RecA Protein and Agar*ACE*® Enzyme

Introduction

RecA Protein and Agar*ACE*® Enzyme are included in this Cloning Enzyme Guide because of their applications for nucleic acid purification or protection in cloning procedures. The RecA protein has the amazing capacity to facilitate the pairing of DNA molecules (for recent reviews see references 1 and 2). Agarases, such as Agar*ACE*® Enzyme, have become a popular tool to liberate DNA from the commonly used electrophoretic separation matrix, agarose.

RecA Protein

RecA forms a helical filament on single-stranded DNA, with a stoichiometry of one RecA monomer per 3 bases of ssDNA. RecA protein binds ATP in addition to DNA, and ATP binding is required for nucleoprotein filament assembly, homology searching, and DNA strand exchange (Figure 1, Panel I). ATP hydrolysis is not required for any of these processes, but it is required for disassembly of the nucleoprotein filament (i.e., disassociation of the RecA protein) upon completion of DNA strand exchange, bypass of structural barriers in DNA during replication, and 4-strand exchange reactions. RecA is also involved in the SOS response of bacteria. As cellular DNA damage levels increase to the point where replication is impeded, RecA protein can assemble a nucleoprotein filament on ssDNA that accumulates as a result of interrupted replication. The nucleoprotein filament facilitates autocatalytic cleavage of the LexA repressor and other proteins, such as the bacteriophage λ cI repressor. This co-protease activity of RecA results in the derepression of over 20 genes regulated by LexA and the induction of bacteriophage λ lysogens (1,2). One of the more useful *in vitro* properties of RecA is its ability to locate and pair a ssDNA sequence to its homologous dsDNA sequence in the presence of ATP[γS] (Figure 1, Panel I). This behavior has been exploited to enrich for specific DNA sequences (3–5), and to protect specific DNA sites from methylation or endonuclease cleavage (RecA-assisted restriction endonuclease, RARE [6], Achilles' heel cleavage [7,8; Figure 9]).

Agar*ACE*® Enzyme

Agarose gels are widely used in the electrophoretic separation of polynucleotides. A highly heterogeneous polysaccharide, agarose is an alternating copolymer of 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose. Agarase enzymes are secreted by a wide variety of organisms as a means of degrading plants containing agaroses, and are classified as α or β agarases, depending on whether they cleave α or β intersaccharide linkages. The majority of reported agarases are of the β type, and most degrade agarose into mixtures of short polysaccharides (i.e., small sugars).

Although a large number of organisms secrete enzyme mixtures that completely hydrolyze unmelted agarose, all reported individual enzymes (and all tested mixtures of individual agarases) require melting of the agarose prior to enzyme treatment in order to obtain complete hydrolysis. As a hot agarose solution cools, it reaches a loose gel state in which agarose chains form double helices joined in loosely associated bundles or fibrils, called the gel I state. As the gel cools further, the bundles further associate with each other to a point where the bundles of agarose chains form an extended network and a hardened agarose gel is formed, called the gel II state, used for running electrophoresis gels (9). Agarases will attack agarose in either the gel I or gel II state, but will not attain complete hydrolysis of the most tightly bundled helices, presumably due to steric considerations.

For this reason, commercially available agarases require that the agarose be melted completely prior to enzyme treatment. For low melting agaroses, this means treatment at 70°C; for regular agarose, it requires 95–100°C. Since the enzymes are typically inactivated at these high temperatures, the agarose must be rapidly equilibrated to lower temperatures prior to enzyme treatment, and care must be taken to ensure that the gel I state agarose does not begin to form. Agar*ACE*® Enzyme, isolated from a *Flavobacterium sp.* from the Dry Tortugas (10), is somewhat more thermostable than other known agarases, thus many of the typical difficulties of this equilibration step are avoided (11). Agar*ACE*® Enzyme also exhibits unusual activity in high concentrations of KI and NaI, which allows protocols to be developed for easy recovery of polynucleotides from regular agarose gels (12). Agar*ACE*® Enzyme also has a broad pH optimum, which allows its use in all common electrophoresis buffers without adjustment of unit addition or presoaking of gels to attain optimum activity. It also exhibits fast reaction rates, so Promega's standard unit definition is based on 15-minute hydrolysis times, as opposed to the industry standard 1 hour.

Since the polynucleotides are typically recovered in the electrophoresis buffer, some downstream applications can be negatively affected by either the high pH or the buffering of the solution. In rare cases, some inhibition has been seen due to residual oligosaccharide, and even more rarely, from the enzyme itself. In the first 2 cases (pH and buffering), soaking the gel prior to hydrolysis generally eliminates the problem. In the first three cases, ethanol precipitation after hydrolysis generally alleviates any problems, and high recoveries are typically obtained due to the fact that Agar*ACE*® Enzyme also precipitates and acts as a carrier.

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RecA Protein

Description

The *E. coli* RecA protein plays a central role in homologous recombination, post-replication repair and the SOS response to DNA damage (for reviews see references 1–6). The purified RecA Protein (Cat.# M1691) binds cooperatively and stoichiometrically to single-stranded DNA (ssDNA) and is a prototype for DNA strand-exchange proteins. The active species in strand exchange is a nucleoprotein filament containing one RecA Protein monomer (38kDa) per 3 bases of ssDNA. ATP hydrolysis is not required for filament formation or paranemic pairing to duplex DNA, but ATP hydrolysis is required for unidirectional DNA strand exchange, dissociation of the protein, bypass of structural barriers in DNA during replication and 4-strand exchange reactions.

One of the more useful *in vitro* properties of RecA is its ability to locate and pair a ssDNA sequence to its homologous dsDNA sequence in the presence of ATP[γS]. This behavior has been exploited for enrichment of specific DNA sequences (7–9), and to protect specific DNA sites from methylation or endonuclease cleavage (RecA-assisted restriction endonuclease, RARE [10], Achilles' heel cleavage [11,12; Figure 9]).

Applications

- Enrichment method for genomic cloning (7–9).
- Sequence-specific cleavage of large fragments of DNA (RecA-assisted restriction endonuclease [RARE10]; Achilles' heel cleavage [11,12]).
- D-loop mutagenesis (13).
- Coating of DNA with RecA Protein to enhance contrast of electron micrographs (14,15).

Enzyme Properties

Requirements: ATP, $MgCl_2$.

Cofactor Concentration: 10mM $MgCl_2$ in the presence of ATP (6).

Optimal Substrate: ssDNA.

Typical Working Concentration: Varies with the application, but at enzyme concentrations above 0.2µM, ATP hydrolysis becomes linear (6).

Optimal pH: Above pH 7, binding to ssDNA is much faster than binding to dsDNA. Binding to dsDNA, pH 6.0 (25°C) (16); DNA-independent ATP hydrolysis, pH 6.0 (25°C) (17), gives a k_{cat} of 0.1 min^{-1} and the k_{cat} drops to 0.015 min^{-1} at pH 7.5; ssDNA-dependent ATP hydrolysis, no dependence on pH between 5.5 and 9.0 (17).

