriate temperature for 2–3 hours.

2. Check that digestion is complete by electrophoresis of a sample (0.3 μ g) on a 1% agarose minigel.
3. If the digest is complete, extract with 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol. Vortex for 1 minute and centrifuge at 12,000 \times g for 5 minutes.
4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge at 12,000 \times g for 5 minutes.
5. Transfer the upper, aqueous phase to a fresh tube. Add 0.1 volume of 3M sodium acetate and 2 volumes of ethanol. Mix well. Centrifuge at 12,000 \times g for 5 minutes.
6. Carefully pour off the supernatant and wash the pellet with 1ml 70% ethanol. Drain the tube; dry the pellet under vacuum and proceed to Section IV.

Table 1. Restriction Enzymes That Generate Exonuclease III-Resistant 3' Overhangs.

Enzyme	Recognition Sequence
Aat II	5'...GACGTC...3' 3'...CTGCAG...5'
Apa I	5'...GGGCC...3' 3'...CCCGGG...5'
Ban II	5'...GPuGCPyC...3' 3'...CPyCGPuG...5'
Bgl I	5'...GCCNNNNNNGGC...3' 3'...CGNNNNNNCCG...5'
BstX I	5'...CCANNNNNNTGG...3' 3'...GGTNNNNNNACC...5'
Hae II	5'...PuGCGCPy...3' 3'...PyCGCGPu...5'
Kpn I	5'...GGTACC...3' 3'...CCATGG...5'
Nsi I	5'...ATGCAT...3' 3'...TACGTA...5'
Pst I	5'...CTGCAG...3' 3'...GACGTC...5'
Sac I	5'...GAGCTC...3' 3'...CTCGAG...5'
Sph I	5'...GCATGC...3' 3'...CGTACG...5'

Notes:

1. The enzymes shown in bold cut in the multiple cloning regions of the pGEM[®]-5Zf or pGEM[®]-7Zf plasmids.
2. *BstX I* and *Bgl I* contain ambiguous bases in their cut sites and may not always be protected sites. The sites are protected in pGEM[®]-5Zf or pGEM[®]-7Zf Vectors.
3. *Hha I*, *Pvu I* and *Sac II* generate 3' overhangs, but these ends are not protected from Exonuclease III digestion.

Table 2. Restriction Enzymes That Generate 5' Overhangs or Blunt Ends in the pGEM[®]-5Zf or pGEM[®]-7Zf Vectors.


Enzyme	Recognition Sequence
BamH I	5'...GGATCC...3' 3'...CCTAGG...5'
Cla I	5'...ATCGAT...3' 3'...TAGCTA...5'
Csp45 I	5'...TTCGAA...3' 3'...AAGCTT...5'
EcoR I	5'...GAATTC...3' 3'...CTTAAG...5'
EcoR V	5'...GATATC...3' 3'...CTATAG...5'
Hind III	5'...AAGCTT...3' 3'...TTCGAA...5'
Nco I	5'...CCATGG...3' 3'...GGTACC...5'
Nde I	5'...CATATG...3' 5'...GTATAC...5'
Not I	5'...GCGGCCGC...3' 3'...CGCCGGCG...5'
Sal I	5'...GTCGAC...3' 3'...CAGCTG...5'
Sma I	5'...CCCGGG...3' 3'...GGGCC...5'
Spe I	5'...ACTAGT...3' 3'...TGATCA...5'
Xba I	5'...TCTAGA...3' 3'...AGATCT...5'
Xho I	5'...CTCGAG...3' 3'...GAGCTC...5'

III.C. Protection of 5' Protruding Ends with α -Phosphorothioate dNTPs

An alternative strategy to using 3' protrusions to block Exo III digestion of vector sequences is to fill in 3' recessed ends with α -Phosphorothioate dNTPs and Klenow DNA Polymerase. This approach allows restriction sites for enzymes leaving 5' protrusions to be used as protecting sites for the generation of deletions with the Erase-a-Base® System. This protecting restriction site must be cleaved and filled in with α -Phosphorothioate dNTPs before the second restriction digestion is performed.

Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.B.)

