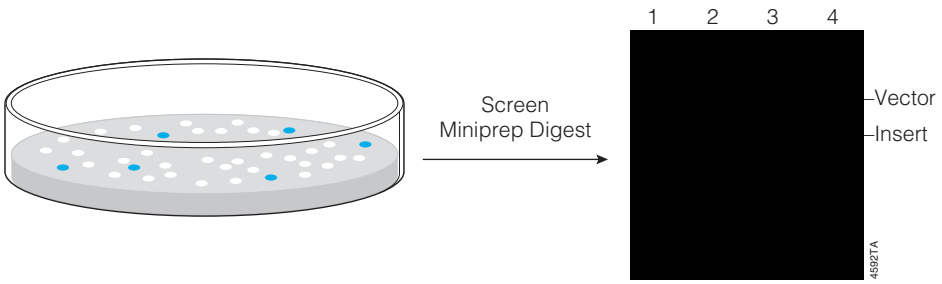
Screening for Recombinants

Introduction

Now that you've transformed your DNA and allowed the colonies to grow overnight, you need to determine if they contain the insert of interest. You can either screen them by colony PCR or the more traditional plasmid miniprep followed by restriction digestion. Colony PCR is the most rapid initial screen. A plasmid miniprep will take an extra day to grow up the culture but will provide a lot of material for further analysis. Some people do both and simply do not put the negative colonies that were identified through the colony PCR through the full miniprep procedure. The choice is yours.



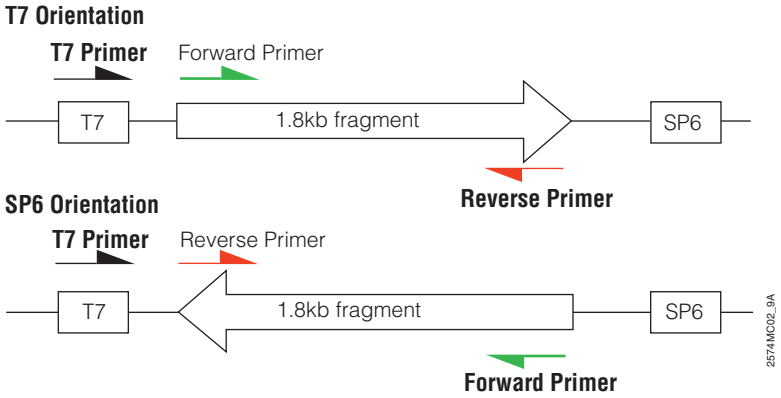
Screening for Recombinants

Colony PCR

Colony PCR involves lysing the bacteria and amplifying a portion of the plasmid. You can use either insert-specific primers or vector-specific primers to screen for recombinant plasmids. If your subcloning scheme will not maintain the orientation of the insert, you can use colony PCR to screen for orientation. Simply combine a vector-specific primer with an insert-specific primer.

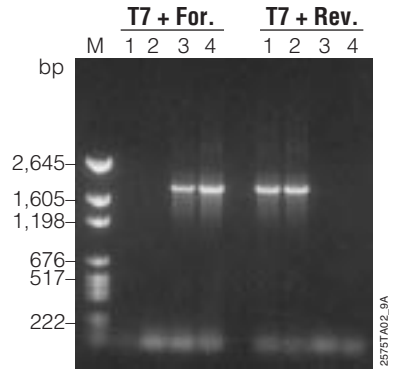
PCR cloning using the A-overhangs left by *Taq* DNA Polymerase(f) and an appropriately T-tailed vector (e.g., pGEM®-T Easy Vector) is not a technique that will retain orientation. The orientation can be rapidly assessed with colony PCR using vector-specific primers and insert-specific primers as detailed below.

This technique was used when screening for orientation of a 1.8kb insert into the pGEM-T Easy Vector. Colony PCR was performed with the T7 Promoter Primer and either the insert-specific forward or reverse PCR primer. Eight white colonies were chosen from the cloning experiment for analysis. Clones with the T7 orientation will produce the fragment only with the T7 primer and reverse PCR primer, and clones in the opposite (SP6) orientation will only produce a product with the forward PCR primer as illustrated below.



