Detection of Cytosine Methylation in RNA Using Bisulfite Sequencing

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Protocol

Detection of Cytosine Methylation in RNA Using Bisulfite Sequencing

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INTRODUCTION

Post-transcriptional RNA modifications are a characteristic feature of noncoding RNAs and have been described for ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and various other small RNAs. However, the biological function of most of these modifications remains uncharacterized. Cytosine-5 methylation (5mC) has been detected in abundant and long-lived RNA molecules such as rRNAs and tRNAs, but, because of technical limitations, the occurrence of base-methylated cytosines in other RNAs is not known. To facilitate the detection of RNA methylation, we have established a method for analyzing base-methylated cytosines in RNA using bisulfite sequencing. Treatment of RNA with bisulfite causes the chemical deamination of nonmethylated cytosines to uracil, while methylated cytosines remain unaffected. cDNA synthesis followed by polymerase chain reaction (PCR) amplification and DNA sequencing allows investigators to reproducibly and quantitatively distinguish unmethylated cytosines (as thymines) from methylated cytosines in tRNAs and rRNAs. Using high-throughput sequencing approaches, this protocol should enable the characterization of 5mC methylation patterns in any RNA molecule, including low abundance RNAs.

RELATED INFORMATION

Additional information on the detection of RNA modifications is reviewed by Kellner et al. (2010). Specific information on the detection of RNA methylation and its biological function is reviewed by Motorin et al. (2010). Additional reagents and techniques are detailed in Recovery of DNA from Low-Melting-Temperature Agarose Gels: Organic Extraction (Sambrook and Russell 2006) and Purification of RNA Using TRIzol (TRI Reagent) (Rio et al. 2010). An article describing Strategies for Cloning PCR Products (D'Arpa 2009) is also available.

MATERIALS

RNase-free materials and equipment must be used throughout the procedure. DEPC (diethyl pyrocarbonate) treatment of solutions is mandatory.

Reagents

Adapter primers (for high-resolution RNA methylation analysis; see Step 31)

Chloroform

dNTPs (2.5 mM each)

DNA protect buffer (EpiTect Bisulfite Kit; QIAGEN 59104)

DNase I, RNase-free (i.e., Turbo DNase; Ambion-Applied Biosystems AM2238)

Ethanol (100% and 75%)

Gel purification kit (QIAquick Gel Extraction Kit; QIAGEN 28704) (for PCR products ≥100 bp; see Steps 28 and 34)

Glycogen (i.e., Glycoblue; Ambion-Applied Biosystems AM9515)

H_2O (RNase-free)

Low-melting agarose gels (native 2%-3%) prepared in TBE buffer (1X)

Micro Bio-Spin 6 columns (Bio-Rad 732-6200)

Phenol (acid):chloroform (Ambion AM9720)

Primers for PCR

It is advisable to design PCR primers that allow recovery of relatively short amplicons, because this method results in considerable RNA degradation, both during the bisulfite reaction and the following desulphonation step at basic pH. This generally limits the recovery of long RNA molecules (>150 nucleotides). Generally, the read-length obtained from state-of-the-art next generation sequencers should allow for collection of sufficient information from short RNA-derived reads.

PCR primer sequences should be designed to avoid containing cytosines, in order to decrease the amplification of deamination artifacts.

Primers for specific reverse transcription (RT; i.e., stem-loop primers) or random primers (Invitrogen-Life Technologies 48190-011) (see Step 20)

Reagents for DNA sequencing (see RNA Methylation Analysis)

Reagents for Recovery of DNA from Low-Melting-Temperature Agarose Gels: Organic Extraction (Sambrook and Russell 2006) (for purification of PCR products <100 bp; see Steps 28 and 34)

Reagents for RNA purification (e.g., TRIzol [Invitrogen-Life Technologies 15596-026]) (see Step 1 and Purification of RNA Using TRIzol (TRI Reagent) [Rio et al. 2010])

Size fractionation using denaturing urea-PAGE or similar procedures after RNA extraction using TRIzol is optional; see Step 2.

RNase inhibitor (recombinant)

Sample containing the RNA of interest (see Step 1)

Avoid repeated freezing and thawing of isolated RNA.

Sodium acetate (pH 5.2)

Sodium bisulfite (EpiTect Bisulfite Kit; QIAGEN 59104)

SuperScript III Reverse Transcriptase (Invitrogen-Life Technologies 18080-044)

SuperScript RT is supplied with the required 5X first-strand buffer and 100 mM DTT (dithiothreitol).

Taq DNA polymerase (with PCR buffer)

TE buffer

TOPO TA Cloning Kit (Invitrogen-Life Technologies K4575-40) (optional; see RNA Methylation Analysis)

Tris-Cl (pH 9.0)

Equipment

Equipment for agarose gel electrophoresis

Equipment for UV gel documentation

Ice

Microcentrifuge, benchtop

Micropipettor and tips

Scalpel

Spectrophotometer

Thermal cycler

Tubes, microcentrifuge (1.5-mL, siliconized, RNase-free)

Tubes, PCR (0.2-mL)

Vortex mixer

Water bath preset to 37°C

METHOD

RNA Extraction

1. Extract total cellular RNA from tissues or cells using TRIzol (see Purification of RNA Using TRIzol (TRI Reagent) [Rio et al. 2010]), or recover RNA from other sources such as density gradients, RNA immunoprecipitates, or size fractionation reactions.
Prepare enough material to recover at least 2 µg of total RNA for the subsequent bisulfite reaction (less RNA may be sufficient following enrichment for specific RNAs).
If recovery of small RNAs is desired, the precipitation mix should contain a carrier such as glycogen (20 µg) to secure efficient recovery.

2. Measure the RNA concentration (260 nm) using a spectrophotometer.
Total RNA extracted from tissues or cells using TRIzol can be processed directly or after size fractionation using denaturing urea-PAGE or similar procedures.

DNase Treatment of RNA

3. Treat 2 µg of RNA with DNase I (2 U) in a total volume of 10 µL for 30 min at 37°C.

4. Add 290 µL of TE buffer and recover the RNA using 1 volume of acidic phenol:chloroform. Vortex briefly and centrifuge at top speed for 5 min at 4°C.

5. Transfer the RNA into a new tube, add 1 volume of chloroform, vortex briefly, and centrifuge at top speed for 5 min at 4°C.

6. Treat the RNA solution as follows:

i. Transfer the RNA solution into a new tube.

ii. Add 3 volumes of 100% ethanol, sodium acetate (pH 5.2) to 0.3 M and 20 µg of glycogen.

iii. Precipitate the RNA at −80°C for ≥1 h.