- 3M sodium acetate (pH 5.2)
 - TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
 - ethanol (100% and 70%)
 - α -phosphorothioate dNTPs (Glen Research Cat.# 80-1000-01, 80-1010-01, 80-1020-01, and 80-1030-01)
1. Prepare covalently closed DNA by standard methods followed by an acid-phenol extraction (reference 2 and Section VIII.A). Digest 5–10 μ g prepared DNA to completion with the enzyme chosen as the vector protecting site.
 2. Add 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1), vortex for 1 minute and centrifuge at 12,000 \times g for 5 minutes.
 3. Transfer the upper, aqueous phase to a fresh tube. Add 0.1 volume of 3M sodium acetate and 2 volumes 100% ethanol. Mix and leave at -20°C or on dry ice for 10 minutes. Centrifuge at 12,000 \times g for 15 minutes.
 4. Remove the supernatant and carefully rinse the pellet in 70% ethanol. Drain the tube and dry the pellet under vacuum.
 5. Resuspend the pellet in 50–100 μ l Klenow 1X Buffer (20mM Tris-HCl [pH 8.0], 100mM MgCl₂). Add sufficient α -phosphorothioate dNTPs to give a final concentration of 40 μ M each.
-  Only use the Klenow 1X Buffer that is supplied with the Erase-a-Base® System. **Do not** use the Reaction Buffer supplied with DNA Polymerase I Large (Klenow) Fragment (Cat.# M2201, M2206, M2181 or M2185) at Step 5.
6. Add dithiothreitol (DTT) to 1mM and Klenow DNA Polymerase to 50u/ml. Incubate for 10 minutes at 37°C.
 7. Heat the sample for 10 minutes at 70°C to inactivate the Klenow DNA Polymerase. Extract the DNA as in Steps 2–4 and resuspend in the appropriate restriction endonuclease digestion buffer. After performing the second restriction digestion, extract the DNA as in Steps 2–4.

IV. Exonuclease III Deletion, Ligation and Transformation Protocol

The following procedure is based on use of 5 μ g of doubly cut plasmid DNA and 25 time points. The amount of each solution used is determined by the amount of starting DNA and the desired number of time points. Reaction volumes can be scaled up or down in proportion to those described below. First-time users should perform the protocol below using the Erase-a-Base[®] Control DNA provided with the system.

The rate of Exo III digestion can be altered simply by changing the incubation temperature (8). Using the recommended amount of Exo III (300–500 units), the digestion rate exhibits temperature dependence as shown in Figure 3. Successive deletions differing in size by 300–400bp are convenient for use with sequencing systems capable of reading 350–450bp, since these allow overlap of 50–150bp.

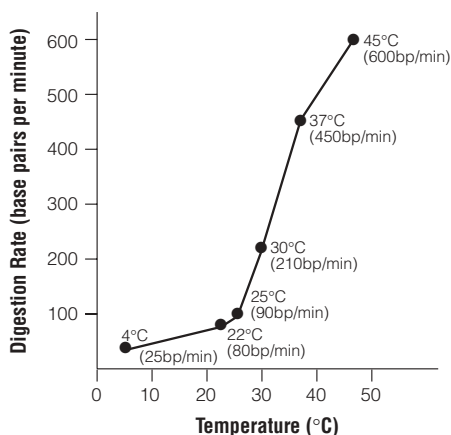


Figure 3. Temperature dependence of Exonuclease III digestion rate.

Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.B.)

- S1 nuclease mix
- TE buffer
- Klenow mix
- ligase mix
- 7.5M ammonium acetate
- competent cells
- SOC medium
- LB plates containing 100µg/ml ampicillin
- ethanol, 100% and 70%

1. Resuspend the pellet of restriction-digested DNA in 10µl TE buffer or sterile deionized water. Run 1-2µl of this DNA on a gel and estimate its concentration by comparison to linear DNA of known concentration. Add 5µg of DNA to 6µl of the 10X Exo III Buffer and bring the volume to 60µl with water. For the Erase-a-Base® Control DNA, use 10µl (5µg) of DNA, 6µl of 10X Exo III Buffer and 44µl of water. Meanwhile, for each DNA deletion series, add 7.5µl of S1 nuclease mix to each of 24 microcentrifuge tubes (or use a conical-bottom 96-well plate) and place on ice.
2. Warm the DNA tube to the digestion temperature in a water bath. Remove a 2.5µl sample to an S1 tube on ice as a time zero control. Add 300-500u Exo III, mixing as rapidly as possible. Remove 2.5µl samples at 30-second intervals into the S1 tubes on ice, pipetting briefly to mix.



Digestion proceeds at about 450 bases/minute at 37°C. There is a 20-30 second lag before the reaction begins.

