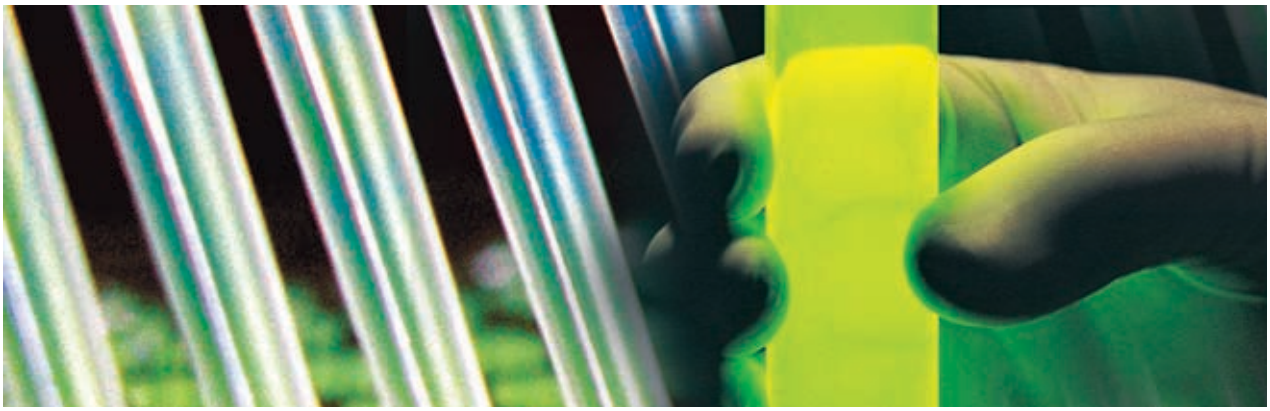


In This Section

Section V: In-Gel Reactions

Overview

Introduction

A variety of enzymatic reactions can be performed in the presence of agarose. In-gel reactions are an alternative approach to standard DNA recovery techniques and provide a multitude of benefits. The use of in-gel reactions will not only save time, but eliminate potential sample loss during DNA recovery from an agarose gel.

Advantages

- Saves time otherwise used for recovery
- Avoids recovery losses
- Avoids recovery damage, especially shearing of higher molecular weight DNA

Applications

Enzymes active in the presence of low melting temperature agaroses include:

- Alkaline phosphatase
- BAL 31 nuclease
- DNA polymerase I
- Klenow fragment
- Restriction endonucleases
- Reverse transcriptases
- T4 DNA ligase
- T4 DNA polymerase
- T4 polynucleotide kinase
- T7 DNA ligase
- T7 DNA polymerase (Sequenase® Polymerase)
- *Taq* DNA polymerase

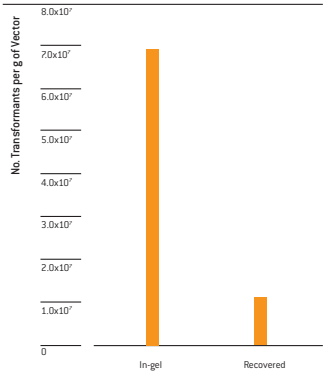
Compatible agaroses

In-gel reactions require the use of a low melting temperature (65°C) agarose. Lonza has developed two high quality Genetic Technology Grade™ (GTG®) Agaroses for this application, specifically NuSieve® GTG® and SeaPlaque® GTG® Agarose. These agaroses are specifically designed and tested for compatibility with in-gel reactions.

- NuSieve® GTG® Agarose is the choice for separation of nucleic acids ≤1,000 bp with a resolving power of 4% - 6% difference in DNA size
- SeaPlaque® GTG® Agarose is the choice for separation of large nucleic acid fragments >1,000 bp

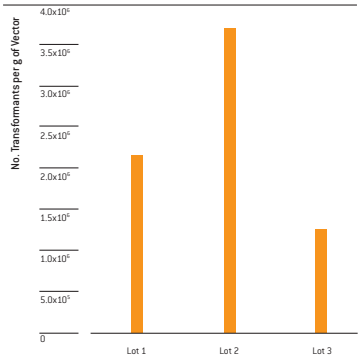
The figures below demonstrate the efficiency of in-gel cloning in our low melting temperature agaroses versus sample recovery and also the consistency between different agarose lots.