K_m: ATP range from 20µM for ssDNA to 100µM for dsDNA based on initial velocity measurement (18).

k_{cat}: When RecA is bound to ssDNA, it hydrolyzes ATP with a k_{cat} of 28–30 min^{-1} at 37°C (17). When RecA is bound to dsDNA, it hydrolyzes ATP with a k_{cat} of 20–22 min^{-1} at 37°C (16).

RecA Filament Assembly: On ssDNA circles over 8,000 nucleotides long, filament formation is completed in less than 2 minutes at 37°C. Assuming a single nucleation event per circle gives a rate of filament extension at 1,100 RecA monomers per minute (19). Extension is polar and proceeds from 5′→3′; therefore, there is a much greater likelihood that the 3′-ends of linear ssDNA will be coated with RecA monomers than the 5′-ends (20).

RecA Filament Disassembly: RecA also displays a polar filament disassembly behavior on linear ssDNA, such that monomers come off at the end opposite to that where filament extension occurs; therefore, both processes proceed 5′→3′ (21). Using dATP instead of ATP can prevent filament disassembly.

Alternative Cofactors and Substrates: dATP, UTP and PTP (purine ribonucleoside triphosphate) serve as cofactors in RecA-mediated reactions (17,22,23). ITP, CTP, dCTP and GTP are hydrolyzed with a nearly normal V_{max} but do not support DNA strand exchange. Reactions with dATP increase hydrolytic rates 20% over ATP (22,24,25).

Inhibitors: ADP, ATP[γS], <25mM NaCl to prevent destabilization of RecA triplexes (26).

K_i: For ATP[γS], 0.6µM (27).

Temperature Stability: RecA Protein is stable at temperatures of 25–45°C as demonstrated by a linear Arrhenius plot (28).

Inactivation: 65°C for 15 minutes (29).

Genetic Locus: *E. coli rec*A.

Promega Product Information

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

Molecular Weight: 37,842 daltons; 352 amino acids.

Typical Working Conditions: RecA Protein reaction conditions vary with the application (see references 7–15), but in general there should be one RecA Protein for every three nucleotides in the ssDNA used for strand invasion protocols (e.g., 160ng oligonucleotide per 6.25µg RecA Protein [26]).

Storage Conditions: Store at –20°C. RecA Protein is supplied in storage buffer containing 20mM Tris-HCl (pH 7.5 at 25°C), 0.1mM EDTA, 1mM DTT and 50% glycerol.

Concentration: RecA concentration is determined by A_{280} using an extinction coefficient of 0.516 at 1mg/ml (30).

Purity: The purity is ≥95% as judged by SDS-polyacrylamide gel electrophoresis with Coomassie® blue staining.

Activity Assays

ATPase Activity: RecA Protein is a ssDNA-dependent ATPase. The rate of ATPase activity is measured as turnover number or apparent k_{cat}. One monomer of RecA protein binds per 3 nucleotides of ssDNA to form an active RecA:ssDNA complex. ATPase activity is determined by monitoring conversion of NADH to NAD in a coupled reaction. The rate of ATPase activity is calculated from the change in absorbance (31). An apparent k_{cat} per minute is calculated from the moles of ATP to ADP per mole of activated RecA:ssDNA.

DNA Strand Exchange: Strand exchange is the ability of RecA Protein to exchange closed circular ssDNA with one strand of linearized dsDNA. The resulting products are open circular dsDNA and linear ssDNA. After 60 minutes of incubation with 15µg of RecA at 37°C ≥50% of the linear dsDNA is converted to open circular DNA (28).

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 1µg of Type 1 supercoiled plasmid DNA, lambda DNA and pGEM® marker DNA[(c)] is incubated with 15µg of RecA Protein in restriction enzyme buffer A 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.

Exonuclease Assay: To test for DNase activity, 50ng of radiolabeled DNA is incubated with 5µg of RecA Protein in restriction enzyme buffer A for four hours at 37°C, and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release of substrate.

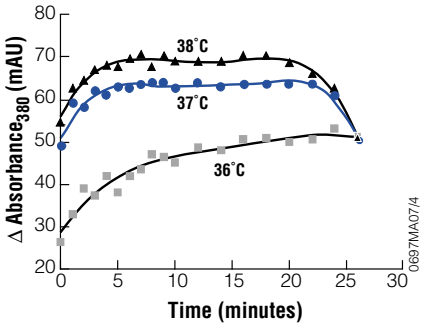

Figure 8. ATPase activity as a function of temperature. ssDNA-dependent ATPase activity was determined spectrophotometrically by monitoring conversion of NADH to NAD in a coupled reaction (31). RecA Protein was slightly in excess of one monomer per 3 bases of M13mp18 ssDNA. Moles of ATP converted to ADP were calculated from the absorbance change of NADH, and the k_{cat} was calculated by dividing the rate of ATP conversion by the amount of activated RecA:ssDNA complex. Since RecA is in excess of ssDNA, the amount of activated RecA:ssDNA complex is equal to the known amount of ssDNA sites in the reaction (31). As seen in the above figure, there is a strong temperature-dependence of the ATPase activity. The measured k_{cat} for 36°C, 37°C, and 38°C was 24, 31, and 34 min^{-1}, respectively.

Table 2. Materials Needed for RecA Protein Protection and Cleavage Protocols.

- Oligonucleotide: Prepared by user to be specific for the intended protected site. Diluted to 160ng/ml.
- RecA: 1-3mg/ml.
- SAM (1.6mM S-adenosyl methionine): Prepared immediately before use from a 32mM stock by dilution with ice-cold 5mM sulfuric acid.
- ATP[γS]: Aliquots of a 10mM solution (in water) are stored at –70°C.
- 80mM magnesium acetate.
- Restriction Enzyme Buffer H (Promega).
- Methylase: In theory, any restriction enzyme/methylase pair could be used. In these protocols, 35u/µl of *Eco*R I methylase was used (Figure 9).
- Restriction Enzyme: In these protocols, 12u/µl *Eco*R I was used (Figure 9).
- Buffer A: 250mM Tris-acetate (pH 7.5 at 25°C), 1mM magnesium acetate.
- Buffer B: 166mM Tris-acetate (pH 7.5 at 25°C), 37mM magnesium acetate, 100mM DTT.
- 250mM potassium acetate.

Table 3. Troubleshooting RecA Protein Cleavage and Protection Protocols.

Symptoms	Possible Causes	Comments
RecA cleavage or protection: extra bands on a gel that look like restriction digest partials.	Nonspecific protection. RecA in excess of oligonucleotide. RecA favors binding to single-stranded DNA; however, it does have a low affinity for double-stranded DNA.	Try a number of different concentrations of oligonucleotide, holding all other reaction components constant. Add 160ng of oligo(dT) immediately after the addition of ATP[γS], which has been described to improve the process (11).
RecA cleavage: uncut DNA. RecA protection: unexpected products that are comigrating with the products of an unprotected restriction digest.	Incomplete protection of the targeted site.	Increase the concentration of RecA and oligonucleotide. Change the reaction buffer to decrease nonspecific cleavage by raising the pH from 7.5 to 7.85 (32). Adjust the oligonucleotide size and/or sequence to target the oligonucleotide more specifically.
Low transformation efficiencies of RecA cleavage products.	Methylation is incompatible with restriction systems of the host.	Check host genotype for incompatible methylation-induced restriction system. Change host if necessary.