7. Recover the RNA by centrifugation at top speed for at least 30 min at 4°C.

8. Wash the RNA pellet with 75% ethanol and briefly air-dry (≤5 min at room temperature).

Bisulfite Treatment of RNA

Work with bisulfite at room temperature, because highly concentrated sodium bisulfite solution precipitates at low temperatures. Bisulfite treatment at high temperatures will deaminate nonmethylated cytosines to uridine sulphonate (see Fig. 1).

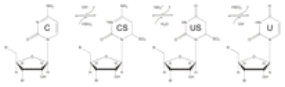

Figure 1. Schematic diagram of the bisulfite conversion reaction in RNA. C, cytidine; CS, cytidine sulphonate; US, uridine sulphonate; U, uridine.

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9. Resuspend the RNA pellet in 10 µL of RNase-free H_2O and transfer into a 0.2-mL PCR tube.

10. Add 42.5 µL of sodium bisulfite solution (EpiTect kit) and mix thoroughly by pipetting.

11. Add 17.5 µL of DNA protect buffer (EpiTect kit) and mix thoroughly by pipetting.

12. Perform the deamination reaction using a thermal cycler to run one of the following programs:

i. For short RNA molecules, use two to three cycles of: 5 min at 70°C followed by 60 min at 60°C.

ii. For long RNA molecules or RNA molecules with high propensity for forming secondary structures, use more than three cycles of 5 min at 70°C followed by 60 min at 60°C.
It is important to keep the reaction at room temperature after deamination, because bisulfite salt solution precipitates in the cold.

13. Desalt the reaction by passage through a Micro Bio-Spin 6 column.

14. Desulphonate the RNA adducts by adding 1 volume of 1 M Tris-Cl (pH 9.0) and incubate at 37°C for 1 h.
Desulphonation at basic pH converts uridine sulphonate to uridine (see Fig. 1).

15. Add sodium acetate (pH 5.2) to 0.3 M, 20 µg of glycogen, and 3 volumes of 100% ethanol. Precipitate the RNA at −80°C for ≥1 h.

16. Recover the RNA by centrifugation at top speed for at least 30 min at 4°C.

17. Wash the RNA pellet with 75% ethanol and briefly air-dry (≤5 min at room temperature).

18. Resuspend the RNA in RNase-free H_2O.

19. Measure the RNA concentration (260 nm) using a spectrophotometer.
Do not store deaminated RNA for periods longer than 1 mo and avoid repeated freezing and thawing of RNA.

cDNA Synthesis

20. To 200–500 ng of bisulfite-treated RNA (from Step 19) in a 0.2-mL PCR tube, add 2 µL of 10 mM dNTPs and 50 nM of RT primer (random hexamer or specific primers). Add RNase-free H_2O to a total volume of 13 µL.
Random priming should be sufficient to reverse transcribe abundant RNAs and qualitatively assess their RNA methylation patterns. For reverse transcription of low abundance RNAs, the use of specific primers is recommended. For reverse transcription of RNAs with known 3' ends (such as small RNAs, i.e., miRNAs), specific stem-loop RT primers can be designed (Fig. 2); this increases the length of the cDNA with the advantage that amplicons can be separated from primer-dimers during the subsequent PCR.

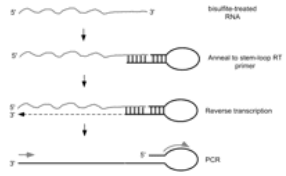

Figure 2. Use of specific stem-loop RT primers. For known 3' ends of RNAs, stem-loop primers can be designed to anneal staggered from the RNA end. PCR primers will amplify the stem-loop structure.

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For quantitative assessment ofRNA methylation, it is advisable to introduce "bar-coding" into the cDNA synthesis. This can be accomplished by including a stretch of random sequence into the RT primer. As a result, each cDNA molecule will carry a unique 5' tag which allows determination of the clonality ofthe sequenced PCR amplicons (Fig. 3).

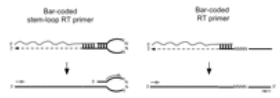

Figure 3. Bar-coded cDNA synthesis. Introduction of a random set of nucleotides in the RT primers (left, stem-loop primer; right, specific primer) allows for tagging/bar-coding of N^4 different RNA molecules (i.e., for N = 4, 4^4 = 256) during cDNA synthesis.

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21. Denature the RNA for 5 min at 70°C.

22. Place the reaction immediately on ice for at least 1 min.

23. Add the following reagents to the reaction tube:

RNase inhibitor	40 U
5X reverse transcription buffer	4 µL
DTT	To 5 mM
SuperScript III reverse transcriptase	200 U

24. Using a thermal cycler, proceed with the following program:

Time	Temperature
10 min	25°C
50 min	50°C
15 min	94°C
hold	4°C

PCR Amplification from First-Strand cDNA

25. Set up a standard PCR using 1–2 µL of first-strand cDNA (from Step 24) per 25-µL reaction and specific forward and reverse PCR primers at 200 nM.

26. Using a thermal cycler, run the PCR program using a low annealing temperature (≥40°C and ≤50°C) for 30–35 cycles.
Keep in mind that deaminatednucleic acid sequences require low primer annealing temperatures (leading to reduced primer specificity) due to the absence ofmost cytosines.

27. Separate the PCR product on a 2%–3% low-melting agarose gel and excise the product using a clean scalpel.
See Troubleshooting.

28. Recover the PCR product using one of the following methods, depending on amplicon length:

i. If the product has a length shorter than 100 bp, recover the PCR product using low-melting agarose/phenol extraction followed by ethanol precipitation using 0.3 M sodium acetate (pH 5.2) and 20 µg of glycogen (see Recovery of DNA from Low-Melting-Temperature Agarose Gels: Organic Extraction [Sambrook and Russell 2006] and Chapter 5 in Sambrook and Russell [2001]).

ii. If longer amplicons are produced, use a gel purification kit.

29. Measure the concentration of the PCR product (260 nm) using a spectrophotometer.

RNA Methylation Analysis

Low-Resolution RNA Methylation Analysis (Small Scale)

30. Clone PCR fragments directly using TOPO TA cloning according to the manufacturer's instructions (see Strategies for Cloning PCR Products [D'Arpa 2009]) followed by Sanger sequencing.
RNA methylation can be assessed qualitatively by comparing sequence reads to the genomic reference sequence. When assessing RNA methylation by mapping sequence reads back to the genome, be aware that an increased number of mismatches can result from reverse transcription errors made by the reverse transcriptase in order to overcome modified nucleotides (Ebhardt et al. 2009).