Efficiency of In-Gel Cloning from SeaPlaque® GTG® Agarose



Two lanes of *Bst* E II digest of lambda DNA were electrophoresed in a 1% SeaPlaque® GTG® Agarose gel prepared in 1X TAE. The 2.3 kb band was excised from each lane. One sample was ligated directly to pUC 19 in the presence of remelted agarose (In-gel) and the other sample was purified using a chaotrope-based recovery protocol (Recovered). Transformation into DH5α® Cells was done following standard protocols.

Efficiency of In-Gel Cloning from NuSieve® GTG® Agarose



A 377 bp fragment was electrophoresed in three separate lots of a 3% NuSieve® GTG® Agarose gel in 1X TAE Buffer then ligated to M13 in the presence of remelted agarose. Transformation into DH5αF′® Cells was done in remelted agarose following standard protocols. Results 1, 2 and 3 are from three separate lots of NuSieve® GTG® Agarose.

Electrophoretic tips

— Use a low EDTA (0.1 mM) 1X TAE Buffer; increases the availability of Mg^{+}, a necessary cofactor in many enzymatic reactions

— Briefly stain the gel **after** electrophoresis with GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide; the presence of ethidium bromide during electrophoresis can result in the degradation of DNA

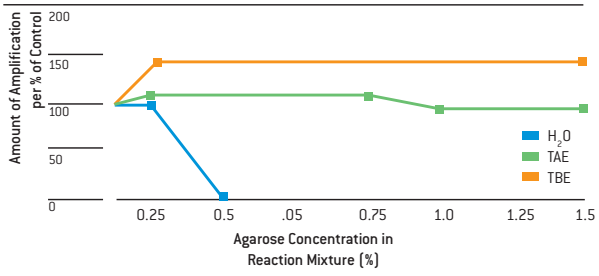
— Visualize your DNA with a UV light source that is ≥300 nm. Short UV wavelength light can damage DNA; alternatively, DNA can be detected using Clare Chemical's Dark Reader® Transilluminator

— Minimize exposure of DNA to UV light to less than 1 minute

— The addition of 1 mM guanosine or cytidine to the gel and electrophoresis buffer is effective in protecting DNA against UV-induced damage; the nucleosides will not impede electrophoresis, detection or purification of DNA.

— Limit the amount of DNA in the band of interest to 100 ng if possible; more DNA may cause smearing and a wide band will result in an unacceptably large piece of gel to be excised and melted.

Effect of Agarose Concentration and Buffer on In-Gel PCR



A 500 bp PCR product was separated on 4% NuSieve® GTG® Agarose in either 1X TAE or 1X TBE Buffer. Following electrophoresis, the PCR product was reamplified with the same primers as in the original amplification. Relative efficiency was measured by comparing the products on an agarose gel.

Reaction tips

— Melt the gel slice at 65°C - 70°C. Do not exceed 70°C as this may cause melting of your DNA

— Thoroughly mix the reaction components at 37°C - 40°C this ensures proper mixing of DNA with reaction components

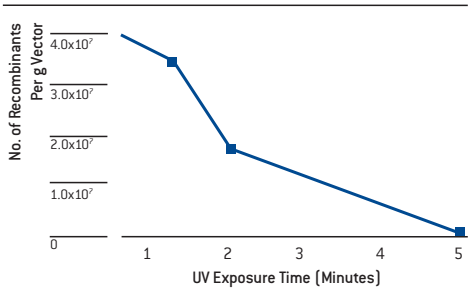
— Do not exceed 0.5% SeaPlaque® GTG® or 1.5% NuSieve® GTG® Agarose in the reaction mix

— Increase the efficiency of blunt end ligations; the addition of larger amounts of vector, insert DNA and T4 DNA ligase or the addition of 5% (w/v) PEG-8000 to the ligation mixture and incubating the ligation reaction mixture for a longer time, can increase the efficiency of the reaction; an alternative strategy is to add linkers with sticky ends to the insert DNA

— When transforming cells, dilute the melted ligation reaction mixture five-fold before adding to cells

— When transforming bacterial cells, dilute the agarose containing the ligated DNA further with warmed (40°C) TAE or sterile distilled water before adding the ligation reaction to the competent cells; it is better to add 10 µl of a five-fold diluted mixture than 2 µl of a more concentrated reaction mixture; by doing this, it is less likely that your ligated DNA will be trapped in the agarose when it is added to the chilled cells; also be sure that you do not add excessive DNA to the competent cells which can decrease the transformation efficiency

Effect of UV Irradiation on In-Gel Cloning



A 2 kb fragment, separated in 1% SeaPlaque® GTG® Agarose and stained with ethidium bromide, was exposed to 300 nm UV irradiation for different lengths of time before excision from the gel, ligation into pBR322, and bacterial cell transformation. The mean of the number of white colonies per microgram vector on two plates is given.