Colony Prep for Colony PCR

1. Pick a well isolated colony and transfer to 50µl of sterile water. Part of the colony may be transferred to LB media containing the appropriate antibiotic for over night culture and miniprep if desired.
2. Boil for 10 minutes.
3. Centrifuge at 16,000 x g for 5 minutes.
4. Use 5µl of the supernatant in a 50µl PCR.



Colony PCR. Colonies were suspended in 50µl sterile water, boiled for 10 minutes, centrifuged at 16,000 × *g* for 5 minutes, and 5µl of the supernatant was used in each amplification. The DNA was amplified by PCR in 50µl volumes with 50pmol of each primer and 1.25 units of *Taq* DNA Polymerase (Cat.# M1661). After an initial denaturation of 2 minutes at 94°C, the amplification profile was 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 1 minute) and extension (72°C for 2.5 minutes); PCR was concluded with 1 cycle of 72°C for 10 minutes. Amplification products (8µl) were analyzed on a 1% agarose gel containing ethidium bromide.

Screening for Recombinants

Go Directly to Gel

GoTaq® DNA Polymerase is the ideal choice for colony PCR applications. The enhanced buffer can handle the "dirty" template better than a conventional reaction buffer, and the Green GoTaq Reaction Buffer allows you to load the PCR products directly onto a gel after amplification. No need to add loading dye.

Colony PCR with GoTaq® DNA Polymerase Typical Reaction.

Nuclease-Free Water	to 50µl
5X Green GoTaq® Reaction Buffer	10µl
PCR Nucleotide Mix (Cat.# C1141)	1µl
GoTaq® DNA Polymerase (5u/µl)	1.25u
Downstream Primer	50pmol
Upstream Primer	50pmol
Colony Lysate	5µl

Assemble the reaction on ice in the order listed. A master mix containing everything but the colony lysate can be prepared and dispensed into reaction tubes ready to accept the colony lysate.

GoTaq® DNA Polymerase

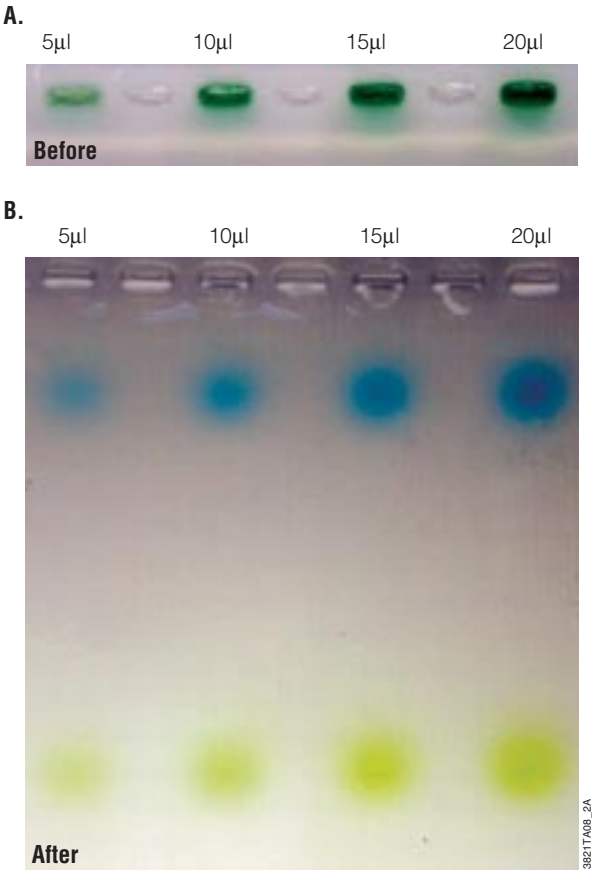
Cat.# M3001 100u; 80 reactions

Cat.# M3005 500u; 400 reactions

Cat.# M3008 2,500u; 2,000 reactions

Supplied with enzyme (5u/µl), 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer. Sufficient to give the indicated number of 50µl reactions using 1.25u of enzyme per reaction.