High-Resolution RNA Methylation Analysis (Large Scale)

31. Use 50 ng of PCR product from Step 29 as DNA template in a 100-µL PCR reaction. Include the following:

Adapter primers (20 µM stock)	1 µL of each primer
dNTPS (10 mM)	10 µL
PCR buffer	1X
Taq DNA polymerase	2.5 U

For quantitative RNA methylation analysis, adapter-containing primers should be designed to match the sequencing platforms available (i.e., 454, Solexa).

32. Using a thermal cycler, run a PCR program using a low annealing temperature (≥40°C and ≤50°C) for 15-20 cycles.

33. Separate the adapter-containing PCR product from the template DNA on a 2%-3% low-melting agarose gel and excise the PCR product using a clean scalpel.

34. Recover the PCR product using one of the following methods, depending on amplicon length:

i. If the product has a length shorter than 100 bp, recover the PCR product using low-melting agarose/phenol extraction followed by ethanol precipitation using 0.3 M sodium acetate (pH 5.2) and 20 µg of glycogen (see Recovery of DNA from Low-Melting-Temperature Agarose Gels: Organic Extraction [Sambrook and Russell 2006] and Chapter 5 in Sambrook and Russell [2001]).

ii. If longer amplicons are produced, use a gel purification kit.

35. Measure the concentration of adapter-containing PCR product and proceed with a standard preparation for high-throughput DNA sequencing.
See Troubleshooting.

TROUBLESHOOTING

Problem: There is no PCR product visible on the agarose gel.

[Step 27]

Solution: Consider the following:

1. Use higher molarities of dNTPs for reverse transcription (Step 20). This allows the reverse transcriptase to overcome blocks due to certain RNA base modifications.

2. Adjust the deamination cycles or denature at higher temperatures in H_2O prior to deamination (tRNAs are stable up to 95°C in a nonbasic environment).

Problem: There is more than one PCR product visible on the agarose gel.

[Step 27]

Solution: Increase the temperature used for cDNA synthesis (Step 24), or increase the annealing temperature during PCR (Step 26) to increase stringency.

Problem: Deamination artifacts are observed.

[Step 35]

Incomplete deamination can occur as a result of extensive secondary RNA structures and leads to many more cytosines in bisulfite-treated RNA than expected. Consider the following:

1. Adjust the deamination cycles or denature at higher temperatures in H_2O prior to deamination.

2. If a disproportionately high number of deamination artifacts persists in the sequence reads (suggesting DNA contamination), verify that the DNase I digest (Step 3) was successful.

DISCUSSION

We used the method presented here for RNA bisulfite sequencing of highly abundant RNA molecules (such as rRNAs and tRNAs) to determine the

we used the method presented here for RNA bisulfite sequencing of highly abundant RNA molecules (such as rRNAs and tRNAs) to determine the exact nucleotide position of methylated cytosines (Schaefer et al. 2009a). Interestingly, we also observed that another modification on cytidine (m4mC) can render this nucleotide refractory to bisulfite-mediated deamination (Schaefer et al. 2009a). This suggests that RNA bisulfite sequencing can be applied to detect m5C and additional cytosine modifications in RNA. In general, this method allows investigation of the cytosine methylation patterns in any candidate RNA region when using specific reverse transcription and PCR primers. Combining the basic methodology with unbiased library preparation (as used in RNA-seq or small RNA sequencing library preparation) should allow analysis of more complex cDNA libraries derived from bisulfite-treated RNA molecules. This could facilitate the identification of new targets of a given RNA methyltransferase and will help to define the biological function of RNA methylation (Schaefer et al. 2009b, 2010).

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Caution

Chloroform ($CHCl_3$)

Chloroform ($CHCl_3$) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

Caution

DEPC (Diethyl pyrocarbonate)

DEPC (diethyl pyrocarbonate) is a potent protein denaturant and is a suspected carcinogen. Aim bottle away from you when opening it; internal pressure can lead to splattering. Wear appropriate gloves and lab coat. Use in a chemical fume hood.

Caution

DTT (Dithiothreitol)

DTT (dithiothreitol) is a strong reducing agent that emits a foul odor. It may be harmful by inhalation, ingestion, or skin absorption. When working with the solid form or highly concentrated stocks, wear appropriate gloves and safety glasses and use in a chemical fume hood.

Caution

Phenol:chloroform

Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol!

Chloroform ($CHCl_3$) is irritating to the skin, eyes, mucous membranes, and respiratory tract. It is a carcinogen and may damage the liver and kidneys. It is also volatile. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood.

Information Panel

Strategies for Cloning PCR Products

Peter D'Arpa

Adapted from PCR Primer, 2nd edition (eds. Dieffenbach and Dveksler). CSHL Press, Cold Spring Harbor, NY, USA, 2003.

INTRODUCTION

Cloning polymerase chain reaction (PCR)-amplified fragments into plasmids offers several advantages. Bacteria containing plasmids can be frozen, providing a ready supply of amplified material. Because of the variety of available plasmids with different promoters and selectable markers, cloning is also useful when mutations are to be introduced into the fragment before expression, or when sequence tags encoded in the vector are to be added in-frame. The ease with which nucleotide sequences can be added to the ends of PCR products has led to the development of a variety of cloning strategies. Because such cloning is typically the first step for generating a reagent that will be used to achieve a specific experimental goal, the efficiency of the cloning procedure is an important consideration: Cloning strategies should be simple in design and execution, requiring a minimum of enzymatic steps. Toward this goal, many companies market and continue to develop reagent kits that improve the ease and rapidity of cloning PCR products. This article focuses on some common and efficient cloning strategies, such as those that use DNA ligase or vaccinia virus topoisomerase I (TOPO), as well as techniques for in vitro and in vivo recombination of PCR products and vectors having homologous duplex ends. Also covered is the production of linear PCR products with defined 5' and 3' functional elements, which enable direct mammalian cell expression or in vitro transcription/translation. We present an overview of these strategies, their molecular basis, and their advantages and disadvantages for specific applications.

RELATED INFORMATION

Specific protocols are available for Purification of PCR Products in Preparation for Cloning (Sambrook and Russell 2006a), Removal of Oligonucleotides and Excess dNTPs from Amplified DNA by Ultrafiltration (Sambrook and Russell 2006b), Blunt-End Cloning of PCR Products (Sambrook and Russell 2006c), Cloning PCR Products into T Vectors (Sambrook and Russell 2006d), Cloning PCR Products by Addition of Restriction Sites to the Termini of Amplified DNA (Sambrook and Russell 2006e), and Genetic Engineering with PCR (Sambrook and Russell 2006f). Additional technical details are available in manufacturers' literature or protocol manuals.

