The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: July 8, 2011
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Electronically completed forms are to be submitted to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190 or to jstanle2@uwo.ca) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety.

Please ensure that all questions are fully and clearly answered. Failure to do so will lead to the form being returned, which will cause delays in your approval and frustration for you and your colleagues on the Committee.

If you are re-submitting this form as requested by the Biohazards Subcommittee, please make modifications to the form in bold print, highlighted in yellow. Please re-submit forms electronically.

PRINCIPAL INVESTIGATOR:	**Dr. Victor Han**
DEPARTMENT:	**Paediatrics**
ADDRESS:	**VRL A5-107**
PHONE NUMBER:	**x58306**
EMERGENCY PHONE NUMBER(S):	**X10215**
EMAIL:	**vhan@uwo.ca**

Location of experimental work to be carried out :

Building : **VRL** Room(s): **A5-108, -111, -114, -116, -120, -124, -129**

Building : **VRL** Room(s): **A5 Bays 1-4**

Building : Room(s):

***For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).**

FUNDING AGENCY/AGENCIES: **CIHR**

GRANT TITLE(S): **Regulation of Fetal Growth, Placental Mesenchymal Stem Cells in Regenerative Therapy**

UNDERGRADUATE COURSE NAME(IF APPLICABLE):

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Steve Dixon	**sdixon5@uwo.ca**	**Oct 26/05**
Caroline Kahiri	**Caroline.Kahiri@schulich.uwo.ca**	**Sept 14/11**
Catherine Currie	**bandccurrie@rogers.com**	**Oct 26/05**
Laura Klein	**kleinlh@yahoo.com**	**Oct 26/05**
Maxim Seferovic	**maxseferovic@hotmail.com**	**Sept 14/09**

Amer Youssef	ayousse3@uwo.ca	May 27/07
Doaa Aboalola	daboalol@uwo.ca	May 15/09
Madhulika Gupta	mbgupta@uwo.ca	Oct 26/05
Majida Abushehab	mabusheh@yahoo.ca	Oct 19/05
Heather Tarnowski-Garner	htarnow@uwo.ca	Aug 25/11

Please explain how the biological agents are used in your project and how they are stored and disposed of. The BARF without this description will not be reviewed.

The self-renewal and differentiation properties of Placental Mesenchymal Stem Cells (PMSCs) are being studied by treating these cells with factors such as hypoxia, exogenous growth factors and over-expression of genes within the Insulin-like Growth Factor (IGF) system. These treatments are also performed on the other cell lines listed to better understand how the IGF system affects development to model Fetal Growth Restriction. In addition, we have generated a number of plasmids with cDNAs coding for the IGF family to be able to probe various tissues for RNA and protein expression.
All biological agents are located on the 5th floor of the Victoria Research Labs which is accessible only by security badge. Cells not being cultured are maintained in a liquid nitrogen dewar or in a -80°C freezer. All tissues and plasmids (stored in glycerol stocks) are kept in a -80°C freezer as well until use. Studies using cultured cells are done in a tissue culture specific room within a certified BSC. All hazardous materials are disposed of as setup through Victoria Hospital (waste bins for either incineration or autoclaving as designated by Stericycle Inc).

Please include a ONE page research summary or teaching protocol in lay terms.
Forms with summaries more than one page will not be reviewed.

Regulation of Fetal Growth: The overall aim of this project is to determine the mechanisms that underlie the short- and long-term effects of FGR by utilizing molecular approaches in mouse models *in vivo* and representative cell cultures *in vitro*. The overall hypothesis is that FGR and developmental programming are the consequence of dynamic interactions between gene and environment that occur in the embryo/fetus and placenta with the IGF system as critical mediator.