Protocol available at:
www.promega.com/tbs/9pim300/9pim300.html



Amplification reactions using GoTaq DNA Polymerase with Green GoTaq Reaction Buffer. Panel A shows loaded wills of an agarose gel. **Panel B** shows the blue and yellow dyes after electrophoresis. Volumes of 5, 10, 15 and 20µl of the amplification reactions were loaded into a 1% agarose gel with TBE buffer and subjected to electrophoresis.

Cycling Conditions For GoTaq® Reactions.

Step	Temp	Time	Cycles
Initial Denaturation	94°C	2	1
Denaturation	94°C	0.5–1.0	25–35
Annealing	42–65°C*	0.5–1.0	
Extension	72°C	1 minute/kb	
Final Extension	72°C	5	1
Soak	4°C	Indefinite	1

Reactions are placed in a thermal cycler that has been preheated to 94°C.

*Annealing temperature should be optimized for each primer set based on the primer melting temperature (T_m). An online calculator for melting temperatures of primers in GoTaq® Reaction Buffer is available at: **www.promega.com/biomath**

The extension time should be at least 1 minute/kilobase of target. Typically, anything smaller than 1kb uses a 1-minute extension.

Screening for Recombinants

Screening by Plasmid Miniprep and RE Digests

The classic method for screening colonies involves performing a plasmid miniprep followed by restriction digestion. Well-isolated colonies are picked from a plate and transferred to culture medium containing the appropriate antibiotic for selection. Proper sterile technique is important. Many different culture media formulations are commonly used for minipreps. Promega recommends LB media supplemented with antibiotics (see page 48) for miniprep cultures to insure that the bacteria do not outgrow the ability of the antibiotic to select for the plasmid. If a rich medium like Terrific Broth is used, the bacteria can grow to very high cell densities and deplete the antibiotic. Once the antibiotic is depleted, the selection pressure to keep the plasmid is removed, and the plasmid may be lost.

You can inoculate the colony into 1–10ml of culture medium. If using a high-copy plasmid, 1–5ml (more typically, 1–2ml) is plenty. If you are using a low-copy plasmid, inoculate 10ml. Aerating the culture is very important for maximum cell density. A 17 × 100mm culture tube is fine for 1–2ml. If growing a larger volume, a 50ml sterile, disposable culture tube is better. Incubate the culture overnight (12–16 hours) with shaking (~250rpm). Remember, the greater the surface area, the greater the aeration. You can even grow miniprep cultures in sterile 25–50ml Erlenmeyer flasks.

Once the DNA is purified, a portion of the plasmid is screened by restriction digestion. For high-copy plasmids, you can obtain 4–10µg plasmid DNA per purification (1–5ml). For low-copy plasmids, you will obtain 1–3µg plasmid DNA per purification (10ml). Use 0.5–1µg of plasmid in your digest. Design the digest so that you can easily determine if your plasmid contains insert.

Note: Be sure to run uncut plasmid on the same gel for comparison.

Antibiotics: Mode of Action and Mechanism of Resistance.

Antibiotic	Mode of Action	Mechanism of Resistance	Working Concentration	Stock Solution
Ampicillin (Amp)	A derivative of penicillin that kills growing cells by interfering with bacterial cell wall synthesis.	The resistance gene (*bla*) specifies a periplasmic enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic.	50–125µg/ml in water	50mg/ml
Chloramphenicol (Cm)	A bacteriostatic agent that interferes with bacterial protein synthesis by binding to the 50S subunit of ribosomes and preventing peptide bond formation.	The resistance gene (*caf*) specifies an acetyltransferase that acetylates, and thereby inactivates, the antibiotic.	20–170µg/ml in ethanol	34mg/ml
Kanamycin (Kan)	A bactericidal agent that binds to 70S ribosomes and causes misreading of messenger RNA.	The resistance gene (*kan*) specifies an enzyme (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents its interaction with ribosomes.	30µg/ml in water	50mg/ml
Streptomycin (Sm)	A bactericidal agent that binds to the 30S subunit of ribosomes and causes misreading of the messenger RNA.	The resistance gene (*str*) specifies an enzyme that modifies the antibiotic and inhibits its binding to the ribosome.	30µg/ml in water	50mg/ml
Tetracycline (Tet)	A light-sensitive bacteriostatic agent that prevents bacterial protein synthesis by binding to the 30S subunit of ribosomes.	The resistance gene (*tet*) specifies a protein that modifies the bacterial membrane and prevents transport of the antibiotic into the cell.	10µg/ml in liquid culture; 12.5µg/ml in plates	12.5mg/ml in ethanol