The choice of PCR product cloning strategy often depends on the subsequent application. When cloning for the purpose of synthesizing the nucleic acid or the protein encoded by the PCR product, the DNA must be cloned into the vector in the proper orientation with respect to the promoter. This is typically accomplished by adding different terminal sequences to the PCR product via the primer tails. In other applications, such as the shotgun cloning of genomic DNA, directional cloning is not required.

The quality of a PCR product's termini is essential to the success of its cloning. The coupling efficiency of the synthesis of oligonucleotide primers is typically ~98%-99%, and therefore 39%-63% of a 50-mer oligonucleotide is truncated on the 5' terminus before purification (Life Technologies 1999). Subsequent purification methods differ in their ability to remove truncated products. Therefore, for certain applications, especially when longer primers are used, high-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE) purification might be necessary. (Consult manufacturers' literature to determine the level of purity required for each method.)

LIGASE-DEPENDENT CLONING

DNA Ligase

One of the most common methods for cloning PCR products uses DNA ligase to covalently join a DNA duplex end having a 5' phosphate to a duplex having a 3' hydroxyl (OH) (e.g., those produced by restriction enzyme cleavage). The reaction proceeds via a phospho-AMP intermediate and occurs only when the 5' phosphate and 3' OH are juxtaposed properly by base-pairing of overhangs or if both molecules have blunt termini. However, PCR products are typically generated using synthetic oligonucleotide primers that lack 5' phosphates. These products can be ligated directly into a vector having 5'-terminal phosphates, as in the case of certain types of T-A and blunt-end cloning vectors (see below). Alternatively, PCR products having 5'-terminal phosphates (produced either by amplification with kinased primers or by kinase treatment or restriction enzyme cleavage of the PCR product) can be ligated to vectors without 5' phosphates that result from phosphatase treatment. Phosphatase treatment of the vector is used in strategies where the vector termini are compatible for ligation (e.g., a vector cut with a single restriction enzyme) to prevent recircularization without insertion of the PCR product.

In cloning reactions, DNA ligase must join both ends of a linear PCR product to a linear vector to form a circular recombinant molecule. As discussed above, this can be accomplished if either the PCR product, the vector, or both have terminal 5' phosphates. When both the insert and vector have 5'-terminal phosphates, the recombinant molecule can be closed circular; when only one of the reactant DNAs has a terminal phosphate, a doubly nicked circular form results. Both types of recombinants transform Escherichia coli, and cloning strategies producing both types are commonly used.

Directional Cloning of a PCR Product Having Noncompatible Termini

In this strategy, the termini of the PCR product have different restriction enzyme cleavage sites that are added to the DNA of interest via the tails of the synthetic oligonucleotide PCR primers. (Neither restriction site should be present within the sequence to be amplified.) Following PCR, about one-tenth of the reaction mix should be electrophoresed on an agarose gel to check whether the expected size has been obtained. The PCR product is then typically purified out of the PCR mix using a silica membrane kit (e.g., QIAquick, QIAGEN; NucleoSpin, Clontech) and then cut with restriction enzymes, gel-purified, and placed into a ligase reaction mixture with a vector having compatible ends. Because both the PCR product

and the vector are cut with restriction enzymes, both the insert and the vector contain 5' phosphates on both termini, and thus closed circular recombinants can be produced. In "sticky end" ligations, the overhangs anneal at a ligation temperature of 16°C, stabilizing the juxtaposition of the 5' phosphate and 3' OH and providing improved ligation efficiency compared to "blunt-end" cloning.

Sticky-end ligations with a vector having noncompatible termini are preferred because self-ligation, which can cause a high background of wrong colonies, is prevented. However, there are pitfalls executing this strategy effectively. Incomplete cutting at one of the restriction sites on the vector can result in a singly cleaved vector with compatible sticky ends that can join during the ligation reaction, producing an unacceptable high background of colonies without the inserted PCR product. Similarly, partial cutting of the PCR product, although less deleterious than partial cutting of the vector, can adversely affect cloning efficiency as a result of mis-estimation of the molar quantity of doubly cut insert and because intermediates consisting of vector linked to a singly cut PCR product are dead ends. The presence of 5'-truncated primers can also result in a PCR product cut on only one end, which can ligate to the vector to produce a dead-end product.

The cutting of PCR products also can be incomplete if the restriction sites are too close to the ends (Zimmermann et al. 1998). Because cleavage efficiency increases as the number of nucleotides flanking the restriction enzyme recognition site increases, it is useful to add more (up to six are required) rather than fewer nucleotides to the ends of a PCR primer's flanking restriction sites and to design cloning strategies using enzymes that cut efficiently close to the ends (see, e.g., http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/cleavage_olignucleotides.asp). Also, it is important to use sufficient amounts of restriction enzymes and longer cutting times.

A high background of the wrong colonies can also be caused by carryover of the supercoiled plasmid that was used as the template for the PCR. Small amounts of supercoiled PCR template can comigrate (and thus copurify) with the linear PCR product. This is more likely to occur if the PCR product is large or if the plasmid preparation contains a low level of deleted plasmids (i.e., not detectable by ethidium bromide staining) that retain the origin of replication and the selectable marker and thus can transform E. coli. One solution to this problem is to cut the template before performing PCR to produce fragments that cannot self-ligate easily and that have different ends from the PCR product (and, of course, use enzymes that do not cut within the insert). Another way to avoid this problem is to use a template containing a different antibiotic-resistance gene from the one used for selecting the recombinants. Some vectors have two selectable markers (e.g., pCR-Blunt II-TOPO contains kanamycin- and zeocin-resistance genes). Thus, if the PCR template plasmid contains a kanamycin-resistance gene, zeocin can be used to select the correct recombinants.

Nondirectional Ligase-Dependent Cloning: Sticky-End Cloning Using a Singly Cut Vector and a Compatible PCR Product with Identical Termini

When only a single restriction site is available on a cloning vector, an additional step, alkaline phosphatase treatment of the vector, is usually used to reduce vector self-ligation. Although this strategy can be successful, it is more prone to failure. Noncompatible sticky-end cloning is the preferred method and should be used wherever possible.

Nondirectional Ligase-Dependent Cloning: Blunt-End Cloning

Any DNA with a blunt end can be joined to any other DNA with a blunt terminus, regardless of the nucleotide sequence, provided that one has a 5' phosphate and the other a 3' OH. PCR products generated with proofreading polymerases (i.e., those having 3'→5' exonuclease activity) are mostly blunt (Lohff and Cease 1992) and can be ligated directly to a blunt-ended vector. However, blunt-end cloning does not stabilize the juxtaposition of the 5' phosphate with the 3' OH, as is provided by the annealing of overhangs in sticky-end cloning. Relative to sticky-end cloning, blunt-end cloning is less efficient, requiring 10-fold greater concentrations of ligase. The potential of the vector to self-ligate (and, thus, the need to remove the phosphate groups with alkaline phosphatase) also reduces efficiency.

