Ligases

Introduction

DNA Ligases are primarily responsible for joining the gaps that form in DNA during replication (i.e., the joining of ''Okazaki'' fragments formed by discontinuous or lagging strand replication; 1), DNA repair, and recombination. The best known RNA ligase is bacteriophage T4 RNA ligase. This enzyme does not appear to have any role in nucleic acid metabolism in bacteriophage T4 infected *E. coli*, but instead appears to be required for the attachment of the bacteriophage's tail fibers to its base plate during bacteriophage assembly (2). However, its activity as a ligase has been used effectively in various molecular biology applications.

Both DNA and RNA ligases catalyze the formation of a phosphodiester bond between adjacent nucleotides with the concomitant hydrolysis of ATP to AMP and inorganic pyrophosphate. Some DNA ligases (such as *E. coli* DNA ligase) use nicotinamide adenine dinucleotide (NAD) instead of ATP as a cofactor and release AMP and nicotinamide mononucleotide (NMN) as a result of phosphodiester bond formation. In general, DNA ligases will only form this covalent linkage in a duplex molecule, for example at a nick in double-stranded (dsDNA) or when joining cohesive- or blunt-ended dsDNAs (Figure 1, Panel A) (3). RNA ligase, on the other hand, has a preference for single-stranded RNA or DNA (Figure 1, Panels B and C) (4). The ligation mechanism is essentially identical for both DNA and RNA ligases, and occurs in three stages (Figure 3). First is the formation of an enzyme-nucleotide intermediate through transfer of an adenylyl group (AMP) from either ATP or NAD to the epsilon-amine group of a lysine residue in the enzyme. This results in the release of pyrophosphate when ATP is the cofactor and NMN when NAD is used. Second, the adenylyl group is transferred from the enzyme to the 5′-phosphate of the DNA (DNA ligases) or donor polynucleotide (RNA ligases), thereby activating it. Third, a phosphodiester bond is formed by nucleophilic attack of the 3′-hydroxyl group of the DNA (DNA ligases) or acceptor polynucleotide (RNA ligases) on the activated 5′-phosphate, with concomitant release of AMP.
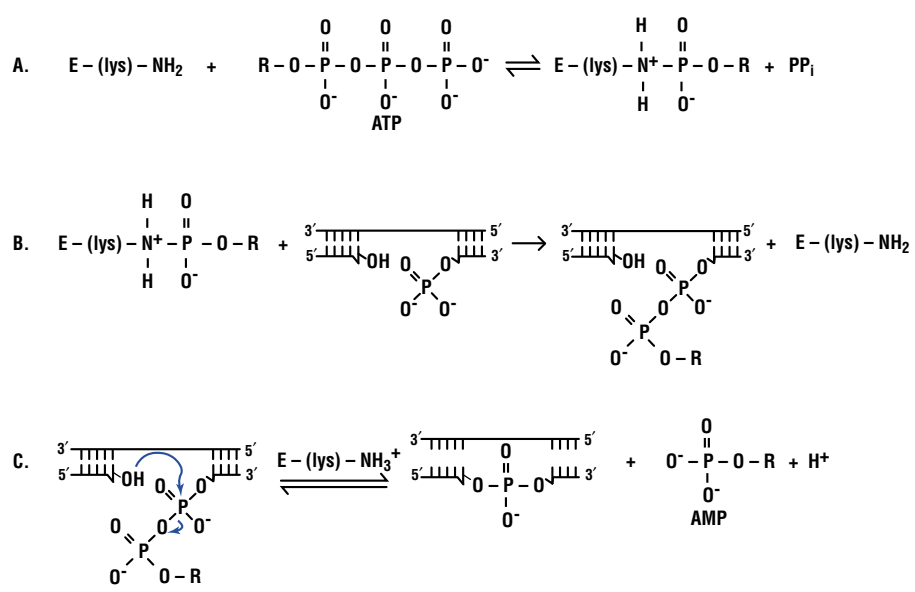
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Figure 3. Ligation mechanism. The three step reaction schematic for ATP-dependent DNA ligases is shown. Differences for NAD-dependent ligases and RNA ligases are noted in this legend. **Panel A:** Transfer of an adenylyl group from ATP (or NAD, such as in the case of *E. coli* DNA ligase) to the epsilon-amine group of a lysine residue in the enzyme with the concomitant release of pyrophosphate (or NMN when NAD is the adenylyl donor). **Panel B:** The adenylyl group is transferred from the enzyme to the 5′-phosphate of the DNA. In the case of RNA ligases, it is transferred to the donor polynucleotide. **Panel C:** Nucleophilic attack by the 3′-hydroxyl group on the activated 5′-phosphate group of the DNA (or acceptor polynucleotide in the case of RNA ligases) forms the phosphodiester bond, with simultaneous release of AMP. For Panels A-C, E = enzyme, lys = lysine residue.