Placental Mesenchymal Stem Cells in Regenerative Therapy: Human adult stem cells from umbilical cord blood and placenta, and a human embryonic stem cell line will be studied *in vitro* for changes in their self-renewal capacity, differentiation and motility by insulin-like growth factors. Control of stem cell fate by manipulating IGF signaling may improve outcomes of stem cell therapy in a variety of human diseases, and may decrease the tumorigenic potential of stem cells.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☐ YES ☒ NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ☐ YES ☐ NO
If YES, please give the name of the species ______________________
What is the origin of the microorganism(s)? ______________________

Please describe the risk (if any) of escape and how this will be mitigated:

Please attach the CFIA permit.

Please describe any CFIA permit conditions:

1.2 Please complete the table below:

Full Scientific Name of Biological Agent(s)* (Be specific)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
E.coli			Yes No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
			Yes No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ No	☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3
	☐ Yes ☐ No	☐ Yes ☐ No	☐ Yes ☐ No			☐ 1 ☐ 2 ☐ 2+ ☐ 3

****Please attach a Material Safety Data Sheet or equivalent from the supplier if the bacterium used is not on this link:*** http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

Additional Comments: ______________________

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☒ YES ☐ NO
(If NO, please proceed to Section 3.0)

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☒ Yes ☐ No	**Placenta (PMSCs)**	Not applicable
Rodent	☐ Yes ☐ No		
Non-human primate	☐ Yes ☐ No		
Other (specify)	☐ Yes ☐ No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Containment Level of each cell line	Supplier / Source of cell line(s)
Human	☒ Yes ☐ No	**HUVEC** **HepG2** **HEK293**	**2** **1** **2**	**LONZA** **ATCC** **ATCC**
Rodent	☒ Yes ☐ No	**CHO** **NIH3T3**	**1** **1**	**ATCC** **ATCC**
Non-human primate	☐ Yes ☒ No			
Other (specify)	☐ Yes ☒ No			

**Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)*

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ☒ 1 ☒ 2 ☐ 2+ ☐ 3

Additional Comments: _____

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☒ YES ☐ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/UNKNOWN**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid	**Pregnant women and cord blood**	☐ Yes ☒ Unknown		☐1 ☒2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid	**Amniotic Fluid**	☐ Yes ☒ Unknown		☐1 ☒2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)	**Placenta, umbilicus**	☐ Yes ☒ Unknown		☐1 ☒2 ☐2+ ☐3
Human Organs or Tissues (preserved)	**Placenta, umbilicus**	Not Applicable		Not Applicable

Additional Comments: _____

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☒ YES ☐ NO If **NO**, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☒ YES, complete table below ☐ NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transformed or	Will there be a change due to transformation	Will there be a change in the pathogenicity of the bacteria after the genetic modification?	What are the consequences due to the transformation of the bacteria?
E coli DH5alpha JM109	**pGem4Z,3Z pBluescript pGEMT pCMV-FLAG pE-GFP pcDNA3 pSuperGFP pRcCMV**				**No**	**Used as probes for in situ hybridizations and IHC**

pCMV-FLAG?

** Please attach a Material*

*** Please attach a plasmid*

****No Material Safety Data*

of E. coli:

http://www.uwo.ca/humanr list.pdf

4.3 Will genetic modificatio ectors be made?

☐ YES, complete table below ☒ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction

** Please attach a Material Safety Data Sheet or equivalent.*

4.3.1 Will virus be replication defective? ☐ YES ☐ NO

4.3.2 Will virus be infectious to humans or animals? ☐ YES ☐ NO

4.3.3 Will this be expected to increase the containment level required? ☐ YES ☐ NO

5.0 Will genetic sequences from the following be involved?

- HIV ☒ NO ☐ YES, specify
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens ☒ NO ☐ YES, specify
- SV 40 Large T antigen ☒ NO ☐ YES
- E1A oncogene ☒ NO ☐ YES
- Known oncogenes ☒ NO ☐ YES, specify
- Other human or animal pathogen and or their toxins ☒ NO ☐ YES, specify

5.1 Is any work being conducted with prions or prion sequences? ☒ NO ☐ YES

Additional Comments: _____

6.0 Human Gene Therapy Trials

6.1 Will human clinical trials be conducted involving a biological agent? ☐ YES ☒ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

6.2 If YES, please specify which biological agent will be used:
Please attach a full description of the biological agent.

