Technically Speaking

Promega

Subcloning Plasmid DNA Constructs

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Abstract

One of the most common applications in the field of molecular biology is the generation of recombinant plasmids for gene expression. However, this seemingly trivial task requires a number of considerations. Here we examine parameters related to the optimization of cloning and screening for plasmid constructs.

Q Is it necessary to dephosphorylate linearized vectors prior to ligating an insert?

If the plasmid vector being used was linearized with a single restriction enzyme (generating either a blunt or overhanging end), then dephosphorylation of the vector is a prerequisite to reduce religated vector background. However, if the vector was cut with two different restriction enzymes that leave incompatible ends (this does not include two different enzymes that both leave blunt ends), then dephosphorylation may be omitted. One exception to this is when the selected restriction sites lie close to one another in the vector. In this case, it is still advisable to dephosphorylate the vector, as you cannot be certain from looking at the digested plasmids on the gel if both enzymes cut the plasmid to completion. The presence of a small amount of singly cut plasmid vector in the subsequent ligation reaction can dramatically increase background, which could make it difficult to identify your desired recombinant.

Q Do I need to dilute Calf Intestinal Alkaline Phosphatase before using it?

Calf Intestinal Alkaline Phosphatase (Cat.# M1821) is supplied at a concentration of 1 unit/µl. Most protocols for using this enzyme stipulate a final activity in the dephosphorylation reaction of 0.01 unit per picomole of DNA ends. (There are two moles of DNA ends for every mole of DNA.) In such protocols we suggest making a fresh dilution of the Calf Intestinal Alkaline Phosphatase (CIAP) down to 0.01 unit/µl in the CIAP 1X Reaction Buffer. CIAP could be used at the higher concentration of 1 unit/µl without any deleterious effect on the DNA itself. However, as CIAP can be difficult to completely inactivate (and as it is a very active enzyme), most protocols using this enzyme use less, rather than more, CIAP to minimize the potential for any carryover into subsequent ligation reactions, where it could severely reduce the number of ligation products.

Q Can I use CIAP in Promega's Restriction Enzyme Buffers?

CIAP absolutely requires zinc for activity, with four zinc ions being bound per dimeric enzyme molecule. In addition, the presence of magnesium ions can stimulate the phosphatase activity of CIAP by as much as 10-fold. The enzyme has an optimal pH range of 9.1 to 10.5, but it is also stable at pH 7.5 to 9.5. It is inactivated at acid pH.

The buffer supplied with CIAP has a pH of 9.3 and contains magnesium chloride and zinc chloride at a final 1X concentration of 1mM and 0.1mM, respectively. As none of Promega's restriction enzyme buffers have a pH below 7.4 and all contain magnesium cations, it is possible to perform a dephosphorylation reaction with CIAP in any of Promega's restriction enzyme buffers, as long as zinc chloride is supplemented into the reaction to a final concentration of 0.1mM.

Promega has just introduced Shrimp Alkaline Phosphatase (SAP) (Cat.# M8201; see page 31 in this issue) as an alternative to CIAP for dephosphorylating DNA for subcloning experiments. Unlike CIAP, SAP will work in Promega's restriction enzyme buffers without adding zinc chloride. This allows the researcher to simultaneously perform restriction digestion and dephosphorylation under the same buffer conditions.

Q How do I inactivate CIAP?

The standard protocol that Promega provides on the Product Information Sheet accompanying the enzyme recommends adding 300µl of a CIAP stop buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA, 200mM NaCl, 0.5% SDS) per 50µl of reaction volume, followed by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation to concentrate the DNA. Inactivation may also be performed by adding EDTA to a final concentration of 10mM and heating to 65°C for 60 minutes or 75°C for 10 minutes prior to extraction with phenol:chloroform:isoamyl alcohol (25:24:1).

Unlike CIAP, SAP is completely and irreversibly inhibited by heating at 65°C for 15 minutes. This is the same condition used to heat inactivate Promega's thermolabile restriction enzymes. Thus, when performing a restriction digestion (using a heat-inactivatable enzyme) simultaneously with dephosphorylation using SAP, both the restriction enzyme and the SAP can be inactivated at the same time.

What options do I have for blunting an overhanging end?