Bacteriophage T4 DNA ligase is the ligase most commonly used in the construction of recombinant DNA molecules for molecular biology applications. It is able to ligate DNA fragments having either complementary cohesive or blunt ends, and has an absolute requirement for ATP as a cofactor; it cannot use NAD. *E. coli* DNA ligase (which, like most prokaryotic DNA ligases uses NAD as a cofactor instead of ATP) can sometimes be used in place of T4 DNA ligase for ligation of single-strand breaks or joining of DNA molecules with cohesive termini. However, unlike T4 DNA ligase, the *E. coli* enzyme does not show blunt-ended ligation activity, except under conditions of molecular crowding with PEG 8000 (5). For this reason T4 DNA ligase has become more widely used in DNA manipulations (Table 1). For intermolecular ligations it is important that at least one of the DNA molecules possesses a 5′-phosphate at either end of the dsDNA in order to form a phosphodiester bond. T4 DNA ligase is also used to join adjacent single-stranded DNA (ssDNA) molecules that have been polymerized on a template from primers annealed at separate sites, such as during site-directed mutagenesis.

The various applications that use bacteriophage T4 RNA ligase are indicated in Table 1. This enzyme has been used for many years to label RNA molecules at their 3′-end with a radiolabeled nucleoside 3′,5′-bisphosphate as a complementary approach to labeling the 5′-end using T4 polynucleotide kinase (6). More recently, this enzyme has proved useful in the cloning of full-length cDNAs by circular and 5′-rapid amplification of cDNA ends (RACE) (7,8). The former method involves circularization of first-strand cDNA followed by inverse PCR, whereas the latter involves ligation of an oligonucleotide linker onto the 3′-end of the first-strand cDNA synthesis product, followed by amplification with a primer complementary to this linker and a gene-specific primer.

T4 DNA Ligase

E.C 6.5.1.1

Description

In vivo, T4 DNA Ligase (Cat.# M1801, M1794) catalyzes the sealing of single-stranded nicks in double-stranded DNA molecules (1,2). It is commonly used for the joining of two strands of DNA between the 5′-phosphate and the 3′-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration (3; Figure 1, Panel A). The enzyme has also been shown to catalyze the joining of RNA to either a DNA or RNA strand in a duplex molecule, but will not join single-stranded nucleic acids (3).

Applications

- Joining blunt-ended double-stranded DNA.
- Joining cohesive-ended double-stranded DNA.
- Sealing nicks on a DNA or RNA strand annealed to a DNA or RNA (4) complementary strand.