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RecA Protein Cleavage and Protection Protocols

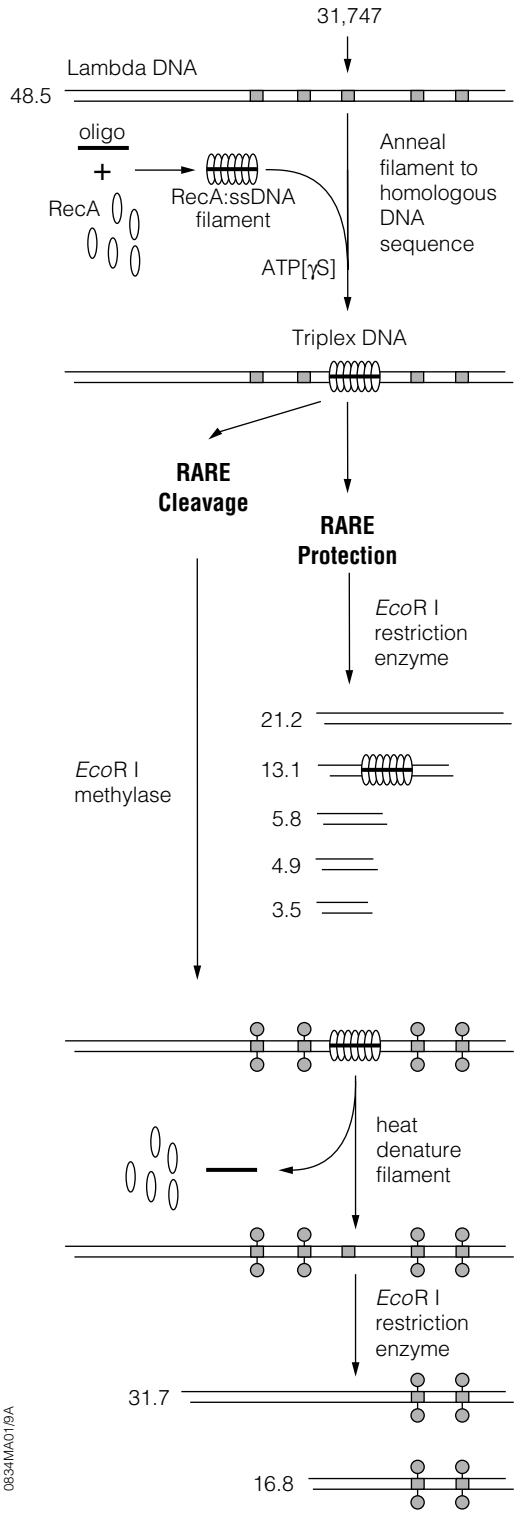

Figure 9. RecA cleavage and protection protocols.

Formation of Triple Helix

1. Add:
   - 1µl 160ng/µl oligonucleotide
   - 6.25µg RecA
   - 1µl buffer A
   - Bring volume to 9µl with water.
2. After 30 seconds, add 1µl of 10mM ATP [γS].
3. Incubate for 10 minutes at 37˚C.
4. Add:
   - 1µg substrate DNA
   - 2.7µl buffer B
   - 2.7µl 1mg/ml BSA
   - Bring volume to 27µl with water.
5. Incubate for 30 minutes at 37˚C.

RecA Protection

Restriction

1. Add:
   - 10µl water
   - 5µl 80mM magnesium acetate
   - 5µl 250mM potassium acetate
   - 20–30u *Eco* RI
2. Incubate at 37˚C for 30 minutes.

RecA Cleavage

Methylation

1. Add:
   - 1.5µl 80mM magnesium acetate
   - 1.5µl 1.6mM SAM
   - 100u *Eco* R I Methylase
2. Incubate for 30 minutes at 37˚C.

Heat Denaturation

1. Heat sample to 65˚C for 15 minutes.
2. Centrifuge the sample at 14,000 x *g* for 5 minutes. Transfer the supernatant to a new tube.

Restriction

1. Add:
   - 10µl water
   - 5µl RE 10X buffer H
   - 20–40u *Eco* RI
2. Incubate for 30 minutes at 37˚C.

RecA Protein Cleavage and Protection Reaction Tips

- **RecA concentration.** 6.25mg RecA in a 10ml reaction works well. To maximize the specificity and efficiency of RecA protections, it may be necessary to manipulate the oligonucleotide:RecA ratio; we recommend holding the RecA concentration constant and titrating the oligonucleotide
- **Oligonucleotide concentration.** The molar stoichiometry (moles of nucleotides to moles of RecA protein) of binding of the oligonucleotide to RecA is 3:1. This ratio is independent of oligonucleotide size and corresponds to 160ng of oligonucleotide per 6.25µg RecA. We recommend using a titration series of 40–280ng in 40ng increments to determine the optimal concentration of oligonucleotide to use with the RecA. If nonspecific protection is a problem, then 160ng of oligo(dT) can be added to the reaction after the addition of ATP[γS].
- **Oligonucleotide design.** An oligonucleotide of 30–36 bases in length is recommended for both RecA cleavage and RecA protection in solution. Locate the protected site in the middle of the oligonucleotide. M. Koob recommends oligonucleotides designed with the restriction site ten nucleotides from the 3′-end (personal communication).
- **Buffer.** The buffers shown in Figure 9 and Table 2 work well with either *Eco*R I methylase or restriction enzyme. If restriction enzymes or methylases other than these are used, it may be necessary to adjust the salt concentration to improve the activity of the enzyme. Acetate salts appear to be less destabilizing to the RecA triplex than chloride salts, and therefore we suggest using potassium acetate rather than potassium or sodium chloride. While Promega's MULTI-CORE™ Buffer contains potassium acetate, we do not recommend its use for these applications.
- **Cloning the products of RecA cleavage.** Because the products of a RecA cleavage reaction are methylated, low transformation frequencies may arise from incompatibilities with the host's restriction/modification system. This will not be a problem when *Eco*R I methylase is used to protect the site, but could be with other restriction enzyme/methylase pairs. If transformation efficiencies are low, compare the genotype of your host to the known methylation-induced restriction systems to determine if this is the cause.

Agar*ACE*® Enzyme

Description

Agar*ACE*® Enzyme (Cat.# M1741, M1743) is a novel, patented agarolytic enzyme produced and extensively tested for the harvest of DNA and RNA from agarose gels (1,2). The harvested polynucleotide is suitable for a variety of downstream applications (page 29).

Applications

- Harvest of DNA and RNA from low-melting agarose gels.
- Harvest of DNA from regular-melting agarose gels (3).

Enzyme Properties

Requirements: Completely melted agarose, thorough mixing after enzyme addition, incubation temperature 42–47°C.

Optimal Substrate: Any agar or agarose, typically 1% (w/v) in electrophoresis buffers (TAE/TBE), melted completely.