A great improvement in the efficiency of blunt-end cloning can be achieved by performing the ligation reaction in the presence of the restriction enzyme used to linearize the vector. In this strategy, self-ligated vectors are continually reopened, which drives the reaction toward the desired product. The PCR-Script Cloning Kits (Stratagene), for example, use the blunt-cutting, eight-base cutter SrfI. In this system, neither terminus of the PCR product can have the sequence 5'-GCCC-3' (the SrfI half-site), and the SrfI cleavage site cannot be present within the amplified region. Stratagene offers vectors with sequencing primer sites and RNA polymerase promoters flanking the insert, and a vector for mammalian expression via a cytomegalovirus promoter.

Nondirectional Ligase-Dependent Cloning: T-A Cloning

PCR products generated with a non-proofreading polymerase contain deoxadenosine (dA) overhangs and can be cloned directly (i.e., without post-PCR enzymatic steps) into vectors having deoxythymidine (dT) overhangs. Vectors for this purpose can be generated by cutting two different restriction sites, leaving a 3' dT overhang on each end of the linearized vector (the recessed strands have 5' phosphates) (Mead et al. 1991). Alternatively, dT can be added with a non-proofreading polymerase in the presence of the deoxynucleotide triphosphate dTTP. The vectors cannot religate because dT cannot pair with dT. Several T-A cloning vectors are available commercially and supplied in linearized form with 3' dT overhangs. The T-A cloning strategy is an efficient one. The PCR products lack 5' phosphates and thus do not ligate with each other. Non-proofreading polymerases such as Taq generally add a dA overhang, although the nucleotide added depends on the preceding nucleotide; the highest cloning efficiencies have been obtained using PCR primers with a 5'-terminal dA (Hu 1993). For efficient addition of dA overhangs to the PCR product, a final extension step of 10 min at 72°C is recommended.

TOPO CLONING

In the TOPO cloning method, vaccinia virus topoisomerase I (TOPO) is used instead of DNA ligase to join DNA molecules (Fig. 1 ; Shuman 1994). Vaccinia TOPO specifically recognizes the duplex sequence 5'-(C/T)CCTTNNN-3' and cleaves after the 3' dT in one strand (see arrow, Fig. 1B). This cleaved intermediate consists of Tyr-274 of topoisomerase I (TOPO) attached covalently to the dT via a 3' phosphodiester bond [i.e., (C/T)CCTT-3'-TOPO], and a free 5' OH on the other side of the break (OH-5'-NNN). This 5' OH can attack the phosphotyrosyl bond between the DNA and TOPO, which results in the religation of DNA and the release of TOPO (i.e., a reversal of the cleavage reaction) (Fig. 1D). However, if the 5'-(C/T)CCTT-3' is located close to the 3' terminus, the single-stranded OH-5'-NNN dissociates from the duplex as a result of insufficient base-pairing, leaving TOPO covalently linked to the 3' end of the duplex. This TOPO-DNA molecule is said to be "activated," because it is highly susceptible to attack by the 5'-OH of a heterologous duplex DNA having a complementary 5' protrusion (Fig. 1C). Because a free 5' OH is required to attack the phosphotyrosyl

bond between the vector and TOPO, PCR products to be cloned must contain 5' OH and therefore must not be phosphorylated; unphosphorylated primers should be used for PCR, and the products must not be kinased. Also, restriction fragments must be treated with alkaline phosphatase before TOPO cloning to generate free 5'-OH termini.

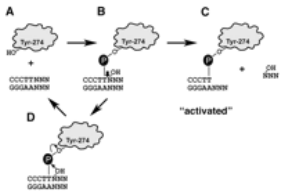

Figure 1. Production of TOPO-activated DNA fragments. (A) Vaccinia virus TOPO with the OH of Tyr-274 is shown with a DNA fragment containing a TOPO cleavage site. (B) TOPO cleaves after the second dT and links to the DNA via a 3' phosphotyrosyl bond, leaving a free 5' OH on the other side. (C) When the cleavage site is located close to the 3'-end of duplex DNA, the single stranded OH-5'-NNN dissociates, and the TOPO-activated molecule can combine with a heterologous DNA duplex having a complementary 5' overhang. (D) The cleavage reaction can reverse if the free 5' OH attacks the phosphotyrosyl bond, religating the DNA and releasing TOPO. (Adapted from Invitrogen 2002b, with permission from Invitrogen, part of Life Technologies © 2002.)

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TOPO cloning is particularly efficient because it does not require post-PCR restriction digestion, and the vector cannot circularize without an insert. The TOPO reaction is rapid, requiring only 5 min for most applications. In addition, gel purification is not required if a single dominant PCR product is obtained, although it is recommended for PCR products >1 kb because TOPO cloning is very efficient for small fragments that can contaminate certain PCRs (Invitrogen 2002a). Further efficiency is obtained through positive (e.g., blue/white tests) and negative (e.g., disruption of the lethal E. coli gene ccdB) selection strategies. Some cloning vectors contain two resistance markers, so that a marker other than the marker on the template can be used to select recombinants. This can eliminate high backgrounds caused by colonies containing the plasmid used as the template to generate the PCR product.

Invitrogen produces cloning vectors (shipped as linearized plasmids with TOPO linked covalently to the 3' termini) that can be used for blunt, T-A, and directional cloning. They contain a variety of DNA functional elements suitable for a wide range of applications. Furthermore, PCR inserts TOPO-cloned into Invitrogen's pENTR system can be transferred via a one-step in vitro reaction using λ phage recombination proteins (i.e., the Gateway method) in the desired orientation into a large number of destination vectors.

TOPO T-A Cloning

As with T-A vectors used for ligase-dependent cloning, TOPO T-A cloning vectors have termini with single 3' dT overhangs and are used to clone PCR products having a single overhanging 3' dA produced by the non-template-dependent terminal transferase activity of polymerases such as Taq, which lack proofreading. As supplied by Invitrogen, the 3' termini of these vectors are linked to vaccinia TOPO. Self-closure of the vector is prevented because the 5'-overhanging dT of one terminus cannot pair with the 3'-overhanging dT of the other end, blocking the 5' OH of one strand from attacking the phosphotyrosyl bond at the other terminus. PCR products with dA overhangs, however, are able to pair with the dT overhangs, juxtaposing the 5'-OH groups and allowing the product to attack the phosphotyrosyl bonds of the vector (Fig. 2). PCR products amplified with polymerase mixtures (Taq plus a proofreading polymerase) should contain a 10-fold excess of Taq to ensure the presence of 3'-dA overhangs on the PCR product (Invitrogen 2002a). PCR products amplified solely with a proofreading polymerase lack these overhangs, which must be added in a post-amplification reaction using Taq polymerase.