6.3 Will the biological agent be able to replicate in the host? ☐ YES ☐ NO

6.4 How will the biological agent be administered?

6.5 Please give the Health Care Facility where the clinical trial will be conducted:

6.6 Has human ethics approval been obtained? ☐ YES, number: ☐ NO ☐ PENDING

7.0 Animal Experiments

7.1 Will live animals be used? ☒ YES ☐ NO If **NO**, please proceed to section 7.0

7.2 Name of animal species to be used **Mus musculus**

7.3 AUS protocol # **2009-063**

7.4 Will any of the agents listed in section 4.0 be used in live animals
☐ NO ☒ YES, specify: **Mice will be given 0.5-1.0X10^6 PMSCs in 1ml (IP injection) to treat induced inflammatory bowel disease (3% dextran sodium sulfate in drinking water *ad libitum* for 5 days)**

7.5 Will the agent(s) be shed by the animal:
☒ YES ☐ NO, please justify:

8.0 Use of Animal species with Zoonotic Hazards

8.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? ☐ YES ☒ NO - If **NO**, please proceed to section 9.0

8.2 Will live animals be used? ☐ YES ☐ NO

8.3 If **YES**, please specify the animal(s) used:

♦ Pound source dogs	☐ YES	☐ NO
♦ Pound source cats	☐ YES	☐ NO
♦ Cattle, sheep or goats	☐ YES, species	☐ NO
♦ Non-human primates	☐ YES, species	☐ NO
♦ Wild caught animals	☐ YES, species & colony #	☐ NO
♦ Birds	☐ YES, species	☐ NO
♦ Others (wild or domestic)	☐ YES, specify	☐ NO

8.4 If no live animals are used, please specify the source of the specimens:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 11.0

11.2 If YES, please give the name of the species.

11.3 What is the origin of the plant?

11.4 What is the form of the plant (seed, seedling, plant, tree...)?

11.5 What is your intention? ☐ Grow and maintain a crop ☐ "One-time" use

11.6 Do you do any modifications to the plant? ☐ YES ☐ NO
If yes, please describe:

11.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:

11.8 Is the CFIA permit attached? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

12.0 Import Requirements

12.1 Will any of the above agents be imported? ☐ YES, country of origin ☒ NO
If **NO**, please proceed to Section 12.0

12.2 Has an Import Permit been obtained from HC for human pathogens? ☐ YES ☐ NO

12.3 Has an import permit been obtained from CFIA for animal or plant pathogens? ☐ YES ☐ NO

12.4 Has the import permit been sent to OHS? ☐ YES, please provide permit # ☐ NO

13.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name Dr.Victor Han ***Date:*** Aug 31/11

14.0 Containment Levels

14.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. ☐ 1 ☒ 2 ☐ 2+ ☐ 3

14.2 Has the facility been certified by OHS for this level of containment?
☒ YES, location and date of most recent biosafety inspection: **Apr 5/2011**
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **BIO-LHRI-0034**

15.0 Procedures to be Followed

15.1 Are additional risk reduction measures necessary beyond containment level 1, 2, 2+ or 3 measures that are unique to these agents? ☐ YES ☒ NO
If **YES** please describe:

15.2 Please outline what will be done if there is an exposure to the biological agents listed such as a needlestick injury or an accidental splash:
SOP for injuries—allow injury to bleed out for 10 minutes and then scrub gently with soap and water. Escort to ER/call EMS if injury severe. Report injury to supervisor(s) and follow up with OHSS
SOP for splashes—rinse affected areas for 10 minutes using appropriate device (tap, eyewash, shower). Escort to ER/call EMS if injury severe. Report incident to supervisor(s) and follow up with OHSS

15.3 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.shs.uwo.ca/workplace/newposition.htm

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Dr. Victor Han**____ ***Date:*** Aug 31/11

15.4 Additional Comments: _____

16.0 Approvals

1) UWO Biohazards Subcommittee:
SIGNATURE: _____
Date: _____

2) Safety Officer for the University of Western Ontario
SIGNATURE: _____
Date: _____