T4 DNA Ligase is a component of the following Promega systems:

- Erase-a-Base® System Plus Vectors (Cat.# E5850)
- Erase-a-Base® System (Cat.# E5750)
- GeneEditor™ *in vitro* Site-Directed Mutagenesis System(g) (Cat.# Q9280)
- Altered Sites® II *in vitro* Mutagenesis Systems(h) (Cat.# Q6210, Q6491, Q6090, Q6501, Q6080, Q6511)
- Altered Sites® II Mammalian *in vitro* Mutagenesis Systems(h) (Cat.# Q5590, Q6600)
- pGEM®-T Vector Systems(d,f) (Cat.# A3600, A3610)
- pGEM®-T Easy Vector Systems(d,f) (Cat.# A1360, A1380)
- pTARGET™ Mammalian Expression Vector System(f,h) (Cat.# A1410)
- PinPoint™ Xa-1 T-Vector Systems(f,i) (Cat.# V2610, V2850)
- Universal RiboClone® cDNA Synthesis System(j) (Cat.# C4360)

Enzyme Properties

Requirements: Mg^{2+}, ATP, DTT (or other reducing agent such as β-mercaptoethanol) (1,2).

Cofactor Concentration: 10mM Mg^{2+}, 10μM-1mM ATP (1), 10mM DTT (1).

Optimal Substrate: *In vivo*, single-stranded nicks in double-stranded DNA molecules (2). *In vitro*, two double-stranded DNAs containing either blunt ("flush") or cohesive ("sticky") ends (1,2,5). In all cases one DNA strand must have a 3′-OH and the other must have a 5′-phosphate.

Typical Working Concentration: 0.1–1.0 units T4 DNA Ligase per 10μl reaction for cohesive-end ligations or nick sealing reactions. Up to ten-fold more ligase may be required for blunt-ended ligations (1).

Optimal pH: The optimum pH range of T4 DNA Ligase has been reported to be 7.0–7.8 (2) or 7.2–7.6 (1).

K_m: 5×10^{-5}–5×10^{-8}M for blunt-ended DNA (1,3,6), 6×10^{-7}M for cohesive ends; 1.5×10^{-9}M for nicks (2); 1.4×10^{-8}M for ATP (1).

Stimulators: Polyethylene Glycol (PEG) 6000 or 8000 at 5% (w/v) (or other macromolecules such as Ficoll®, albumin, or glycogen) can stimulate ligation of blunt-ended DNAs over 1,000-fold, as judged by shifts in electrophoretic mobility. Cohesive-ended DNA ligation is stimulated by PEG to a far lesser extent. T4 RNA Ligase greatly stimulates blunt-end ligations (6).

Monovalent cations at low concentration (~20mM) can give slight stimulation (~30%), but at high concentrations they will inhibit ligation (1,7). Polyamines such as spermidine have been reported to both stimulate (1) and inhibit (7) ligation reactions at low (<1mM) concentrations. At higher concentrations polyamines inhibit ligation. HMG 14 has been shown to stimulate blunt-ended ligation by as much as 50% when present at a 50:1 molar ratio of HMG 14:DNA (1).

Alternative Cofactors and Substrates: Double-stranded DNA with blunt ends, single-stranded nicks in DNA/RNA hybrids or RNA/RNA hybrids. In all cases, one DNA or RNA strand must have a 3′-OH and the other must have a 5′-phosphate. Mn^{2+} can substitute for Mg^{2+} with reduced efficiency (1).

Inhibitors: dATP is a competitive inhibitor of ATP in ligation reactions (2). Monovalent cations inhibit ligations with almost no activity seen at greater than 200mM (1,7).

There are conflicting reports concerning polyamines such as spermidine, which are reported to both stimulate (1) and inhibit (7) ligation reactions at low (<1mM) concentrations. At higher concentrations they inhibit ligation (1,7). Ethidium bromide inhibits ligations with an ID_{50} of approximately 4.3µM (1). Anions are reported to inhibit blunt-end ligations at concentrations greater than 25mM for phosphates and greater than 50mM for salts in general (1). Hexaminecobalt chloride and Cibacron blue F3GA act as inhibitors (1). Excess ATP has been reported to inhibit blunt-ended ligations at high concentration (5mM) (5).

K_i: 3.5×10^{-5}M for dATP (2).