Reference

Gründemann, D. and Schömig, E., *BioTechniques 21:* 898 - 903, 1996.

Timetable

1. (Day 1) Preparation of vector. Preparation of insert. Preparation of competent cells.
2. (Day 2) Ligation reaction. Transformation reaction.
3. (Day 3) Assess the results obtained from the ligation-transformation reaction.

Tips

— Use 1X TAE Buffer with 0.1 mM EDTA.

— Electrophorese the DNA without ethidium bromide in the gel.

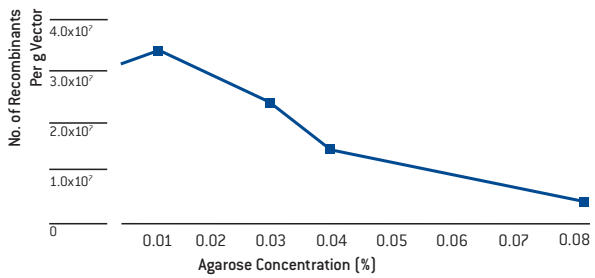
— Minimize the exposure time of the DNA to less than 1 minute under UV light.

— Do not exceed a final concentration of 0.5% SeaPlaque® GTG® or 1.5% NuSieve® GTG® Agarose in the reaction mixture.

— Dilute the agarose solution when running in-gel transformations. Do not exceed >0.02% agarose in the transformation reaction.

NOTE: Electroporation can not be used with in-gel ligation/transformation procedures.

Effect of Agarose Concentration on Transformation



Aliquots of agarose solutions (0.25%, 0.5%, 1.0% and 2.0% in 1X TAE) were added to pUC19 monomer DNA which had been placed in labeled tubes. Aliquots (6 µl=0.01 ng DNA) of the samples containing agarose were added to 100 µl of (Invitrogen, Inc.) frozen competent cells on ice. The agarose concentrations shown are those in the tubes of competent cells after samples were added. Transformations were carried out following standard protocol. Points shown on the graph represent the mean of three transformations.

- Materials
  - Sterile microcentrifuge (≥1 ml) and polypropylene tubes (17 mm x 100 mm)
  - Horizontal electrophoresis chamber
  - Scalpel or razor blade
  - Heating block or water bath
  - Ice bucket and ice
- Reagents
  - SeaPlaque® GTG® or NuSieve® GTG® Agarose
  - GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide
  - Distilled water
  - T4 DNA ligase
  - Competent cells
  - Restriction enzymes
  - Calf intestine alkaline phosphatase
  - 1X TAE (0.1 mM EDTA) Gel and Running Buffer
  - Reagents for phenol extraction (Section VI)
  - 10 mM Tris-HCl, pH 7.5
  - Ethanol
  - 10X T4 ligation buffer

    500 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 50 mM Dithiothreitol; 10 mM ATP pH 7.6; 200.0 µg/µl nuclease-free BSA (bovine serum albumin)

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Preparing the vector

1. Cut vector with appropriate restriction enzymes.
2. Dephosphorylate vector with calf intestine alkaline phosphatase.
3. Phenol extract vector.
4. Ethanol precipitate vector.

Preparing the insert

1. Cut DNA to be used as insert with appropriate restriction enzymes.
2. Electrophorese DNA (100 ng) in a low melting temperature agarose gel prepared in 1X TAE Buffer (0.1 mM EDTA).
3. Briefly stain DNA with GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide.
4. Excise gel band containing insert and place in a sterile, preweighed tube.
5. Estimate the volume of the gel slice based upon the weight of the slice (e.g., 100 mg = 100 µl).
6. Determine the concentration of the DNA in the gel slice by assuming 100% recovery of DNA in the slice.
7. Store excised band at 4°C until ready for use.
8. Prepare agarose plates with the appropriate selective media for the next day's use.