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: _____
Date: SEPTEMBER 16, 2011

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

Product Information Sheet for ATCC®CRL-1730™

Cell line info

Cell Line Designation: HUV-EC-C
ATCC Catalog No. CRL-1730™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: umbilical vein; vascular endothelium
Morphology: endothelial
Growth Properties: adherent
Tumorigenic: the cells were not tumorigenic in immunosuppressed mice, but did form colonies in semisolid medium.
DNA profile (STR analysis):

Amelogenin: X
CSF1PO: 11,12
D13S317: 9,11
D16S539: 11,12
D5S818: 11,12
D7S820: 8,12
TH01: 6,9.3
TPOX: 8,11
vWA: 16

Products: factor VIII
Depositors: H. Hoshi
Comments: Endothelial Cell Growth Supplement (ECGS) and unidentified factors from bovine pituitary, hypothalamus or whole brain extracts are mitogenic for this line.
The cells have a life expectancy of 50 to 60 population doublings.
Karyotype: Karyology performed for one batch of CRL-1730™ .in 1996 reflected a hypodiploid human cell line with a modal chromosome number of 45 occurring in 72% of the cells counted, all of which had monosomic N13. The rate of polyploid cells among this population was 15.8%. This karyology differed from earlier work-ups performed on the cells that showed approximately 60% of the cells retained 2 chromosomes 13. The apparent clonal variation in cultures of CRL-1730 (most likely dependent upon passage and growth conditions) has also been noted in STR profiles with unstable alleles at D13S317 allele #9, D13S317 allele #11, and D7S820 allele #12. Other coexisting subclones include those with 46,XX,-11,-13,i(11p),i(11q) and 46,XX,+11,-13 karyotypes. For all karyotypes performed, both X chromosomes appear normal.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 5th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 2007. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Use Restrictions

These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of contaminated cell lines.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium. and spin at approximately 125 xg for 5 to7 minutes.

American Type Culture Collection
P.O. Box 1549
Manassas, VA 20108 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
Or contact your local distributor.

4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio). and dispense into

5. a new culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6). pH (7.0 to 7.6).*

6. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all of the growth medium except for approximately 5 to 10 ml to cover the floor of the flask. The old medium can be saved for reuse. Incubate the cells at 37°C in a 5% carbon dioxide gas phase until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes to spin down the suspended cells into a soft pellet. Remove all but 5 ml of supernatant medium, then resuspend the cells in the remaining medium and add back to a 25 cm^2 flask. The old medium can be saved for reuse. Incubate at 37°C in a 5% carbon dioxide gas phase until they are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum, which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels.
Subcultivation ratio : 1:2 to 1:3.

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in **Culture Of Animal Cells: A Manual Of Basic Technique** by R. Ian Freshney, 5th edition, published by Wiley-Liss, N.Y., 2005.

Medium Renewal

Two to three times weekly.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated of F-12K Medium, Catalog No. 30-2004.
To make the complete growth medium, add the following components to the base medium:

- 0.1mg/ml heparin
- 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS)
- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% carbon dioxide gas phase.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020.
Note: A high quality ECGS prepared from bovine neural tissue (Sigma Cat # E-2759 or equivalent) should be used to propagate CRL-1730™. It is best to initiate the cells with the highest recommended concentration of ECGS.

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Technical Information site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references may be available in the catalog at www.atcc.org)

Hoshi H. And McKeehan W.L. (1984), **Brain- and liver cell-derived factors are required for growth of human endothelial cells in serum-free culture.** Proc. Natl. Acad. Sci. USA 81:6413-6417. PubMed: 6333682.

Zahedi K. (1997), **Characterization of the binding of serum amyloid P to laminin**. J. Biol. Chem. 272:2143-2148. PubMed: 97152982.

Soker S. et al. (1996), **Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-endoded domain**. J. Biol. Chem. 271:5761-5767. PubMed: 96215040.