3. After all the samples have been taken, move the tubes to room temperature for 30 minutes. Add 1µl of S1 Nuclease Stop Buffer to each tube and heat at 70°C for 10 minutes to inactivate the S1 Nuclease. Centrifuge briefly to collect the liquid at the bottom of the tube.
4. To determine the extent of digestion, remove 2-3µl samples (40-60ng DNA) from each time point for analysis on a 1% agarose gel.
5. While the gel is running, precipitate the samples by adding 0.3 volumes 7.5M ammonium acetate and 2 volumes 100% ethanol. Mix well. Incubate at -20°C for 15 minutes to overnight. Centrifuge at 12,000 × g for 5 minutes. Carefully remove the supernatant and wash the pellet with 0.5ml of 70% ethanol. Centrifuge again. Drain the tube and dry the pellet under vacuum. Resuspend the pellet in 9µl TE buffer.

Note: After precipitation the pellet will be very small and may be invisible.

6. Transfer the samples from each time point to a 37°C water bath and add 1µl of Klenow mix to each sample. Incubate for 3 minutes and then add 1µl of the dNTP mix. Incubate for an additional 5 minutes at 37°C. Heat inactivate the Klenow at 65°C for 10 minutes.

7. Transfer the samples to room temperature and add 40µl of ligase mix to each sample. Mix well and incubate at room temperature for 1 hour.
8. Thaw on ice the required aliquots of JM109 competent cells or another suitable strain. Use 200µl low efficiency ($>10^7$ cfu/µg) or 20µl high efficiency ($>10^8$ cfu/µg) cells for each transformation. For each time point, add 10µl of ligation products to the competent cells, mix gently and incubate for 30 minutes on ice (9). Heat to 42°C in a water bath for 45 seconds, then place on ice for 2 minutes. Add 200µl of room temperature SOC medium, warm to 37°C for 5 minutes, and shake at 37°C for 1 hour (10). Plate the entire mixture on appropriate selective media. Promega's pGEM® Vectors and other plasmids may be plated on LB plates containing 100µg/ml ampicillin. Using competent cells giving 10^7 - 10^8 transformants/µg supercoiled DNA, this procedure should result in dozens to hundreds of colonies per deletion time point.

Note: Other ligation conditions and transformation protocols also can be used. For example, polyethylene glycol (PEG) can be omitted from the ligase mix, but the incubations should then be performed for several hours to overnight. If cells giving 10^6 or fewer transformants/µg are used, expect few colonies.

Use of SOC medium results in greater numbers of transformants than LB medium.

V. Screening of Deletion Subclones

Screen 10–20 recombinants from each time point to select those containing deletions of appropriate sizes for further analysis. This can be accomplished by a number of rapid screening methods, such as colony PCR. Colony PCR is a rapid method for determining if a specific DNA fragment is inserted in plasmid vectors. PCR amplification is performed on single bacterial colonies grown on selective media plates (for additional information see references 11 and 12). After selecting the desired subclones, grow these up in overnight culture and perform a plasmid miniprep to obtain enough DNA for sequencing. Promega's Wizard® *Plus* SV Minipreps DNA Purification System offers a rapid alternative (15 minutes or less) to the standard miniprep procedure. Use of the Wizard® *Plus* SV Minipreps with a vacuum manifold allows the simultaneous processing of numerous samples, decreasing screening time further.

VI. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
No apparent deletions of experimental DNA occur when analyzed by gel electrophoresis	<p>If the restriction enzyme leaving the Exo III-sensitive end failed to cut completely, those singly cut molecules will remain the same size as the original starting DNA throughout the deletion time-course. Check the activity of the appropriate restriction enzyme. Repeat the initial digestion with more enzyme for a greater time period.</p> <hr/> <p>There may not be sufficient nucleotides left near the recognition site of the second enzyme to allow cleavage (6). Reverse the order of use of the restriction enzymes.</p> <hr/> <p>The NaCl concentration after restriction digestion may be too high; NaCl concentrations as low as 20mM are known to affect the Exo III digestion rate (7). Ethanol precipitate the DNA, wash the pellet with 70% ethanol and resuspend in TE buffer or water.</p> <hr/> <p>The EDTA concentration may be too high, binding the Mg²⁺ ions necessary for the reaction. Ethanol precipitate the sample and resuspend in water.</p> <hr/> <p>Inactive Exo III and/or S1 Nuclease enzymes. Perform a deletion series with the Erase-a-Base® Control DNA.</p>
Deletion series appears degraded when analyzed by gel electrophoresis	<p>The original vector preparation contains nicked molecules; Exo III will recognize nicks within the DNA template and will start to generate deletions from those nicks, resulting in a smear. Check vector DNA on a gel to confirm that it is at least 80% supercoiled. If not, prepare the DNA again, or see Section VIII.A for acid-phenol removal of nicked plasmid DNA.</p>
No DNA appears on gel of deletion series	<p>DNA loss occurred during extraction and precipitation of DNA following restriction digestion, and insufficient DNA was loaded on the gel for detection. DNA should be quantified prior to deletion as stated in Section IV, Step 1. If DNA is visible in the zero-time sample but not at other time points, see potential causes of DNA degradation (above).</p>