Preparing competent cells

If you prepare your own competent cells, set up a small overnight culture of cells. High transformation efficiencies can be obtained with frozen competent cells that are either prepared in the laboratory or purchased. Similar transformation efficiencies of 10^7 transformants/µg pUC18 DNA with insert have been obtained using the Hanahan Procedure (see citation at end of section); transformation efficiencies are reduced when using the $CaCl_2$ method.

NOTE: Electroporation can NOT be used with in-gel ligation/transformation procedures.

Ligation reaction

1. Remelt the agarose slice by heating to 68°C for 10 minutes.
2. Place the remelted gel slice at 37°C until needed.
3. For a final reaction mixture of 50 µl, add the following components in the following order:

Volume	Component
18 µl	10 mM Tris-HCl, pH 7.5 or distilled H_2O
5 µl	10X T4 DNA ligation buffer
1 µl	Vector (amount sufficient to obtain a molar ratio of insert to vector of 3 - 4:1)
1 µl	T4 DNA ligase (between 1 - 2 units)

4. Mix the components gently with a pipette.
5. Add up to 25 µl of the remelted agarose gel slice (25 ng of insert DNA). Do not exceed a final concentration of 0.5% SeaPlaque® GTG® or 1.5% NuSieve® GTG® Agarose in the reaction mixture.
6. Mix components by resuspending with a pipette.
7. Ligate at room temperature for 2 - 3 hours.

Transformation reaction

The transformation procedure outlined below is a modification of the Invitrogen procedure included with their DH5α® Competent Cells. When using other frozen competent cells follow manufacturer's directions.

1. Prepare competent cells if working with fresh cells. If using frozen competent cells, remove cells from storage and thaw on ice.
2. Heat the ligation reaction at 68°C for 5 minutes to remelt the agarose.
3. Add competent cells into a prechilled polypropylene tube (17 mm x 100 mm). We use 100 µl of thawed DH5α® cells.
4. Add 1 µl - 2 µl of diluted ligation reaction (70 pg - 140 pg vector) to competent cells.
5. Mix and incubate on ice for 30 minutes.

NOTE: Exceeding 0.02% SeaPlaque® GTG® or NuSieve® GTG® Agarose concentration in the final mixture may reduce transformation efficiencies.

6. Heat shock the cells by placing the tubes in a 42°C water bath for 45 seconds.
7. Place the cells back on ice for 2 minutes.
8. Add 0.9 ml of SOC medium to cells.
9. Incubate at 37°C with shaking for 30 - 60 minutes.
10. Spread 1 µl - 200 µl of the transformation mixture onto an agar plate with the appropriate selective medium.
11. Allow the liquid to soak in.
12. Invert the plates.
13. Incubate overnight at 37°C.

■ Reagents

- SOC medium
- 2.0 g Bacto-tryptone, 0.5 g Yeast extract, 1.0 ml 1 M NaCl 0.25 ml 1 M KCl
- Dissolve in a final volume of 100 ml distilled water; autoclave
- Add aseptically:
  - 1.0 ml 2 M Mg^{2+} (1 M $MgCl_2$, 1 M $MgSO_4$, filter sterilized)
  - 1.0 ml 2 M glucose (filter sterilized)
- LB Agar Plates Supplemented with Ampicillin and X-gal (1 L)
  - 10.0 g tryptone
  - 5.0 g yeast extract
  - 10.0 g NaCl
  - 15.0 g agar
  - 800 ml distilled water
  - Stir to dissolve components
  - Adjust to pH 7.5 with NaOH
  - Adjust volume to 1 L with distilled water
  - Carefully heat while stirring until agar is dissolved
  - Remove stir bar and sterilize the medium by autoclaving 25 minutes
  - Place the flask of medium in a water bath set at 50°C and let the medium cool for at least 30 minutes
  - While medium is cooling, prepare ampicillin and X-gal stock solutions.
  - Add 1 ml of ampicillin stock and 2 ml of X-gal stock to the tempered medium and pour into plates
  - Allow plates to sit on bench overnight to dry; then package and store at 4°C
- Ampicillin stock
  - 100.0 mg of ampicillin to 1 ml of sterile distilled water
- X-gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside) stock
  - 40.0 mg of X-gal to 2 ml of dimethylformamide