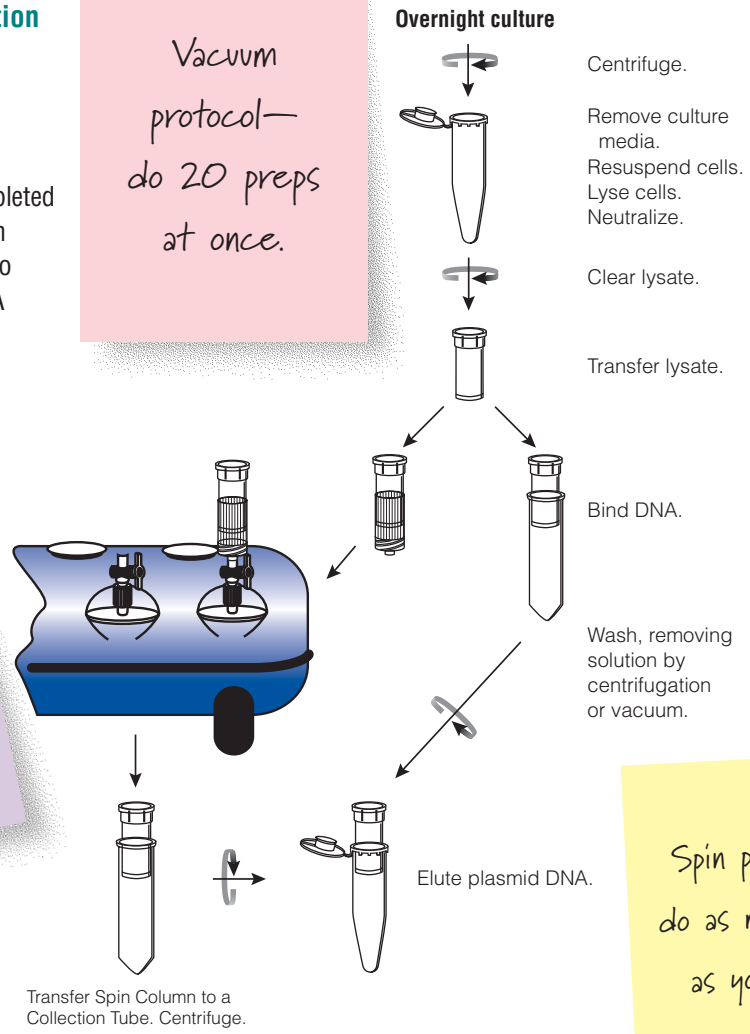
Screening for Recombinants

Plasmid Minipreps

Wizard® *Plus* SV Minipreps DNA Purification System

The Wizard *Plus* SV Minipreps DNA Purification System[h,i], a simple membrane-based system, provides a reliable method for rapidly isolating plasmid DNA. The entire procedure can be completed in 45 minutes or less. Work with up to 5ml of an overnight culture of a high-copy plasmid or up to 10ml of low-copy plasmid. Use the isolated DNA directly for applications such as automated fluorescent sequencing and restriction digests.

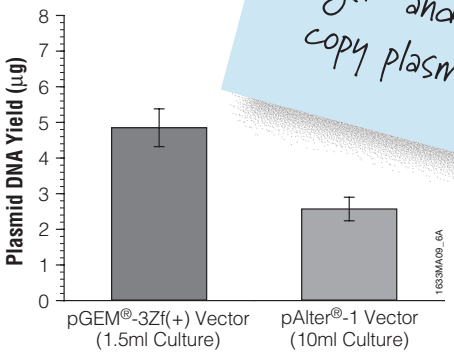
Vacuum protocol— do 20 preps at once.