Typical Working Concentration: One unit per 200mg of 1% gel.

Optimal pH: Approximately 6.75; however, Agar*ACE*® Enzyme exhibits ≥80% activity at pH 6.0–8.3. The unit activity is determined at pH 8.3, where the enzyme is least active, so under all conditions that researchers are likely to use, the activity will be at or greater than the expected activity (Figure 15).

Inhibitors: 0.3% SDS inhibits 35% in 15 minutes, urea, formaldehyde and guanidine-HCl at ≥0.2M inhibit completely. **Note:** The latter 3 inhibitors can be soaked out of the gel prior to digestion (Table 5, Figure 16).

Half-Life: Approximately 15 minutes at 57°C, about 10 minutes at 60°C and >6 minutes at 65°C.

Temperature Stability: No significant loss of activity after approximately 90 days at 4°C or 3 days at 20–23°C.

Inactivation: 70°C for 15 minutes.

Promega Product Information

Source: *Flavobacterium* strain NR19.

Molecular Weight: 42kDa.

Typical Working Conditions: One unit per 200mg melted 1% agarose in 1X TAE (pH 8.3) at 42–47°C.

Storage Conditions: Store at –20°C. Agar*ACE*® Enzyme is supplied in storage buffer containing 20mM Tris (pH 7.3).

Unit Definition: One unit is defined as the amount of Agar*ACE*® Enzyme required to completely degrade 200µl of 1% SeaPlaque® agarose in 1X TBE (pH 8.3) in 15 minutes at 42–47°C (Figure 11). Agar*ACE*® Enzyme degradation of agarose is measured by monitoring sugar release (4).

Purity: >90% as determined by visual inspection of a Coomassie® blue-stained SDS-polyacrylamide gel.

Activity Assays

Functional Assay (DNA Recovery): >95% recovery from 2µg of a DNA ladder ranging from 0.4 to 3.0kb in length.

Contaminant Assays

Endonuclease Assay: To test for endonuclease activity, 1µg of supercoiled plasmid DNA is incubated with 5 units of Agar*ACE*® Enzyme for 4 hours at 42°C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel. There must be no visible nicking or cutting of the DNA.

Exonuclease Assay: To test for DNase and RNase activity, 50ng of radiolabeled DNA or RNA is incubated with 5 units of Agar*ACE*® Enzyme for 4 hours at 42°C in 1X TAE buffer (pH 8.3) containing 6.6mM Mg^{2+}. The release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is <1% release for DNase and <3% release for RNase.

Single-Stranded DNase: 5 units of Agar*ACE*® Enzyme are tested with 50ng denatured substrate DNA in 1X TAE buffer with 6.6mM Mg^{2+} for 4 hours at 42°C. Minimum passing specification is <5% release.

Blue/White Cloning: pGEM®-3Zf(+) Vector[d] is digested with *Hin*c II or *Eco*R I, separated on a 1% gel and the agarose digested using Agar*ACE*® Enzyme. The harvested DNA is then religated and transformed into JM109 cells, which are then plated on X-Gal/IPTG/Amp plates. A minimum of 400 colonies is counted. White colonies result from transformation with ligated plasmids with damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs, such as *Eco*R I, must produce fewer than 2% white colonies, and blunt-cutting enzymes, such as *Hin*c II, must produce fewer than 5% white colonies (5). Transformation efficiency must be ≥1 x 10^5 cfu/µg DNA.

Additional Quality Control Assays

T-Vector Cloning: At least 100 colonies must be obtained, ≥60% of which are white, when JM109 cells are transformed with the pGEM®-T Vector[d,f] containing an insert purified using Agar*ACE*® Enzyme. 80% of the white colonies generated must contain inserts.

Labeling Efficiency: Using the Prime-a-Gene® Labeling System, a labeling efficiency of ≥1 x 10^9cpm/µg DNA must be obtained.

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DNA Recovery Tips

- TBE can be used instead of TAE for the gel running buffer. However, fewer downstream applications can be performed directly from a TBE gel digest. (See Table 8 and #TB228 for more information.)
- To visualize the DNA, ethidium bromide may be added to the gel directly (0.5µg/ml) or the gel can be stained after electrophoresis. To stain the gel after electrophoresis, soak in a solution of 0.5µg/ml ethidium bromide for 30 minutes at room temperature. Ethidium bromide added to the gel is lost during electrophoresis. As such, small fragments may not be visible. This can be avoided by adding ethidium bromide to the gel running buffer or staining the gel after electrophoresis. **Caution:** Ethidium bromide is a carcinogen. Wear gloves and use appropriate precautions when working with solutions and gels that contain ethidium bromide.
- It is important that the gel slice is completely melted. It will take approximately 10–15 minutes for 200µl of 1% LMP agarose to melt completely in a 1.5ml polypropylene microcentrifuge tube. If the gel slice is over 300mg it should be broken up using a spatula or razor blade prior to melting. Unless the sample contains large (>10kb) shearable DNA, monitor melting by gently pipeting the sample. **If the agarose is completely melted, it should pipet like water.**
- Use of a thermal cycler is convenient for isolation of DNA from many samples. Simply load the samples and program the melting and digestion temperatures into the unit. **Note:** Add the Agar*ACE*® Enzyme after the temperature has been reduced to 42–47°C to avoid denaturation of the enzyme.
- Agar*ACE*® Enzyme performs equally well in TAE (pH 7.3, 7.8 or 8.3) and TBE (pH 8.3) and performs adequately in 20mM phosphate and MOPS buffers across the pH range of 6.5–8.5. Note that the buffer selection may affect subsequent applications in which the isolated nucleic acid will be used (Table 8; #TB228).
- SDS (>0.3% w/v) and formaldehyde will inactivate Agar*ACE*® Enzyme. (See Table 5 and Technical Bulletin #TB228 for more information.)
- In some cases, components of the running buffer may interfere with downstream applications. In many cases, these effects can be reduced by soaking the gel slice in water before digestion with Agar*ACE*® Enzyme (Figure 16).

DNA Recovery Protocol

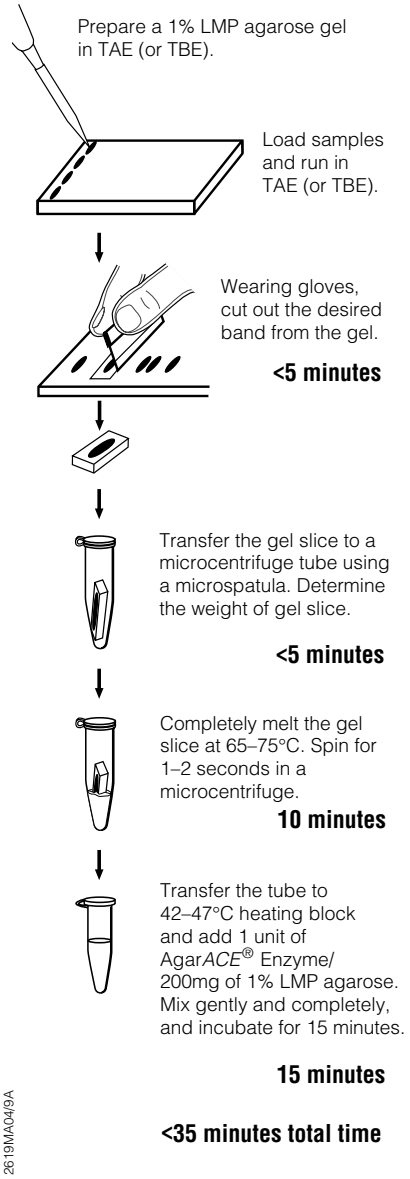


Figure 10. DNA recovery protocol.