T4 DNA Polymerase (Cat.# M4211, M4215) is probably the most versatile enzyme for blunting both 5´ and 3´ overhanging single-stranded sequences. This is due to the potent 3´→5´ exonuclease activity of T4 DNA Polymerase in addition to its 5´→3´ polymerization activity. The 3´→5´ exonuclease activity enables it to efficiently trim back single-stranded 3´ overhangs generated by restriction enzymes, whereas its 5´→3´ polymerization activity can fill in 5´ overhangs. Moreover, as dNTPs are required when blunting a 3´ overhang, 5´ and 3´ overhangs can be blunted simultaneously in the same reaction. (dNTPs allow the 5´→3´ polymerization activity to fill in any 5´ overhangs generated by the 3´→5´ exonuclease activity removing bases from the double-stranded DNA end.)

DNA Polymerase I Large (Klenow) Fragment (Cat.# M2201, M2206) can also be used to blunt 5´ and 3´ overhangs. However, the 3´→5´ exonuclease activity of this enzyme is not as potent as that of T4 DNA Polymerase. Therefore it is not the enzyme of choice for blunting 3´ overhangs.

How fast can I perform a ligation reaction?

Typical ligation reactions with blunt or complementary overhanging ends can be performed either overnight at 4°C or for 4–18 hours at 15–20°C. However, ligation of DNA fragments with complementary overhanging ends can be completed in as little as 3 hours at room temperature. This is because annealing between the complementary overhanging sequences increases ligation efficiency by stabilizing the DNA with ends close to one another.

The ligation time can be reduced further for both blunt and complementary overhanging ends by using ligation buffers containing molecular crowding reagents. One such system is Promega's LigaFast™ Rapid DNA Ligation System (Cat.# M8221, M8225). Using this system, it is possible to perform a ligation reaction at room temperature in 5 minutes for DNA fragments with complementary overhanging ends and 15 minutes for blunt-ended DNA fragments. The resulting number of colonies obtained following transformation is comparable to that obtained after a standard ligation at 4°C overnight.

What is the largest insert I can clone into a typical pGEM® Vector?

We have not determined an absolute upper size limit for an insert that can be cloned into any standard plasmid cloning vector such as one of the pGEM® Vector[a] series. The largest insert that we have cloned into the plasmid pGEM®-3Z Vector was 21kb (i.e., about seven times the size of the vector) (1). However, the size limit for an insert will depend on the sequence of the insert being cloned. That is to say certain inserts may not be tolerated well by *E. coli* when present on a high copy number plasmid. Reasons for this include the cryptic expression of a toxic protein encoded by the insert, or in the case of blue/white screening vectors, the insert may generate a fusion protein with the LacZ alpha peptide that reduces the host strain's viability. The insert may also contain repeat sequences that result in regions of the insert or vector becoming deleted. However, these factors are not limited to large inserts and can equally apply to smaller ones.

How can I screen for my inserts?

Blue/white screening on plates containing X-gal and IPTG is probably the easiest way to screen for the presence of an insert. This is only possible when cloning into the *lac*Z alpha peptide sequence of a plasmid containing this gene, such as pGEM®-3Zf(+) Vector (Cat.# P2271) and transforming this construct into an *E. coli* strain that allows so-called alpha complementation (e.g., JM109). White colonies are indicative of a cloned insert, while blue colonies suggest religated vector. However, this screening only tells you if an insert is present. It does not inform you of the orientation. (When cloning into a single restriction site, the insert can be in one of two orientations.) Moreover, it is possible to clone an insert into the *lac*Z alpha peptide gene that will result in a fusion protein with full or partial alpha complementation activity. In these cases, you may see blue or light blue colonies instead of white colonies.

For these reasons, and in cases where a vector is being used that does not allow blue/white screening, it is necessary to screen for inserts by other means. One such method involves restriction digestion of miniprep plasmid DNA using enzymes giving a cleavage pattern that will indicate the presence as well as the orientation of the insert. Another quick, convenient alternative is colony PCR[b]. In this method, a sterile toothpick is used to transfer some of the colony directly to a PCR mix containing buffers, dNTPs, primers and thermostable DNA polymerase. No prelysis of the *E. coli* is required, as the first denaturation step provides sufficient lysis. It is possible to distinguish one insert orientation from another by designing primers that anneal to certain regions of the vector and insert such that an amplification product will only arise when the insert is cloned in one direction. A third method that is usually used for screening a large number of colonies on many plates for a rare cloning event is colony hybridization. This method uses a labeled nucleic acid probe to screen for the presence of a particular sequence (2).

References

1. Kobs, G. (1990) *Promega Notes* **28**, 9.
2. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Laboratory, Cold Spring Harbor, New York, 6.9.

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[a]U.S. Pat. No. 4,766,072.

[b]The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.