Temperature Stability: T4 DNA Ligase is inactivated at temperatures above 37°C.

Inactivation: Incubate at 70°C for 10 minutes or add EDTA to 25mM concentration.

Genetic Locus: Bacteriophage T4 gene 30 (1).

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone.

Molecular Weight: 68kDa.

Typical Working Conditions: A typical ligation of cohesive-ended DNA contains 10–200ng of vector DNA and sufficient insert DNA to make a 1:1, 1:3 or 3:1 insert:vector molar ratio. The DNA is incubated in buffer containing 30mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 10mM DTT and 1mM ATP in a final volume of 10µl. 0.1–1 unit of T4 DNA Ligase is added. For cohesive-end ligations, incubate at 20–25°C for approximately 3 hours or at 4–8°C overnight. For blunt-end ligations incubate at 15–20°C overnight.

Storage Conditions: Store at –20°C. T4 DNA Ligase is supplied in storage buffer containing 10mM Tris-HCl (pH 7.4), 50mM KCl, 1mM DTT, 0.1mM EDTA and 50% glycerol.

Unit Definition: 0.01 Weiss unit of T4 DNA Ligase is the amount of enzyme required to catalyze the ligation of greater than 95% of the *Hin*d III fragments of 1µg of Lambda DNA at 16°C in 20 minutes. (One Weiss unit is the amount of enzyme that catalyzes the conversion of 1nmol of ^{32}PPi into a charcoal-absorbable form in 20 minutes at 37°C in an ATP-PPi exchange form.)

Purity: The purity is ≥90% as judged by SDS-polyacrylamide gels with Coomassie® blue staining.

Activity Assays

Lambda Packaging Efficiency: A control insert is ligated to 1µg of EMBL3 Vector Arms with 2.5 units of T4 DNA Ligase. The ligated DNA is packaged using Packagene® Extract (Cat.# K3151). The packaged phage is diluted 1:10,000 and used to infect bacterial strain LE392. After an overnight incubation at 37°C, the phage titer and the packaging efficiency are measured. The minimum packaging efficiency must be 5×10^6pfu/µg DNA.

Contaminant Assays

Endonuclease Assay: 1µg of pGEM®-5Zf(+) Vector is incubated with 5 units of T4 DNA Ligase in T4 DNA Ligase 1X Buffer (Tables 12,15) for 16 hours at 37°C. Following incubation the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

Single-Stranded and Double-Stranded DNase Assay: To test for DNase activity, 50ng of radiolabeled single-stranded or double-stranded DNA is incubated with 20 units of T4 DNA Ligase in 1X Buffer (Tables 12,15) for 16 hours at 37°C. Minimum passing specification is <2% release of single-stranded and <1% release of double-stranded radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

RNase Assay: To test for RNase activity, 50ng of radiolabeled RNA is incubated with 20 units of T4 DNA Ligase in 1X Buffer (Tables 12,15) for 5 hours at 37°C. Minimum passing specification is <3% release of radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

Blue/White Cloning Assay: This assay is performed to demonstrate that T4 DNA Ligase is free from contaminating activities, which can affect the efficiency and integrity of plasmid cloning. Any exonuclease or polymerase activity that alters the termini of linearized plasmids during ligation will result in a proportion of white-colored colonies above background levels.

A pGEM® series Vector is linearized with three different restriction enzymes in separate reactions to generate three different types of termini: *Eco*R I for 5′-overhangs, *Kpn* I for 3′-overhangs, and *Hin*c II for blunt ends. Linearized plasmids are purified, and ligations are performed using 12 units of T4 DNA Ligase in overnight incubations at 4°C. Competent JM109 cells are transformed with ligated plasmids and plated on X-Gal/IPTG/Amp plates. A minimum of 750 colonies is counted. White colonies result from transformation with ligated plasmids that have damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs must produce fewer than 2% white colonies and blunt-cutting enzymes must produce less than 5% white colonies. The minimum transformation efficiency must be 1×10^5cfu/µg DNA. Figure 4 includes a diagram of the blue/white color selection protocol.