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
Gel electrophoresis indicates smaller secondary bands are present at each time point	If the protecting restriction enzyme failed to cut completely, the singly cut molecules will be Exo III digested at twice the rate of doubly-cut molecules, so a second, smaller species will be present at each time point. Check the activity of the appropriate restriction enzyme. Repeat the initial digestion for a longer time and using more enzyme.
Low number of transformants	<p data-bbox="495 472 894 522">Too much Exo III used. Use 300–500 units Exo III per reaction tube.</p> <p data-bbox="495 541 894 720">Transformation efficiency of competent cells may be too low. Competent cells must surpass 1×10^7 colonies/μg of supercoiled DNA transformed in order to obtain successful results with the Erase-a-Base® System. Check the transformation efficiency of the competent cells by transforming intact supercoiled plasmid DNA.</p> <p data-bbox="495 740 894 1047">Unsuccessful ligation. Digest some plasmid DNA with a restriction enzyme that generates blunt ends. Ligate several hundred nanograms of digested vector to itself using the components provided with the system. Load a sample on an appropriate percentage agarose gel next to a sample of unligated linear vector and observe if there is an apparent shift in mobility of the ligated sample. If the ligation is unsuccessful, the DNA ends may not be blunt. Repeat the deletion using two- to threefold more S1 Nuclease.</p>
Transformed colonies do not contain an appropriate size insert	It has been observed that 25–50% of the clones do not correlate with the sizes indicated from the original deletion ladder (1,4); the cause of this observation is not known. Screen several colonies (10–20) in order to detect the correct ones.

VII. References

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VIII. Appendix

VIII.A. Removal of Nicked DNA Molecules by Acid-Phenol Extraction

Materials to Be Supplied by the User

(Solution compositions are provided in Section VIII.B.)

- 50mM sodium acetate (pH 4.0)
- 3M sodium acetate (pH 5.2)
- 2M sodium acetate (pH 4.0)
- 1M Tris-HCl (pH 8.6)
- 2M NaCl
- redistilled phenol
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)
- TE buffer

Preparation of Acid-Phenol



It is important that the pH of the phenol be 4.0; no selective removal is seen at pH 4.2. In addition, it is necessary to maintain low ionic strength because the closed circular DNA partitions into the phenol phase as the NaCl concentration is raised above 125mM.

1. Add 10ml 50mM sodium acetate (pH 4.0) to 10g phenol and stir to dissolve (approximately 3–4 hours at room temperature).
2. Let the phases separate and remove the upper, aqueous phase by aspiration. Add 10ml 50mM sodium acetate (pH 4.0) and stir to emulsify. Let the phases separate overnight.
3. Remove a small sample of the aqueous phase and determine the pH using a phenol-resistant pH probe.
4. Repeat Step 1 2–3 times or until the pH of the aqueous buffer is less than 4.1. Store at 4°C for one month or less.

Acid-Phenol Extraction



It is important to minimize the time that the DNA is exposed to low pH since this can result in depurination, causing losses in the recovery of closed circular molecules. The extraction procedure will work either at 4°C or room temperature, but Zasloff *et al.* (5) recommend 4°C to reduce the rate of depurination. Their results indicate that, using the recommended conditions, nicking occurred at a rate of less than one purine per 30kb per hour.

1. Prepare plasmid or replicating form DNA by standard methods so that the DNA is free of RNA and protein (4).
2. Add 0.1 volume 3M sodium acetate (pH 5.2) and precipitate with ethanol. Rinse the pellet with 70% ethanol and dry under vacuum.
3. Dissolve the pellet in deionized water and then add 2M sodium acetate (pH 4.0) to give a final concentration of 50mM, and 2M NaCl to give a final concentration of 75mM.
4. Add an equal volume of acid-phenol (prepared above) and mix thoroughly. Centrifuge at $10,000 \times g$ for 5 minutes and recover the aqueous phase. The appearance of the lower phase can vary from a turbid white emulsion to a thick white band near the interface. Two or three extractions may be needed to remove most of the contaminating nicked and linear DNA.
5. When the extraction is complete, add 0.05 volume of 1M Tris-HCl (pH 8.6) and extract with 1 volume of chloroform:isoamyl alcohol (24:1).
6. Recover the aqueous phase, add 0.1 volume of 2M NaCl and precipitate with ethanol. Rinse the pellet with 70% ethanol, dry under vacuum and dissolve the DNA at 0.5–1.0 $\mu\text{g}/\mu\text{l}$ in TE buffer for storage.