Brief Protocol

1. Prepare a 1% low melting point agarose gel in TAE or TBE.
2. Load samples and DNA size standard(s). Run gel until bands are well separated.
3. Wearing gloves, refer to the DNA size standards to locate and cut out the desired band.
4. Transfer the gel to pre-weighed microcentrifuge tube using a clean spatula. Determine the weight of the gel slice. 1mg of gel equals approximately 1µl of volume.
5. Completely melt the gel slice at 65–75°C. Centrifuge for 1–2 seconds in a microcentrifuge to collect melted gel to bottom of tube.
6. Transfer the tube to a 42–47°C heating block or water bath and add 1 unit of Agar*ACE*® Enzyme/200µl (200mg) of melted gel. Mix gently and completely, and incubate for 15 minutes.

   The activity of Agar*ACE*® Enzyme is linear with respect to agarose concentration. That is, 1 unit of Agar*ACE*® Enzyme will digest 200µl of 1% agarose in 15 minutes, whereas 2 units of enzyme are required to digest an equivalent volume of 2% agarose in 15 minutes. Likewise, 200µl of 1% agarose will be completely digested in 1 hour using 0.25 units of Agar*ACE*® Enzyme (Table 4 and Figure 13).

   Check for complete digestion by gently pipetting the reaction. The reaction should have the viscosity of water.
7. The DNA can be used in many applications without further purification. If desired, the DNA can also be ethanol precipitated before use. Some downstream applications require ethanol precipitation of the DNA (Table 8). Promega Technical Bulletin #TB228 contains an application compatibility guide for determination of what further purification, if any, is needed.

For a more detailed protocol on the use of Agar*ACE*® Enzyme refer to Promega's *Protocols and Applications Guide,* Third Edition (pp. 84–86). Further information on downstream applications using Agar*ACE*® Enzyme-purified DNA can be found in *Promega Notes* 53, 54 and 61, Table 8 and Figures 17–20.

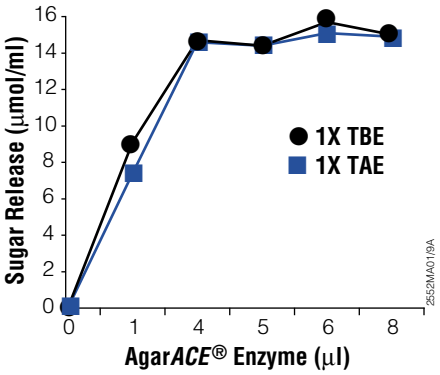


Figure 11. Determination of Agar*ACE*® Enzyme activity. Melted agarose is incubated with a titration of enzyme. The point at which the maximal amount of sugar is released through agarose hydrolysis is measured. The same amount of enzyme is required for either TAE or TBE gels.

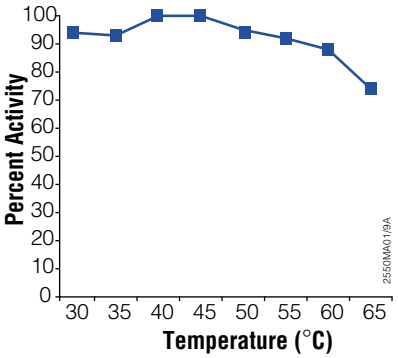


Figure 12. Temperature profile for Agar*ACE*® Enzyme. The activity of Agar*ACE*® Enzyme, added to a reaction at the indicated temperatures and allowed to act for 15 minutes, is shown. Note that although the temperature profile is fairly broad, 100% agarose hydrolysis (required for good DNA recovery) is only obtained at 42–47°C.

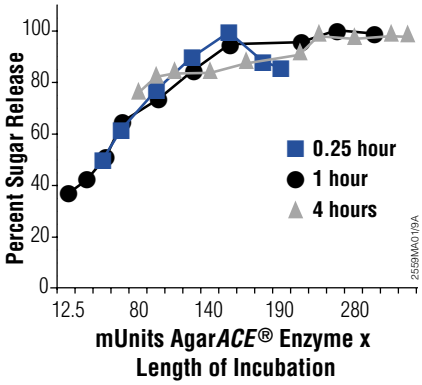


Figure 13. Agar*ACE*® Enzyme performance at longer digestion times. In this experiment, various amounts of Agar*ACE*® Enzyme were tested at incubation times of 15 minutes, 1 hour, and 4 hours. The graph shows that the enzyme titration maximizes at nearly the same value of enzyme x time for all three incubation times. This means that if the use of less enzyme is desired, the incubation time can be increased proportionately (up to a 10-fold enzyme dilution/4 hour incubation) (see Table 4 for precise values; 3).

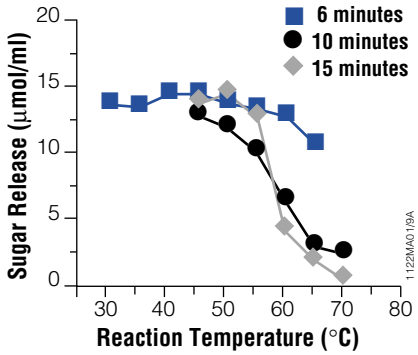


Figure 14. Agar*ACE*® Enzyme survival at various temperatures. Agar*ACE*® Enzyme is relatively stable for a few minutes at temperatures of 65–70°C, which allows the hydrolysis protocol to be performed without a lower temperature equilibration step. By the time a sample is taken from a block and the Agar*ACE*® Enzyme is added, the temperature is usually at 60–65°C, and is dropping (4).

Table 4. Digestion Time Versus Units of Enzyme.

Digestion Time (at 42–47°C, unless noted)	Units of Agar*ACE*® Enzyme
15 minutes	1.0
1 hour	0.25
4 hours	0.1
16 hours (overnight)*	0.025

*Perform overnight digestions at 37°C in an oven.

Agar*ACE*® Enzyme Activity Assays:

Agarase activity assays are generally based on the fact that hydrolysis of the agarase polymer liberates free reducing sugar, which is then measured colorimetrically. Two types of assays are commonly used; one in which the amount of enzyme is varied and the maximal sugar release attained is measured (Figure 11). The other type of assay is a fixed enzyme/fixed time format, where the amount of sugar released is compared to that obtained for a known standard condition (Figures 12–14).

Table 5. Effects of Buffer Additives on Agar*ACE*® Enzyme (4).