Cloning in the Presence of Agarose — continued

Controls

We recommend the following controls be run in parallel with the test plates:

Vector	Ligase	# of blue colonies	# of white colonies	Reason for control
pUC 18-native	-	+++++	-	Cell competency test
pUC18-singly digested efficiency	-	+	-	Background for ligation efficiency
pUC18-singly digested	+	++++	-	Determines ligation efficiency
pUC18-doubly digested background	-	+	-	Unrestricted vector background
pUC18-doubly digested	+	+	-	Determines the efficiency of double digestion
pUC18-native	+	+++++	-	Reagent check

Assessing the results

Count colonies and assess the results of ligation-transformation. Colonies containing recombinant plasmids should be white. Blue colonies result from non-recombinant pUC18 plasmid.

References

Hanahan, D., *J. Mol. Biol.* **166**: 557 - 580, 1983.
Crouse, G.F., *et al., Meth. Enzymol.* **1**: 78 - 89, 1983.
Frischauf, A.M., *et al., Nucl. Acids Res.* **8**: 5541 - 5549, 1980.
Majumdar, D., *et al., BioTechniques* **7**: 188 - 191, 1989.
Murray, J.A.H., *Nucl. Acids Res.* **14**: 10118, 1986.
Struhl, K., *BioTechniques* **3**: 452 - 453, 1985.

Procedure

NOTE: The urea sample buffer prevents the samples from regelling after digestion. It will also result in good separation of the DNA during electrophoresis.

1. Electrophorese DNA (several µg) in a low melting temperature agarose prepared in 1X TAE Buffer (0.1 mM EDTA).
2. Briefly stain the gel with GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide.
3. Excise the gel slice containing the DNA of interest and place in a preweighed microcentrifuge tube.
4. Estimate the volume of the gel slice based upon the weight and determine the concentration of the DNA in the gel slice (e.g., 100 mg = 100 µl). Assume 100% recovery of the DNA.
5. Store excised band at 4°C until ready for use.
6. Add sterile distilled water to bring volume of the gel slice to 200 µl (200 mg).
7. Remelt the gel slice by heating to 68°C for 10 minutes.
8. Mix the melted gel slice by pipetting.
9. Remove a volume containing the quantity of DNA needed for restriction digestion.
10. Maintain the sample at 37°C to prevent gelling.
11. Dilute the restriction endonuclease with the appropriate reaction buffer.
12. Digest DNA at the appropriate temperature and time for your particular enzyme.
13. Stop the reaction by adding 30 µl of urea sample buffer to 50 µl of the digestion mixture and mixing.
14. Heat the sample to 65°C for 10 minutes if it has regelled.
15. Load the sample onto appropriate agarose gel for analysis.

- Materials
  - Horizontal electrophoresis chamber
  - Scalpel or razor blade
  - Microcentrifuge tubes (≥1 ml)
  - Water bath or heating block
- Reagents
  - SeaPlaque® GTG® or NuSieve® GTG® Agarose
  - 1X TAE (0.1 mM EDTA) Gel and Running Buffer
  - Sterile distilled water
  - GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide
  - Restriction endonuclease(s)
  - Urea sample buffer
    - 8% Ficoll® (Type 400) Polymer
    - 27 mM EDTA
    - 0.27% bromophenol blue
    - 5 M urea
    - Adjust to pH 8.0

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

References

Hermann, R.G. and Whitfeld, P.R., *Methods in Chloroplast Molecular Biology,* Elsevier Biomedical Press, 1982.
Hermann, R.G., *et al., Gene* **8**: 179 - 191, 1980.
Parker, R.C. and Seed, B., *Meth. Enzymol.* **65**: 358 - 363, 1980.
Peacock, A.C., *et al., Anal. Biochem.* **149**: 177 - 182, 1985.

Procedure 1

1. Electrophorese PCR products in low melting temperature agarose prepared in 1X TAE (0.1 mM EDTA) Buffer.
2. Briefly stain the gel with GelStar® or SYBR® Green I Gel Stain or ethidium bromide.
3. Excise a gel slice containing the template DNA and place into a preweighed microcentrifuge tube.
4. Store the gel slice at 4° C protected from light until ready for use.
5. Melt the agarose gel slice containing template DNA at 65°C for 10 minutes.
6. Dilute with 65°C, sterile distilled water to a final DNA concentration of 0.1 ng/µl.
7. Add a portion of the DNA into the amplification reaction mixture (do not exceed 1 ng DNA/reaction).
8. Combine the components of the reaction mixture.
9. Vortex the reaction mixture to mix the contents.
10. Spin briefly in a microcentrifuge.
11. Overlay the mixture with mineral oil if necessary.
12. Perform amplification reactions with conditions appropriate for the template DNA and primers.
13. Remove mineral oil.
14. Remelt the reaction mixture at 65°C prior to analyzing.