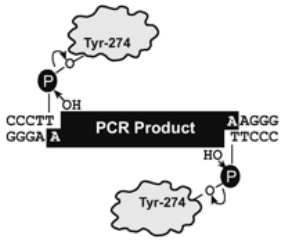

Figure 2. TOPO T-A cloning. The non-template-dependent terminal transferase activity of Taq polymerase adds a single dA to the 3'-ends of PCR products. The linear TOPO T-A cloning vector contains overhanging 3'-dT residues and is "TOPO-activated" (i.e., TOPO is attached via a 3' phosphotyrosyl linkage), enabling it to ligate efficiently with PCR products having single 3'-dA overhangs on both sides. The 5' OH of each end of the PCR product can attack the phosphotyrosyl bond between the vector DNA and TOPO, resulting both in the release of TOPO molecules and the production of a doubly nicked, circular, recombinant molecule (not shown). (Reproduced from Invitrogen 2002a, with permission from Invitrogen, part of Life Technologies © 2002.)

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TOPO Blunt-End Cloning

As with conventional ligase-mediated cloning of blunt-ended DNA fragments, recircularization of the vector is a potential problem. In TOPO cloning, the 5' OH of each blunt end is not sterically hindered from attacking the phosphotyrosyl bond on the other end, permitting recircularized vector lacking the insert to be produced. To overcome this, Invitrogen has designed TOPO cloning vectors in which ligation of the insert disrupts the lethal E. coli gene ccdB (which is toxic because it traps gyrase) (Van Melderen 2002). Thus, cells having plasmids that self-ligate without an insert are killed when plated.

TOPO Directional Cloning

The TOPO cloning vectors for directional cloning have one blunt terminus and one with a 4-bp 5' protrusion (GTGG). The PCR product can be cloned directionally into the vector by adding four bases to the forward primer that are complementary to the vector's protrusion (i.e., CACC). The vector's protrusion invades the blunt-ended PCR product, annealing with the added bases. This orients the PCR product correctly and positions its 5' OH to attack the phosphotyrosyl bond (Fig. 3). Correct orientation is obtained in >90% of recombinant clones (Invitrogen 2002a).

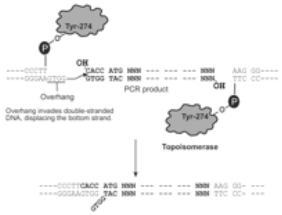

Figure 3. Directional TOPO Cloning. A linear vector (i.e., Invitrogen's pENTR) is shown as two independent TOPO-linked DNA molecules: One end (left) has a 5'-GTGG protrusion; the other terminus (right) is blunt. Directional insertion of the PCR product occurs via complementarity between the vector's protrusion and the four bases (CACC) added to the 5'-terminal of the duplex PCR product's forward primer. The vector protrusion accesses the product via strand invasion. Annealing these base pairs juxtaposes the 5' OH on the PCR product to attack the phosphotyrosyl bond. The other terminus of the PCR product is blunt, and its 5' OH is not hindered from attacking the phosphotyrosyl bond on the blunt-ended side of the vector. (Reproduced from Invitrogen 2002a, with permission from Invitrogen, part of Life Technologies © 2002.)

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PRODUCTION OF LINEAR CONSTRUCTS WITH DEFINED 5' AND 3' FUNCTIONAL ELEMENTS THAT ARE USED DIRECTLY FOR MAMMALIAN CELL EXPRESSION OR IN VITRO TRANSCRIPTION/TRANSLATION

Using either the TOPO Tools (Invitrogen) or TAP Express (Genlantis) systems, linear constructs can be produced in a single day. This might be preferable to making plasmid clones in certain circumstances, especially in high-throughput situations.

TOPO Tools

The TOPO Tools $P_{CMV/TetO}$ 5' Element Kit (Invitrogen) enables the TOPO-mediated joining of functional elements (such as promoters, tags, and terminator regions) to PCR-amplified DNA. After adding the 5' and 3' functional elements, the construct is PCR-amplified, and the linear product is used directly for in vitro transcription (RNA probe and antisense) and transcription/translation (protein analysis). The product can also be transfected for in vivo expression and protein analysis (e.g., in protein-protein interaction analyses by coprecipitation and mammalian two-hybrid systems).

To create constructs having specific 5'-side and 3'-side functional elements, different 11-bp sequences are incorporated into the primers for amplifying a DNA of interest (Fig. 4). This creates a PCR product having one terminus complementary to a 5'-TOPO-adapted functional element and the other end complementary to a 3'-TOPO-adapted element. The terminal six bases of the primers' 5' 11-base sequences complement the 5' overhang sequence of the TOPO-adapted functional element, whereas the internal five bases comprise the TOPO recognition sequence. Cleavage of the recognition sequences on the PCR product creates ends with a 3'-phosphotyrosyl-linked TOPO and a 5' six-base overhang complementary to the overhang of a 5' or 3' functional element. Thus, both ends of the PCR product and both functional elements have TOPO linked to the 3' strand and a six-base overhang on the 5' strand terminating with an OH. When the 6-bp overhang of the PCR product anneals with the 6-bp overhang of a functional element, each 5' OH attacks the other molecule's phosphotyrosyl bond. Both strands of the functional element ligate to both strands of the PCR product, and two TOPO molecules are released on each functional side.

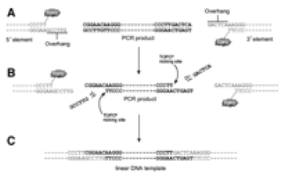

Figure 4. TOPO Tools ($P_{CMV/TetO}$ 5' Element) for producing linear DNA constructs with defined 5' and 3' functional elements. (A) The 5' and 3' functional elements and the PCR product to which they are to be added are shown. Different 11-bp sequences (bold) are added to the ends of the PCR product via forward and reverse primers. (B) TOPO cleaves the PCR product, creating TOPO-activated overhangs (and the release of single-stranded 6-mers) complementary to the overhangs of the TOPO-activated functional elements. (C) Annealing the overhangs juxtaposes the 5' OH of each to attack the phosphotyrosyl bond of the other, ligating both strands of each functional element to both strands of the PCR product, releasing two TOPO molecules, and creating a linear recombinant template for direct use in vitro and in vivo. (Reproduced from Invitrogen 2002b, with permission from Invitrogen, part of Life Technologies © 2002.)