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T4 RNA Ligase
E.C. 6.5.1.3

Description

T4 RNA Ligase (Cat.# M1051) catalyzes the ATP-dependent formation of either an intra- or intermolecular 3′,5′-phosphodiester bond between a donor poly- or oligonucleotide containing a 5′-phosphate group and an acceptor poly- or oligonucleotide containing a 3′-hydroxyl group (1,2). The minimal length of a polyribonucleotide for circularization is 8 bases (3). For an intermolecular reaction the minimum size for an acceptor is a trinucleotide, whereas the donor can be as small as a nucleoside 3′,5′-bisphosphate (4–6). This enzyme is used to circularize RNA molecules or join them to other RNAs. DNAs can also be used in intra- and intermolecular reactions, although with less efficiency than RNA (7–9).

T4 RNA Ligase is used to specifically label the 3′-end of RNA molecules with 5′-^{32}P-radiolabeled nucleoside 3′,5′-bisphosphate (e.g., [5′-^{32}P]pCp) (10,11). The resulting 3′-end labeled RNAs can be used for enzymatic or chemical sequencing studies or for studies of ribonuclease activity and RNA/protein interactions (12,13). T4 RNA Ligase can also be used in 5′-RACE (Rapid Amplification of cDNA Ends) to ligate a specific oligodeoxyribonucleotide to the 3′-end of the first strand of cDNA synthesis. The oligodeoxyribonucleotide serves as a primer binding site for the upstream primer in 5′-RACE (14). Alternatively, the cDNA may be circularized in an intramolecular reaction for use in circular RACE (cRACE) (15).

T4 RNA Ligase has also been used for the site-specific incorporation of unnatural amino acids into protein (16,17). This involves using T4 RNA Ligase to ligate a CA dinucleotide modified with an unnatural amino acid onto the 3′-terminus of a 3′-CA deficient amber suppressor tRNA. Using this method, it is possible to introduce a variety of labels at specific sites in a protein (18,19).

Another useful application of T4 RNA Ligase involves the blunt-end ligation of double-stranded DNA. Although T4 RNA Ligase cannot by itself catalyze this reaction, it can stimulate the activity of T4 DNA Ligase in joining blunt ends by as much as 20-fold (20).

Applications

- Labeling the 3′-end of RNA with cytidine 3′,5′-bisphosphate (10,11).
- Cloning of full-length cDNAs/5′-RACE (14,15).
- Intra- and intermolecular ligation of single-stranded DNA, RNA and oligonucleotides (2,7–9,21).
- Incorporation of unnatural amino acids and labels into proteins (16–19).
- Stimulation of blunt-ended ligation efficiency (20).

Enzyme Properties

Requirements: Both Mg^{2+} and ATP are required for enzyme activity.

Cofactor Concentration: A concentration of 5–10mM Mg^{2+} is optimal for activity. Concentrations above 10mM are inhibitory (9,22). Although a final ATP concentration of 1mM is used in the unit activity assay (circularization of 5′-[^{32}P]-rA$_{14–20}$) of T4 RNA Ligase, 100µM ATP is sufficient for ligase reactions (1,9,22).

Optimal Substrate: For intramolecular circularization reactions, the optimal substrate is a polynucleotide with a 5′-phosphate and a 3′-hydroxyl group. For intermolecular reactions, the donor should possess a 5′-phosphate and the acceptor a 3′-hydroxyl. To limit the reaction to a single ligation product, the acceptor should possess a hydroxyl group at both the 5′- and 3′-termini, whereas the donor should possess a 5′- and 3′-phosphate. For a single nucleotide donor, the reaction can be driven towards high yields of product by incubating with a molar excess of donor over acceptor (7,10). In contrast, the use of oligonucleotide donors requires an excess of acceptor to donor. A molar ratio of 5:1 is usually optimal (7,8).