Additive	Effect on Agar*ACE*® Enzyme
Ethidium Bromide (0.5–5mg/ml)	None detected
Glycerol (0–50%)	None detected
DTT (0–10mM)	None detected
NaCl (0–200mM)	None detected
NaCl (200–500mM)	Causes variation in the digestion rate, not recommended
EDTA (0–10mM)	None detected
SDS (<0.1% w/v))	None detected
SDS (>0.3% w/v)	Inactivates enzyme
Formaldehyde	Inactivates enzyme

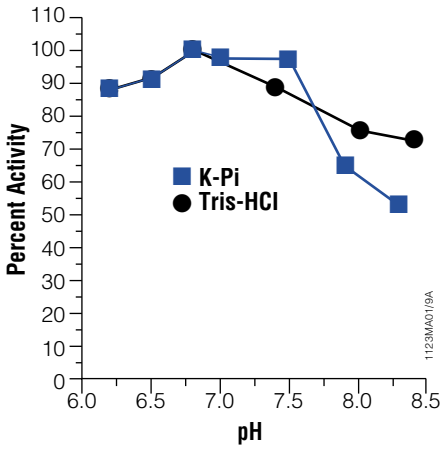


Figure 15. The effect of pH on Agar*ACE*® Enzyme activity. Although the pH optimum for Agar*ACE*® is 6.75, the pH activity profile is fairly broad. The enzyme is assayed for quality control purposes under the low activity condition (pH 8.3), so that any pH deviations on the acidic side will result in higher activity than that indicated on the product label (4).

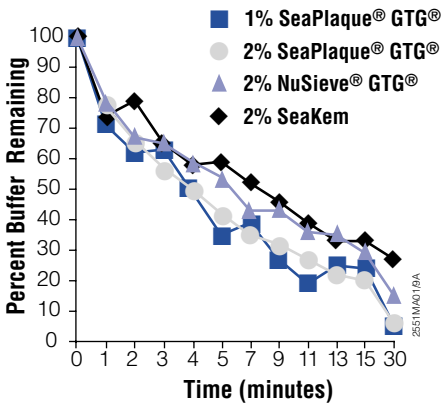


Figure 16. Buffer removal by gel soaking. About 70–80% of the 1X TBE buffer in slices of several types of gels can be removed by a 15-minute soak in 10 volumes of water. Other experiments (data not shown) demonstrate that DNA fragments as short as 200bp are largely retained in the gel under these conditions.

Ethanol Precipitation of DNA

1. Hydrolyze the agarose completely as described in the standard procedure.
2. For sodium acetate precipitation, add 0.1 volume of 3M NaOAc (pH 5.2), mix, then add 2 volumes of room temperature 95% ethanol (2X the volume after NaOAc addition). For ammonium acetate precipitation, add 0.5 volume of 7.5M NH_4OAc, mix, then add 2.5 volumes of room temperature 95% ethanol (2.5X the volume after NH_4OAc addition).
3. Allow the mixture to stand 1–2 hours (2 hours for small amounts of DNA in NH_4OAc) at room temperature (do not incubate in the cold), then centrifuge for 15 minutes at room temperature in a microcentrifuge at maximum speed.
4. Immediately decant the supernatant, orienting the tube so that the pellet is on the upper side of the tube. Centrifuge the tube for 1–2 seconds in a microcentrifuge to bring down residual supernatant. Remove the remaining supernatant.
5. Wash the pellet by adding 200–500µl of cold (<10°C) 70% ethanol, agitating briefly, centrifuging for 5 minutes and decanting as above.
6. Dry the pellet in a Speed-Vac® instrument if desired, then reconstitute the pellet in an appropriate volume of water or buffer. Allow at least 15 minutes for the nucleic acid to redissolve.

Table 6. Troubleshooting Agar*ACE*® Enzyme.

Symptom:

Large quantities of white or translucent precipitate in tube when ethanol precipitated; pellet resuspends poorly

Possible Causes:	Comments:
Incomplete digestion of agarose prior to precipitation; possible loss of enzyme activity.	Resuspend the sample, remelt residual agarose and retreat with Agar*ACE*® Enzyme.
Incomplete digestion of agarose prior to precipitation; agarose concentration higher than intended.	Resuspend sample, remelt residual agarose and retreat with Agar*ACE*® Enzyme.
Incomplete digestion of agarose prior to precipitation; possible buffer incompatibility.	Repeat using a different buffer, and a longer incubation time.

Table 7. DNA Recovery from Agar*ACE*® Enzyme-Hydrolyzed Agarose after Ethanol Precipitation (6).

	DNA Recovery (%)		
DNA Size (bp)	Sodium Acetate, 1 hour at 25°C	Ammonium Acetate, 2 hours at 25°C	Ammonium Acetate, 1 hour at 25°C
296	122 ± 4	105 ± 2	39 ± 3
344	115 ± 4	95 ± 3	27 ± 2
395	nd	100 ± 1	54 ± 2
500	104 ± 4	99 ± 1	74 ± 2
1,000	115 ± 4	96 ± 1	65 ± 2
1,600	107 ± 3	94 ± 1	80 ± 4
2,000	94 ± 3	83 ± 1	62 ± 3
3,000	106 ± 1	77 ± 3	78 ± 3

Recovery, using Agar*ACE*® Enzyme, of 100ng total DNA from a DNA ladder, as quantified by image analysis of a SYBR® Green I-stained gel. Starting amounts of DNA for the smaller bands (296bp, 344bp) were approximately 2–4ng and apparent recoveries >100% were due to gel contributions. Results demonstrate DNA recoveries at or near 100% for sodium and ammonium acetate precipitations after 1 and 2 hours, respectively, but lower recoveries with ammonium acetate for 1 hour.

Compatibility of Agar*ACE*® Enzyme with Downstream Applications

Table 8. Summary of the Compatibility of Nucleic Acid Isolated Using Agar*ACE*® Enzyme With Subsequent Applications.

Application (running buffer)	Can Be Used Directly in Application?	Required Pre-Treatment	Comments
Ligation			
(TAE)	Yes	None	TBE lowers transformation efficiency (6).
(TBE)	Yes	None	
Cloning of DNA Fragments			
(TAE)	Yes	None	TBE can greatly lower transformation efficiency (Table 10; 6).
(TBE)	Yes	None	
Transformation of *E. coli*			
(TAE)	No	Add ligase buffer	For TBE, the DNA volume is important. Tables 9 and 10 and Figure 20 show that addition of ligase buffer is important.
(TBE)	No	Add ligase buffer	
Electroporation of *E. coli*			
(TAE)	No	Ethanol precipitation	Slightly fewer colonies obtained.
(TBE)	No	Ethanol precipitation	
pGEM®-T Vector Cloning			
(TAE)	Yes	None	The number of colonies is somewhat variable.
(TBE)	Yes	Soak gel in water	
Labeling with T4 PNK			
(TAE)	Yes, but lower labeling	Add 2X kinase (optional)	Soaking the gel in water can improve labeling (Figure 18).
(TBE)	Yes, but lower labeling	Add 2X kinase (optional)	
Dephosphorylation (AP)			
(TAE)	Yes	None	Borate ions inhibit CIAP (Figure 17).
(TBE)	No	Ethanol precipitation	
Digestion with Restriction Enzymes			
(TAE)	Yes, but more enzyme	Yes, in certain cases	Important! Varies with restriction enzyme used (Table 11).
(TBE)	Yes, but more enzyme	Yes, in certain cases	
Prime-a-Gene® Labeling			
(TAE)	Yes	None	See reference 7.
(TBE)	Yes	None	
Nick Translation			
(TAE)	Yes	None	See reference 7.
(TBE)	Yes	None	
***fmol*® DNA Sequencing**			
(TAE)	Yes	Ethanol precipitation	Ethanol precipitation before sequencing is useful for eliminating "hard stops" (Figure 19; 6).
(TBE)	No	Ethanol precipitation	
PCR			
(TAE)	Yes	None	See Figure 19 and reference 6.
(TBE)	Yes, but 40–60% yield (variable)	Ethanol precipitation	