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This contrasts with standard TOPO cloning, wherein only a single strand on each side of the insert is ligated to the vector, producing the doubly nicked, circular form when transformed. The addition of the functional elements is directional because different 6-nt sequences are added to each end of the primers incorporated into the PCR product. This has the advantage of allowing different 5' functional elements (such as promoters) and 3' functional elements (e.g., epitopes, purification tags, polyadenylation sequences) to be added to the proper sides of the PCR product in a single reaction. After the functional elements are added, another round of PCR is used to produce enough of the linear construct for in vitro reactions or in vivo transfection. Overall, production of the construct requires one PCR step to add different 11-bp sides to the gene of interest, a TOPO reaction to simultaneously add the 5'-side and 3'-side functional elements, and another round of PCR for amplification. These steps can be accomplished in less than a single day, as compared to the several days often required for ligation of insert with vector, transformation, and the testing of colonies for the correct recombinants (Invitrogen 2002b). A major advantage of the system is its adaptability to high-throughput analysis of multiple DNAs of interest. HPLC purification of PCR primers is recommended to ensure that they are full-length.

TAP Express T7 IVT Rapid Gene Expression Kit

In this system, a promoter and a terminator are added to the respective sides of a PCR product using two PCR steps. The first step amplifies the gene of interest using a 5'-side primer with a tail complementary to the promoter and a 3'-side primer with a tail complementary to the terminator. In the second step, each strand of the product of the first PCR reaction becomes a primer for extension on the promoter or terminator. This reaction also includes a primer identical to the 5'-most end of the promoter and another that is complementary to the very 3'-end of the terminator. These primers have modified 5'-end bases, and the resulting linear PCR products are believed to show better expression after transfection than do PCR products not having the 5'-modified bases.

LIGASE-INDEPENDENT CLONING

Uracil DNA Glycosylase

In the uracil DNA glycosylase (UDG) method (Nisson et al. 1991; Varshney and van de Sande 1991; Rashtchian et al. 1992), specific primer tails are synthesized with dUMP instead of dTMP. The resulting PCR products contain dUMP residues in the primer region, which are susceptible to deglycosylation by UDG, rendering the dUMP residues abasic and unable to base-pair. This creates 3' overhangs on the PCR product that anneal with complementary 3' overhangs, which have been designed on a commercially supplied vector. The deglycosylation and the annealing with the vector occur simultaneously in a 30-min reaction, which is then transformed into E. coli, where the insert-vector junctions are repaired. Directional cloning is accomplished by using different 5'-side and 3'-side PCR primers to create different 3' overhangs on each side of the PCR product (Fig. 5). The advantages of the system are the elimination of the time-consuming tasks associated with some other systems for cloning PCR products (restriction endonuclease digestion, PCR product purification, ligation, or end-polishing). The disadvantage is the limited number of vectors available that use this cloning system.

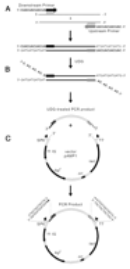

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Figure 5. UDG for creating single-stranded overhangs on the PCR product for annealing with complementary overhangs on the vector; repair and covalent joining of the vector-insert junctions occur in vivo. The UDG method relies on the incorporation of dUMP residues in place of dTMP in the 5'-end of each amplification primer. (A) Target DNA is amplified using primer tails synthesized with dUMP residues instead of dTMP; the resulting PCR products have dUMP-containing sequence at their 5' termini. (B) UDG treatment of the PCR product renders dUMP residues abasic and unable to base-pair, producing 3' overhangs. (C) The single-stranded 3' overhangs of the PCR product are annealed with complementary 3' overhangs of the commercially supplied, linearized vector (pAMP1). The creation of single-strand tails on the PCR product (via deglycosylation of dUMP residues by UDG) and the annealing of the PCR product to the vector occur in a single 30-min reaction. (Reproduced from Invitrogen 2002a, with permission from Invitrogen, part of Life Technologies © 2002.)

In-Fusion

In the In-Fusion system (Clontech), the PCR product is recombined with the linearized vector of choice in an in vitro reaction catalyzed by a proprietary enzyme (Fig. 6). Directionality of cloning is achieved by making each side of the PCR product homologous to each side of the linearized vector through addition of different 15-base tails to each primer (dA overhangs do not affect the reaction, so a proofreading or a non-proofreading polymerase can be used). The linearized vector and the PCR product are mixed with the proprietary In-Fusion enzyme, and in a single 30-min reaction, the enzyme catalyzes the alignment and strand displacement of the homologous ends of the PCR product with the vector, while a 3'-exonuclease activity removes the single-stranded region; the nicks are repaired after transformation of E. coli.

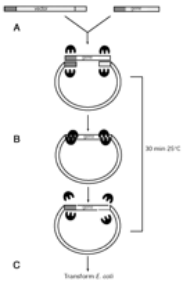

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Figure 6. Ligation-independent cloning using In-Fusion (Clontech). (A) The reaction mixture contains a vector of choice linearized by a restriction cut, a PCR product generated with primers containing 15-bp 5'-ends homologous to the ends of the linear vector (gene), and the proprietary In-Fusion enzyme. (B) The enzyme catalyzes the alignment and strand displacement of the homologous ends of the PCR product with the vector, while a 3'-exonuclease activity removes single-stranded regions. (C) The nicks are sealed after transformation of E. coli.

This system is simple, requiring only the design of 15-nt tails for each primer, and universally applicable to any vector and any restriction site within a vector that can be used for linearization. There is no need to digest the PCR product with restriction enzymes, and when a single predominant product is obtained, minimal cleanup is required. The system is useful when only a single restriction site is available for insertion because it nonetheless provides an efficient method for directional cloning. For expression cloning, no additional amino acids are added to the expressed protein, and epitope tags can be added via PCR. The procedure is especially useful for high-throughput applications because, once multiple PCR products are in hand, all subsequent steps are the same (i.e., different combinations of restriction enzymes are not required to cut different PCR products). Another advantage is that linearized vectors are available in the Clontech kits, and these vectors are adapted for the direct transfer of the insert to a variety of other types of functional plasmids via Cre-loxP-based in vitro recombination. Although 15-nt tails are required on the PCR primers, these are smaller than the 25+-nt tails required for either Xi-cloning (Genlantis) or BP Clonase (Invitrogen) (see below).