Typical Working Concentration: 100 units per milliliter of reaction (10).

Optimal pH: pH 7.5–8.2 (Tris-HCl, 25°C) (1).

K$_m$: For ATP, 12µM (22).

Isoelectric Point: pI = 6.1.

Stimulators: PEG 8000 increases the ligation efficiency of single-stranded DNA 30-fold at a concentration of 25%, whereas the addition of dimethyl sulfoxide (DMSO) to a final concentration of 10–20% increases the yield of RNA ligations 2- to 3-fold (8,11).

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Alternative Cofactors and Substrates: T4 RNA Ligase can utilize a wide variety of modified nucleoside 3′,5′-bisphosphates as donors in ligation reactions. Examples include 5-bromodeoxyuridine, 2′-O-methylcytidine, and 1-methylguanosine (2).

Inhibitors: The nucleoside 2′,5′-bisphosphate, 2′,5′-ADP inhibits T4 RNA Ligase activity in the presence of magnesium (23).

Inactivation: 65°C for 15 minutes or 100°C for 2 minutes (1,9,22).

Genetic Locus: Bacteriophage T4 gene 63 (24).

Promega Product Information

Source: Purified from an *E. coli* strain expressing a recombinant clone.

Molecular Weight: T4 RNA Ligase is a monomeric enzyme with a molecular weight of 43.5kDa (24).

Typical Working Conditions: For ligation of single-stranded nucleic acids to each other use the T4 RNA Ligase 10X Buffer supplied with the enzyme, diluted 1:10 (50mM Tris (pH 7.8), 10mM $MgCl_2$, 5mM DTT and 1mM ATP) and BSA at a final concentration of 10µg/ml. Incubate at 17–25°C for 10–16 hours (8,14,15,21). Labeling the 3′-end of RNA molecules with cytidine 3′,5′-[5′-^{32}P] bisphosphate can be carried out in the same buffer, but reactions are incubated at 5°C for 6–24 hours and DMSO is added to 10% final concentration (10,11).

Storage Conditions: Store at –20°C. T4 RNA Ligase is supplied in storage buffer containing 10mM Tris (pH 7.5), 50mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol and 0.1% Tween® 20.

Unit Definition: One unit is defined as the amount of enzyme required to catalyze the formation of 1 nanomole of 5′-[^{32}P]-rA$_{14-20}$ into a phosphatase-resistant form in 30 minutes at 37°C at a 5′-terminal concentration of 10µM. The reaction conditions are specified below under Functional Assay.

Purity: T4 RNA Ligase is determined to be >90% homogeneous, as judged by SDS-polyacrylamide gels with Coomassie® blue staining.

Activity Assay

Functional Assay: The RNA substrate (5′-[^{32}P]-rA$_{14-20}$, 10µM of 5′-termini) is ligated in the presence of T4 RNA Ligase 1X Buffer (Table 15) and T4 RNA Ligase for 15 minutes at 37°C. After ligation, the reaction is terminated by heating at 100°C for 2 minutes. The ligated substrate is then treated with 10 units of Calf Intestinal Alkaline Phosphatase (Cat.# M1821) for 10 minutes at 37°C. The amount of phosphatase-resistant substrate is monitored by scintillation counting of the TCA-precipitable material.

Contaminant Assays

DNase Assay: To test for the absence of DNase activity, 50ng of radiolabeled DNA is incubated with 20 units of T4 RNA Ligase in T4 RNA Ligase 1X Buffer (Tables 12, 15) for 3 hours at 37°C. The minimum passing specification is <1% release of radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

RNase Assay: To test for the absence of RNase activity, 50ng of radiolabeled RNA is incubated with 20 units of T4 RNA Ligase in T4 RNA Ligase 1X Buffer (Tables 12, 15) for 3 hours at 37°C. The minimum passing specification is <1% release of radiolabeled nucleotides as monitored by scintillation counting of TCA-soluble material.