Labeling with either Prime-a-Gene® Labeling System or Nick Translation System

Use Agar*ACE*® Enzyme-isolated DNA directly, or if it is necessary, concentrate the DNA by performing an ethanol precipitation. Cool DNA solution to room temperature before adding labeling reactants (7).

fmol® DNA Sequencing System

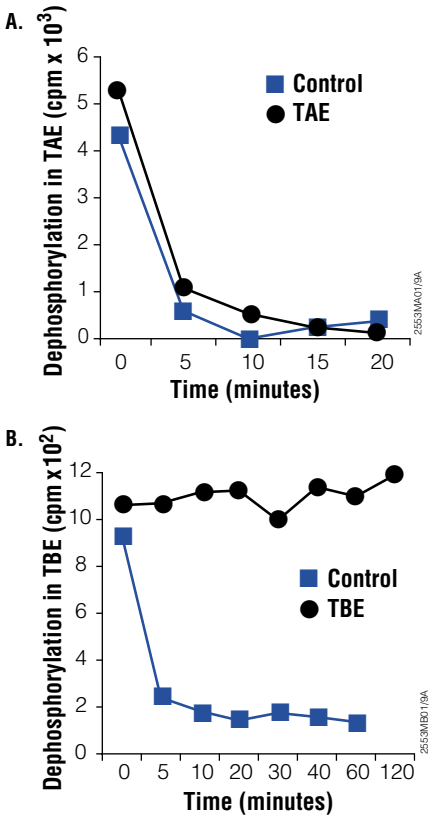
Use Agar*ACE*® Enzyme-isolated DNA directly if TAE is used as the running buffer, or if TBE is the buffer or it is necessary to concentrate the DNA, perform an ethanol precipitation. In some cases "hard stops" have been observed at various locations when the DNA is not first ethanol precipitated (6).

Transformation of Cells by Electroporation

Before using the DNA for electroporation, ethanol precipitate it using sodium acetate, and wash the DNA pellet at least once with cold (<10°C) 70% ethanol to remove traces of the sodium acetate. The total number of colonies observed will be approximately 2–3-fold lower than observed for DNA in water.

Compatibility of Agar*ACE*® Enzyme with Downstream Applications (continued).

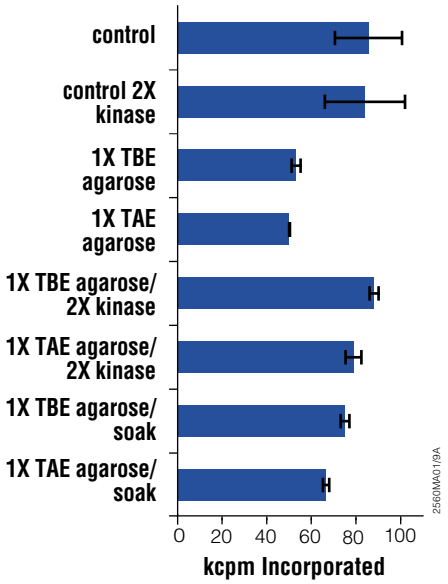
Figure 17. Performance of Agar*ACE*® Enzyme-harvested DNA in dephosphorylation reactions with calf intestinal alkaline phosphatase (CIAP). For TAE gels (Panel A), the DNA is efficiently dephosphorylated relative to control, but the borate ion in TBE gels (Panel B) completely inhibits the CIAP enzyme.



Recommendation: Dephosphorylation with Calf Intestinal Alkaline Phosphatase

Use isolated DNA directly if TAE is used as the running buffer. If it is necessary to concentrate the DNA or if TBE is used, perform an ethanol precipitation. If ethanol precipitation is not done for samples from TBE, the borate in the TBE will completely inhibit the CIAP.

Figure 18. Kinase reactions with Agar*ACE*® Enzyme-harvested DNA. The figure demonstrates that while performing a kinase reaction with control DNA in water, using twice the normal amount of kinase does not increase labeling. However, it does increase labeling of Agar*ACE*® Enzyme-harvested DNA (from TBE or TAE gels) up to control levels. Alternatively, soaking the gel to remove some of the buffer has a similar benefit.

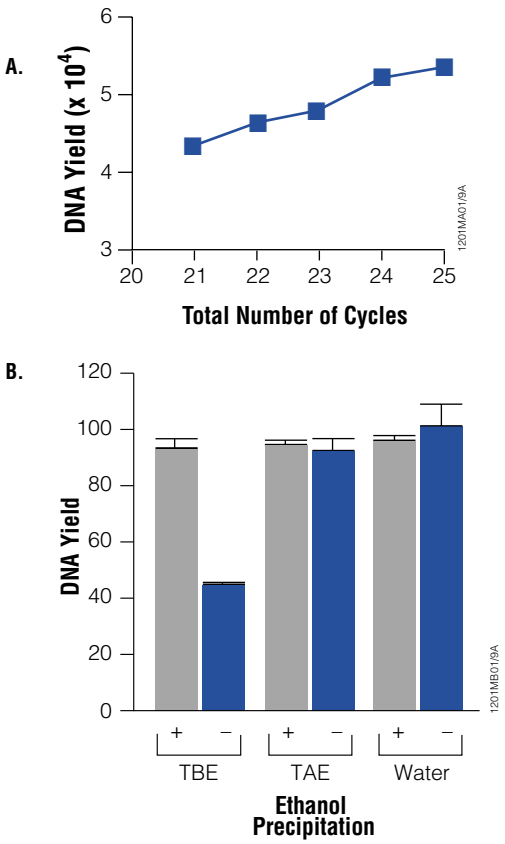


Recommendation: 5′ End-Labeling with T4 Polynucleotide Kinase

Perform either an ethanol precipitation (using sodium acetate; ammonium ion inhibits kinase) prior to labeling or double the amount of kinase added to obtain the highest specific activity possible. Alternatively, soak the gel in water (Figure 16) prior to digesting with Agar*ACE*® Enzyme to get DNA that can be labeled to at least one-half the specific activity of DNA in water. DNA harvested from unsoaked gels and not ethanol precipitated will label to at least one-fourth to one-third the specific activity of DNA in water.

Compatibility of Agar*ACE*® Enzyme with Downstream Applications (continued).