Gateway Clonase System

This in vitro site-specific recombination system adds specific sequences directionally to the 5'-ends of PCR primers. These sequences are homologous to sequences on the vector, and recombination between the PCR product and the vector is mediated by specific recombinases in vitro. The Gateway BP Clonase enzyme mix (Invitrogen) is based on the bacteriophage λ site-specific recombination system (Landy 1989) and uses the bacteriophage λ recombination protein integrase (Int) and the E. coli-encoded protein, integration host factor (IHF). The sequences added to the PCR product are 25-bp attB sequences (plus four terminal Gs); directionality is provided by different attB sequences (attB1 and attB2) added on the

5' and 3' sides of the PCR product, respectively (Fig. 7). (These attB sequences can also be added by cloning the PCR product between two attB sites in a vector by traditional restriction cleavage/ligation, or by directional TOPO cloning.) Subsequent in vitro recombination of the attB sequences flanking the PCR product with the attP sequences flanking the ccdB gene (a negative selection marker) (Bernard and Couturier 1992) on the "donor" vector causes excision of the ccdB gene and insertion of the PCR product to generate an "entry" clone. This recombinant entry clone is flanked by ~100-bp attL sequences, which result from recombination of attB with attP, and thus is not used for protein expression. Instead, the original PCR product can be transferred from this entry clone into a variety of expression vectors using another in vitro recombination reaction (mediated by the Gateway LR Clonase enzyme mix) in which the attL-flanked PCR product recombines with attR on the "destination" expression vector. This produces an expression clone in which the PCR product is again flanked by 25-bp attB sites. The Clonase system is not affected by 3'-dA overhangs, so either a proofreading or a non-proofreading polymerase can be used, and digestion with restriction enzymes and ligation are not required. Recombination can be achieved in as little as 1 h. One potential disadvantage of the system is that the peptide encoded by the attB sequence (8 amino acids) is added onto the expressed protein. However, insertion of a protease cleavage sequence (e.g., TEV protease) can permit removal of amino-terminal peptides from the expressed protein.

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Figure 7. Cloning PCR products by in vitro recombination using the Gateway system (Invitrogen). (A) The target sequence (gene) is amplified by PCR with primers containing the 25-bp attB sequences (+ 4 terminal Gs). BP Clonase catalyzes recombination between the attB sites of the PCR product and the attP sites of the donor vector. The E. coli gene ccdB in the donor vector is replaced with the PCR product to create an entry clone in which the PCR product is flanked by the attL sequences created by the recombination. (B) From the entry clone construct, the PCR product can be transferred to a variety of expression vectors by in vitro recombination with the destination vector, mediated by LR Clonase (a mix of λ phage integrase and excisionase proteins, and E. coli-encoded IHF), to produce an expression clone. (Reproduced from Invitrogen 2002a, with permission from Invitrogen, part of Life Technologies © 2002.)

RECOMBINATION IN VIVO

Xi-Clone High Speed Cloning System

In the Xi-Clone High Speed Cloning System (Genlantis), a PCR product is generated using PCR primers having nucleotide tails 28-32 nt in length that are homologous to the termini of a linearized vector. These two linear molecules are then transformed together into a proprietary recombinase-positive strain of E. coli (SmartCells), where they recombine in vivo, producing a circular plasmid. Linear vectors are available commercially; alternatively, the Genlantis kits include the materials needed to adapt any vector of choice for Xi-cloning, as well as instructions on primer design. Using this system can save considerable time because restriction digestion and ligation are not required. Also, additional amino acids are not added onto expressed proteins. The major disadvantage of the technique is the need to add 25+ nt to the PCR primers, which adds to the expense of the procedure; the extra nucleotides also can be a potential source of amplification difficulties.

SUMMARY AND PERSPECTIVE

Cloning PCR products typically is the first step to generate a reagent for an experiment, and new ligase-independent techniques have been developed to increase the efficiency of this procedure. Traditional methods involve restriction digestion of the PCR product and vector to make their termini compatible, enabling ligase-mediated production of the desired recombinant. Such methods, although effective, require multiple post-PCR enzymatic steps and are less applicable to high-throughput cloning. Also, the presence of restriction sites within the PCR product can limit the strategies that can subsequently be used. Nevertheless, traditional methods continue to be used extensively for historical reasons, because they are tried and true, and because most molecular biology laboratories have large inventories of restriction enzymes.

Newer ligase-independent methods, including TOPO cloning and in vitro recombination, are being increasingly adopted because of their efficiency. These methods are more amenable to high-throughput cloning and offer the ability to insert the PCR product into any restriction site on a plasmid that can be used for linearization; the presence of restriction sites within the PCR product is not limiting because the product is not digested. Most importantly, these methods can be more efficient than traditional methods because they require fewer enzymatic steps.

TOPO-activated vectors (Invitrogen) are provided with vaccinia topoisomerase I covalently linked via a phosphotyrosyl bond to each 3'-end, and are used to ligate PCR products directly without restriction digestion. Cloning PCR products into TOPO vectors is highly efficient, and a variety of vectors are available for cloning PCR products that have blunt termini or dT overhangs. Directional cloning is also available, and, once cloned directionally into the so-called "entry" vector, the PCR product can be transferred efficiently via in vitro recombination to a range of "destination" vectors for expression. Other methods involve adding termini (15-32 bp) to the PCR product that are used for ligase-independent recombination with the vector in vitro (Clontech or Invitrogen). The In-Fusion system (Clontech) uses a proprietary enzyme for recombining PCR products with termini homologous to the ends of any linearized vector; no additional bases need be added to the PCR product beyond the region of homology with the vector. Vectors are also available that can transfer PCR products cloned in this way into a variety of expression systems via additional in vitro recombination reactions.

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Recipe

TBE buffer

Prepare a 5X stock solution in 1 L of H_2O:

54 g of Tris base

27.5 g of boric acid

20 mL of 0.5 M EDTA (pH 8.0)

The 0.5X working solution is 45 mM Tris-borate/1 mM EDTA.

TBE is usually made and stored as a 5X or 10X stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution. Some investigators prefer to use more concentrated stock solutions of TBE (10X as opposed to 5X). However, 5X stock solution is more stable because the solutes do not precipitate during storage. Passing the 5X or 10X buffer stocks through a 0.22-µm filter can prevent or delay formation of precipitates.

Recipe

TE buffer

Reagent	Quantity (for 100 mL)	Final concentration
EDTA (0.5 M, pH 8.0)	0.2 mL	1 mM
Tris-Cl (1 M, pH 8.0)	1 mL	10 mM
H_2O	to 100 mL	

Recipe

Tris-Cl

Tris base

HCl

To prepare a 1 M solution, dissolve 121.1 g of Tris base in 800 mL of H_2O. Adjust the pH to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 mL
7.6	60 mL
8.0	42 mL

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H_2O. Dispense into aliquots and sterilize by autoclaving.

If the 1 M solution has a yellow color, discard it and obtain Tris of better quality. The pH of Tris solutions is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5°C, 25°C, and 37°C, respectively.