VIII.B. Composition of Buffers and Solutions

7.5M ammonium acetate (100ml)

57.81g ammonium acetate
 Dissolve the ammonium acetate in 100ml nuclease-free water (final volume). Sterilize by filtration (0.2µm filter).

dNTP mix

0.125mM each of dATP, dCTP, dGTP and dTTP

Exo III 10X Buffer

660mM Tris-HCl (pH 8.0)
 6.6mM MgCl₂

Klenow mix

30µl Klenow 1X Buffer
 3–5u Klenow DNA polymerase

Make fresh for each experiment.

Klenow 1X Buffer

20mM Tris-HCl (pH 8.0)
 100mM MgCl₂

LB (Luria-Bertani) medium (per liter)

10g Bacto®-tryptone
 5g Bacto®-yeast extract
 5g NaCl

LB plates with ampicillin (per liter)

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.0 with NaOH. Autoclave. Allow the medium to cool to 55°C before adding ampicillin (100µg/ml final concentration). Pour 30–35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

ligase mix

790µl deionized water
 100µl Ligase 10X Buffer
 100µl 50% PEG
 10µl 100mM DTT
 5u T4 DNA Ligase

Ligase 10X Buffer

500mM Tris-HCl (pH 7.6)
 100mM MgCl₂
 10mM ATP

2M NaCl

116.9g NaCl

Add nuclease-free water to a final volume of 1L. Sterilize by autoclaving.

S1 nuclease mix (for 25 timepoints)

172µl deionized water
 27µl S1 7.4X Buffer
 60u S1 Nuclease

Make fresh for each experiment.

S1 Nuclease Stop Buffer

0.3M Tris base
 0.05M EDTA

S1 7.4X Buffer

0.3M potassium acetate (pH 4.6)
 2.25M NaCl
 16.9mM ZnSO₄
 45% glycerol

SOC medium (per liter)

10g Bacto®-tryptone
 5g Bacto®-yeast extract
 5g NaCl
 10mM MgSO₄
 10mM MgCl₂

Adjust to pH 7.0 with NaOH. Autoclave. Add filter-sterilized glucose to a final concentration of 20mM.

VIII.B. Composition of Buffers and Solutions (continued)

2M sodium acetate (pH 4.0)

Dilute glacial acetic acid to 2M.
Adjust pH to 4.0 with NaOH.

3M sodium acetate (pH 5.2)

40.8g sodium acetate • 3H₂O

Dissolve sodium acetate in 80ml water. Adjust the pH to 5.2 with glacial acetic acid and add water to final volume of 100ml.

TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA

TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol, phase with 1 part of chloroform:isoamyl alcohol (24:1).

VIII.C. Related Products

Erase-a-Base® System Components Available Separately

Product	Size	Cat.#
Exonuclease III*	5,000u	M1811
	25,000u	M1815
S1 Nuclease	10,000u	M5761
DNA Polymerase I Large (Klenow) Fragment*	150u	M2201
	500u	M2206
T4 DNA Ligase* (Weiss units)	100u	M1801
	500u	M1804

*For Laboratory Use

Vectors and Competent Cells

Product	Size	Cat.#
pGEM®-5Zf(+) Vector	20µg	P2241
pGEM®-5Zf(-) Vector	20µg	P2351
pGEM®-7Zf(+) Vector	20µg	P2251
pGEM®-7Zf(-) Vector	20µg	P2371
JM109 Competent Cells >10 ⁸ cfu/µg*	1ml (5 x 200µl)	L2001
JM109 Competent Cells, >10 ⁷ cfu/µg	1ml (5 x 200µl)	L1001

*For Laboratory Use

Sequencing Systems

Product	Cat.#
SILVER SEQUENCE™ DNA Sequencing System	Q4130

Contains sufficient reagents for 100 sets of DNA sequencing reactions and staining reagents for 10 gels.

Product	Cat. #
<i>fmol</i> ® DNA Sequencing System	Q4100

For Laboratory Use. Contains sufficient reagents for 100 sets of DNA sequencing reactions.

DNA Purification

Product	Cat. #
Wizard® Plus SV Minipreps DNA Purification System	
+ Vacuum Adapters	50 preps A1340
	250 preps A1470

For Laboratory Use.

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