Endonuclease Assay: To test for endonuclease activity, 1µg of lambda or pGEM®(d) DNA is incubated with 20 units of T4 RNA Ligase in T4 RNA Ligase 1X Buffer (Tables 12, 15) for 3 hours at 37°C. Following incubation, the DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

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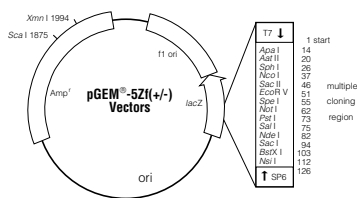
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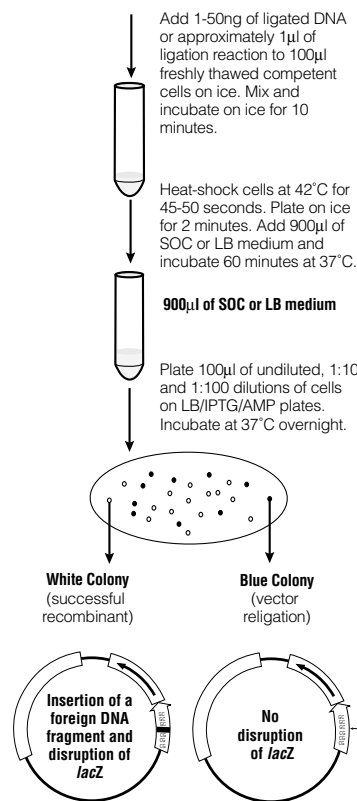Precision Design… *for Life*

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Blue/White Color Selection

Figure 4. Blue/white color selection protocol.

1. Pre-chill sterile 17 x 100mm polypropylene culture tubes (e.g., Falcon 2059) on ice, one per transformation.

2. Remove frozen competent cells from –70°C and place on ice for 5 minutes or until just thawed. Competent cells will quickly lose their competency if warmed above 4°C.

3. Gently mix the cells by flicking the tube and transfer 100µl of the thawed competent cells to each of the pre-chilled culture tubes.

4. Add 1–50ng of ligated DNA (in a volume not greater than 10µl) to competent cells. Transformation efficiency will decrease with greater amounts of DNA. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times. Do not pipet or vortex to mix.

5. Immediately return the tubes to ice for 10 minutes.

6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. DO NOT SHAKE.

7. Immediately place the tubes on ice for 2 minutes.

8. Add 900µl of cold (4°C) SOC or LB medium to each transformation reaction then incubate for 60 minutes at 37°C with shaking (approximately 225rpm).

9. For each transformation reaction, we recommend diluting the cells 1:10 and 1:100 in medium, and plating 100µl of the undiluted, 1:10 and 1:100 dilutions on antibiotic plates. The 1:100 dilution may be omitted for transformations from ligation reactions. Plate the transformed cells on LB plates containing100µg/ml ampicillin, 0.1mM IPTG and 40µg/ml X-Gal.

   Most plasmids used for routine subcloning contain the gene for β-lactamase (Amp^r) and should be selected for using liquid and solid medium containing 100µg/ml ampicillin. Other common selectable markers include tetracycline (use at 12.5µg/ml on plates, 10µg/ml in liquid culture), chloramphenicol (use at 20µg/ml) and kanamycin (use at 30µg/ml). Antibiotics are heat sensitive; do not add to culture medium above 55°C.

10. Incubate the plates at 37°C for 12–14 hours (overnight).

11. Colonies that contain recombinant plasmid (disruption of *lacZ* α-peptide) will appear white while colonies that contain nonrecombinant plasmid (intact *lacZ* α-peptide) will appear blue.

Further information on bacterial transformation and blue/white color selection can be found in Promega's *Protocols and Applications Guide,* Third Edition (pp. 46, 51, 383), and in Promega Technical Bulletin #TB095.