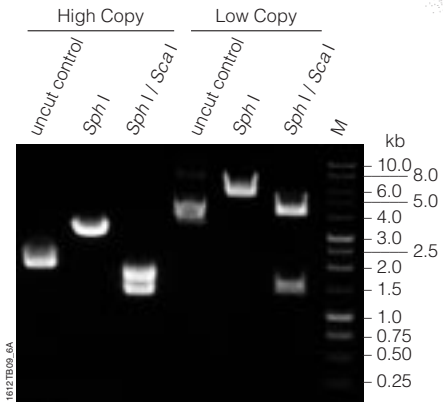
Wizard® Plus SV Minipreps
Ready for spin protocols:
A1330 50 preps
A1460 250 preps
Ready for spin or vacuum protocols:
A1340 50 preps
A1470 250 preps
Protocol available at:
www.promega.com/tbs/tb225/tb225.html

Spin protocol— do as many preps as your rotor can hold.

Works with high- and low-copy plasmids!



Plasmid DNA yield from high and low copy plasmids using the Wizard *Plus* SV Minipreps DNA Purification System. *E. coli* DH5α™ cells were transformed with either the pGEM®-3Zf(+) Vector (high-copy number plasmid) or the pALTER®-1 Vector (low-copy number plasmid) and grown in LB medium containing 50µg/ml of ampicillin (16 hours at 37°C, 200rpm). Plasmid DNA was isolated in sets of 42 on three consecutive days (126 total samples each) from 1.5ml (pGEM-3Zf(+) Vector) and 10ml (pALTER-1 Amp^r Vector) cultures using the Wizard® *Plus* SV Minipreps DNA Purification System.



Restriction enzyme digestion of high- and low-copy number plasmids. The plasmid vectors pGEM-3Zf(+)(high-copy) and pALTER-1 (low copy), isolated from *E. coli* DH5α using the Wizard *Plus* SV Minipreps DNA Purification System, were digested with 10 units of the indicated enzymes for 1 hour at 37°C. The digested samples were resolved on a 1% agarose gel and stained with ethidium bromide. The marker is the 1kb DNA Ladder (Cat.# G5711).

Troubleshooting Subcloning Experiments

Symptoms	Possible Causes	Comments
Few or no colonies obtained after transformation	Cells not competent	Competent cells may exhibit lower transformation efficiencies 5–6 weeks after preparation. To verify that bacteria are competent, perform a test transformation using a known amount of a standard supercoiled plasmid (see page 47).
	Unsuccessful ligation	Analyze samples of a linearized vector and the vector + insert ligation on an 0.8% agarose gel. If ligation was successful, the banding pattern of the ligation products should be different from that of the unligated sample.
	Inactive T4 DNA Ligase	Verify that the T4 DNA Ligase is active; perform a control ligation reaction with linear plasmid DNA.
	Inactive T4 DNA Ligase Buffer	Store T4 DNA Ligase 10X Reaction Buffer in small aliquots at –20°C to minimize freeze-thaw cycles of the buffer. Multiple freeze-thaw cycles may degrade the ATP in the buffer.
	Digested vector ends are not compatible with the fragment	Restriction enzyme sites that are adjacent within the multiple cloning region or near the ends can prove difficult to digest completely. See page 40 for a method to create PCR products with restriction sites in the primers.
	Excess ligation products added to competent cells	The added ligation products should not exceed 0.5% of the transformation reaction volume. Excess DNA (>10ng) may also inhibit the transformation.
High Background	Unsuccessful dephosphorylation of vector DNA	Attempt to religate the dephosphorylated vector. It should religate with low efficiency.
	Plates lack the correct antibiotic; the antibiotic is inactive	Perform a mock transformation with no DNA added. If colonies grow, discard the plates.
	Ratio of linearized, phosphorylated vector to insert DNA is too high	Reduce the amount of linearized vector in the reaction. Religation of the vector is favored when the vector:insert ratio is too high.