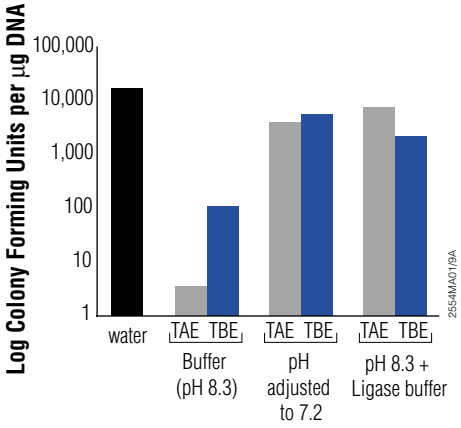
Figure 19. Performance of Agar*ACE*® Enzyme-harvested DNA in PCR. Panel A demonstrates that for template DNA harvested from a TAE-buffered gel using Agar*ACE*® Enzyme, the yield of PCR product (measured in a fluorescent dye assay) increased linearly with increasing numbers of cycles. **Panel B** demonstrates that template DNA harvested from TAE gels using Agar*ACE*® Enzyme resulted in PCR product yields similar to those observed with control DNA resuspended in water. Template DNA harvested from TBE gels needed to be ethanol-precipitated to perform well in PCR.



Recommendation: PCR from Isolated Fragment

Agar*ACE*® Enzyme-isolated DNA may be used directly if TAE is used as the running buffer. If TBE is used or if it is necessary to concentrate the DNA, an ethanol precipitation should be performed. TBE results in 2–3-fold lower PCR yields unless DNA is first ethanol-precipitated.

Figure 20. Transformation efficiency is greatly dependent on the pH of the gel-running buffer. When DNA is used in buffer at pH 8.3 (TAE or TBE) low transformation efficiency results, but when the pH is adjusted to 7.2 the transformation efficiency increases dramatically. Addition of ligase buffer decreases the interference caused by high pH electrophoresis buffers, provided the volume used in a 100µl transformation is minimal.



Recommendation: Transformation of *E. coli.*

We recommend using DNA harvested from TAE, setting up a normal ligation and transforming cells with ≤5µl DNA per 100µl competent cells. If TBE is used, run the gel in 0.5X buffer or soak the gel in water prior to digestion, and then use ≤8µl of DNA per 100µl cells. In all cases, for high colony yields ethanol precipitation of DNA will remove the buffer and concentrate the DNA.

Table 9. Transformation Efficiency of DNA Isolated from Agarose Gels using Agar*ACE*® Enzyme (6).

Treatment	cfu/µg
water control	6.4 x 10^7
TAE (pH 8.3)	7.5 x 10^7
TAE (pH 8.3) + Agar*ACE*® Enzyme	8.1 x 10^7
TAE (pH 8.3) + agarose + Agar*ACE*® Enzyme	1.3 x 10^8
TBE (pH 8.3)	2.2 x 10^7
TBE (pH 8.3) + Agar*ACE*® Enzyme	2.5 x 10^7
TBE (pH 8.3) + agarose + Agar*ACE*® Enzyme	4.6 x 10^7
TAE (pH 7.2)	6.5 x 10^7
TAE (pH 7.2) + Agar*ACE*® Enzyme	6.8 x 10^7
TAE (pH 7.2) + agarose + Agar*ACE*® Enzyme	7.7 x 10^7

pGEM®-3Zf(+) Vector DNA was treated in one of three ways: 1) suspended in 1X gel buffer; 2) diluted in 1X gel buffer and Agar*ACE*® Enzyme (1u/200µl) added; or 3) embedded in 1% SeaPlaque® GTG® agarose cast in 1X gel buffer, melted and digested with Agar*ACE*® Enzyme as described in the DNA Recovery Protocol (Figure 10). Pseudoligations contained 5ng of plasmid in 5µl of one of the above mixtures, 1µl of 10X Ligase Buffer and 4µl water. In each case, 2µl of the pseudoligation (1ng DNA) was transformed into low efficiency competent JM109 Cells and plated on LB/Amp/IPTG/X-Gal plates. Colonies were counted and cfu/µg were calculated.

Table 10. Luciferase Activity and Transformation Efficiency of pBEST-*luc*™ Vector DNA After Ligation (6).

Treatment	µl DNA Used in Ligation	cfu/µg	% Colonies Expressing Luciferase
TAE (pH 8.3)	3µl	1 x 10^6	97%
TAE (pH 8.3)	8µl	3.1 x 10^4	95%
no ligase control	8µl	<limits	—
TBE (pH 8.3), water-soaked	3µl	1 x 10^5	100%
TBE (pH 8.3), water-soaked	8µl	7.4 x 10^3	98%
no ligase control	8µl	<limits	—
TBE (pH 8.3)	3µl	<limits	—
TBE (pH 8.3)	8µl	<limits	—
no ligase control	8µl	<limits	—

The pBEST *luc*™ Vector(1) was digested with *Bam*H I and *Cla* I and 2µg was loaded onto a 1% SeaPlaque® GTG® gel cast in either 1X TAE or 1X TBE (pH 8.3). The *luc* gene and vector fragments were excised from the gel, pre-soaked in water where noted and the DNA isolated as described in the DNA Recovery Protocol (Figure 10). Equal molar ratios of the two fragments were mixed adding either 3 or 8µl of the hydrolyzed agarose (20–22ng DNA and 51–54ng DNA, respectively) to the ligation. Fragments were ligated in a 10µl reaction using 3 units of T4 DNA Ligase, overnight at 15°C. Part of the ligation reaction (5µl) was transformed into 50µl Subcloning Efficiency JM109 Competent Cells, plated on nitrocellulose filters on LB/Amp plates and incubated at 37°C overnight. Colonies were counted for transformation efficiency. Luciferase activity was assayed by transferring the nitrocellulose filter to a dish containing 1mM luciferin in 0.1M sodium citrate buffer (pH 5.5) at 45–50°C and observing in the dark at room temperature. The limits of detection were 4.5 x 10^2 cfu/µg.

Table 11. Effect of Agar*ACE*® Digestion on Restriction Enzyme Activity.

Enzyme	Units Required if Isolated from 1X TBE*	Units Required if Isolated from 1X TAE*	Notes
Acc I	3.5X	7X	Purify with Wizard® PCR Preps**
Apa I	3X	3X	
*Bam*H I	4X	4X	
*Eco*R I	6X	6X	Purify with Wizard® PCR Preps**
*Hin*d III	8.5X	8.5X	Purify with Wizard® PCR Preps**
Kpn I	4X	4X	
Not I	3.5X	2X	
Pst I	7X	17X	Purify with Wizard® PCR Preps**
Sac I	1X	1.5X	
Sal I	1.5X	2X	
Sph I	1X	1X	

*The amount of restriction enzyme required (X-fold) to digest the DNA relative to a control digestion containing DNA in water. The DNA was isolated from *Promega LMP* (Cat.# V2831, V3841), Preparative Grade Agarose; other agaroses may give different results.

**The use of TBE buffer is not recommended with the Wizard® PCR Preps DNA Purification System.