Procedure 2

1. Follow steps 1 - 2 in Procedure 1.
2. Turn the gel upside down while it is on the UV transilluminator.
3. Stab the band of interest with a glass Pasteur pipette. When the pipette is removed from the gel, a small plug of agarose will be contained in the tip.
4. Remove the plug of agarose.
5. Add a portion of the plug of agarose to the amplification reaction. The plug does not require melting; it will melt during the first denaturing step.
6. Follow steps 8 - 14 in Procedure 1.

References

Bjourson, A.J. and Cooper, J.E., *Nucl. Acids Res.* **20**: 4675, 1992.
Fors, L., *et al., Nucl. Acids Res.* **18**: 2793 - 2799, 1990.
Nichols, W.C., *et al., Genomics* **8**: 318 - 323, 1990.
Wilkie, T.M. and Simon, M.I., *Methods: A Companion to Methods in Enzymology* **2(1)**: 32 - 41, 1991.

Amplification Reaction Mixture

If the reaction buffer does not contain magnesium ion, add sufficient amount for your template/primer.

Component	Volume (µl)	FINAL Concentration
10X Reaction Buffer	5	1X
dNTP mix (1.25 mM each)	8	200 µM each
Primer #1, 10 µM	2.5	0.5 µM or 1.0 µM
Primer #2, 10 µM	2.5	0.5 µM or 1.0 µM
Template DNA + Agarose	5 - 10 (0.3 ng - 1.0 ng DNA)	
Polymerase	0.5 (2.5 units)	
Sterile distilled water	to 50 µl	

- Materials
  - Horizontal electrophoresis chamber
  - Scalpel or razor blade
  - Vortex mixer
  - Microcentrifuge
  - Thin-walled microcentrifuge tube
  - Microcentrifuge tubes (≥1 ml)
  - Thermal cycler
- Reagents
  - SeaPlaque® GTG® or NuSieve® GTG® Agarose
  - 1X TAE (0.1 mM EDTA) Gel and Running Buffer
  - DNA amplification kit
  - Forward and reverse primers
  - Sterile distilled water
  - GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

Restriction Digestion in the Presence of Agarose — continued

References for other in-gel reactions

Cycle Sequencing
Fraser, G.J., *Resolutions* **9(3):** 1, 1993.
Gorman, K.B. and Steinberg, R.A., *BioTechniques* **7:** 326 - 329, 1989.
Kretz, K.A. and O'Brein, J.S., *Meth. Enzymol.* **218:** 72 - 79, 1993.
Random Prime Labeling
Daum, H.A. III, *et al., BioTechniques.* **11:** 784 - 790, 1991.
Feinberg, A.P. and Vogelstein, B., *Anal. Biochem*. **137:** 266 - 267, 1984.
Rose, E.A., *et al., Cell.* **60:** 495 - 508, 1990.
Soreum, H., et al., *App. Environ. Microbiol.* **56:** 1033 - 1037.

Blunt-end Ligation
Hemsley, A., *et al., Nucl. Acids Res.* **17:** 6545 - 6551, 1989.
Majumdar, D., *et al., BioTechniques* **7:** 188 - 191, 1989.
Murray, J.A.H., *Nucl. Acids Res.* **14:** 10118, 1986.
Translation & Transcription of RNA
Brandt, T.L. and Hackett, P.B., *Anal. Biochem.* **135:** 401 - 408, 1983.
Gallitelli, D. and Hull, R., *J. Virol. Methods* **11:** 141 - 144, 1985.
Person, D.A. and Finn, O.J., *BioTechniques* **4:** 398 - 399, 1986.

Attaching Linkers
Crouse, G.F., *et al., Meth. Enzymol.* **101:** 78 - 89, 1983.
Gap Filling
Majumdar, D., *et al., BioTechniques* **7:** 188 - 191, 1989.