Troubleshooting Subcloning Experiments—Deletions of the Insert

During screening of your recombinant plasmids, you may encounter a situation where part of your insert is deleted. Perhaps careful examination of the subcloning strategy has not identified steps that may have led to this deletion. You attempt to sequence these clones and find that a portion of the insert has been deleted along with part of the vector. How did this happen? Two possibilities come to mind that could lead to such an event.

Symptoms	Possible Causes	Comments
Recombinants are isolated but contain deletions (usually unidirectional deletions of insert and part of vector)	Insert is unstable in the host strain	The insert may have been a substrate for recombination by recombinases in the most bacterium (remember most common laboratory strains are *rec*A minus, but there are other recombinases present). You can transform the plasmid into an *E. coli* strain deficient in more recombinases than just the recA. Some strains like SURE® cells from Stratagene are deficient in recombinases *rec*B and *rec*J and may allow you to propagate the unstable insert. Also try growing at a lower temperature.
	Insert is toxic to the host strain	Certain inserts may produce toxic gene products. The bacteria responds by deleting a portion of either the plasmid, the insert or both. High copy-number plasmids will tend to produce more toxin and thus be more prone to deletion or rearrangement. Most cloning plasmids carry the modified ColE1 origin of replication derived from pUC vectors, which maintain the copy number of the plasmid as high as 100–400 copies per bacterium. Low-level, leaky transcription in this high copy number plasmid can yield significant quantities of the toxic product. One solution is to transfer the insert to a different vector with a lower copy number. Promega has some vectors with lower copy numbers available like the pALTER®-1 (based on the pBR322 ColE1 origin; as few as 25 copies per cell) and the pALTER®-*Ex*2 (based on the pACYC origin of replication; ~10 copies per cell). This solution requires moving to a new vector. If you need to stay with the same vector (e.g., a mammalian expression vector) there are *E. coli* strains that have mutations that limit the copy number of pUC-based ColE1 origin of replication. The ABLE® strains from Stratagene will reduce copy number four- to ten-fold compared to more common laboratory strains.

Screening for Recombinants: Ordering Information

Competent Bacteria

Product	Size	Cat.#
Select96™ Competent Cells (>10^8cfu/µg)	1 × 96 reactions	L3300
JM109 Competent Cells, >10^8cfu/µg*	5 × 200µl	L2001
JM109 Competent Cells, >10^7cfu/µg	5 × 200µl	L1001

*For Laboratory Use.

Chemicals for Blue/White Screening

Product	Size	Conc.	Cat.#
IPTG, Dioxane-Free	1g	—	V3955
	5g	—	V3951
	50g	—	V3953
X-Gal	100mg	50mg/ml	V3941

For Laboratory Use.

Reagents for Colony PCR

Product	Conc.	Size	Cat.#
GoTaq® DNA Polymerase	5u/µl	100u	M3001
	5u/µl	500u	M3005
	5u/µl	2,500u	M3008
PCR Nucleotide Mix	10mM	200µl	C1141
	10mM	1,000µl	C1145
Set of dATP, dCTP, dGTP and dTTP	100mM	10µmol	U1330
	100mM	25µmol	U1420
	100mM	40µmol	U1240
	100mM	200µmol	U1410

For Laboratory Use.

Plasmid DNA Purification

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System[l,m]*	50 preps	A1330
	250 preps	A1460

Ready for use as a spin prep requiring only a microcentrifuge.

Product	Size	Cat.#
Wizard® *Plus* SV Minipreps DNA Purification System plus Vacuum Adapters[l,m]*	50 preps	A1340
	250 preps	A1470

Ready for use as a spin prep or a vacuum prep. Spin protocol requires only a microcentrifuge. Vacuum protocol requires Vac-Man® Laboratory Vacuum Manifold and a microcentrifuge.

Product	Size	Cat.#
Vac-Man® Laboratory Vacuum Manifold, 20-sample capacity	1 each	A7231

*For Laboratory Use.