Hay, R. J., Caputo, J. L., and Macy, M. L., Eds. (1992), **ATCC Quality Control Methods for Cell Lines**. 2nd edition, Published by ATCC.

Caputo, J. L., **Biosafety procedures in cell culture**. J. Tissue Culture Methods **11**:223-227, 1988.

Fleming, D.O., Richardson, J. H., Tulis, J.J. and Vesley, D., (1995) **Laboratory Safety: Principles and Practice**. Second edition, ASM press, Washington, DC.

ATCC Warranty

The viability of ATCC products is warranted for 30 days from the date of shipment. If you feel there is a problem with this product, contact Technical Services by phone at 800-638-6597 (U.S., Canada, and Puerto Rico) or 703-365-2700 (elsewhere) or by e-mail at tech@atcc.org.

Disclaimers

This product is intended for laboratory research purposes only. It is not intended for use in humans.

While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.

This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.

Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

Cell Line Designation: Hep G2
ATCC Catalog No. HB-8065™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: liver; hepatocellular carcinoma
Age: 15 years
Gender: male
Ethnicity: caucasian
Morphology: epithelial
Growth properties: adherent
Tumorigenic: the cells were not tumorigenic in immunosuppressed mice, but did form colonies in semisolid medium.
Cellular Products: alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen;
complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein)
Receptors expressed: insulin; insulin-like growth factor II (IGF II)
Depositors: Wistar Institute
Comments: The cells express 3-hydroxy-3-methylglutaryl-CoA reductase and hepatic triglyceride lipase activities. The cells demonstrate decreased expression of apoA-I mRNA and increased expression of catalase mRNA in response to gramoxone (oxidative stress). There is no evidence of a Hepatitis B virus genome in this cell line
Karyotype: Modal number = 55 (range = 50 to 60); has a rearranged chromosome 1.
Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.
Purified DNA from this line is available as ATCC HB-8065D™ (10µg)

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Use Restrictions

These cells are distributed for research purposes only. Cell lines and hybridomas deposited for patent purposes are not always screened for contamination, antibody production or characterized by the ATCC. Release of a culture, the use of which may be claimed in a patent, from the ATCC during the effective term of any such patent is not meant to carry with it, and does not grant any license, express or implied, under any patent, or the right to use a culture in any process described in a patent.
The above culture was deposited in the ATCC in connection with a **patent** application. Copies of U.S. Patents may be obtained from the Commissioner of Patents, U.S. Patent and Trademark Office, Box 9, Washington, D.C. 20231.
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Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium. and spin at approximately 125 xg for 5 to7 minutes.

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Or contact your local distributor.

4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio). and dispense into a 25 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6). pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum, which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels.
Subcultivation Ratio: 1:4 to 1:6.

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in **Culture Of Animal Cells: A Manual Of Basic Technique** by R. Ian Freshney, 5th edition, published by Wiley-Liss, N.Y., 2005.

Medium Renewal

Fluid change twice weekly

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020 and ATCC Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

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(additional references are available in the catalog at www.atcc.org)

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

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Product Information Sheet for ATCC® CRL-1573™

Cell Line Designation: 293 (HEK293)
ATCC® Catalog No. CRL-1573™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: kidney; transformed with adenovirus 5 DNA
Age: fetus
Morphology: epithelial
Growth properties: adherent
Doubling time: about 19 hours
Tumorigenic: tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with 10(7) cells.
Receptors expressed: vitronectin
Virus susceptibility: human adenoviruses
DNA profile (STR analysis)

Amelogenin: X
CSF1PO: 11,12
D13S317: 12,14
D16S539: 9,13
D5S818: 8,9
D7S820: 11,12
TH01: 7,9.3
TPOX: 11
vWA: 16,19

Depositors: F.L. Graham
Comments: Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome, it is now clear that only left end sequences are present. The line is excellent for titrating human adenoviruses.
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached. The cells will re-attach to the flask over a period of several days in culture at 37C.
The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit.
The Ad5 insert was cloned and sequenced, and it was determined that a colinear seqment from nts 1 to 4344 is integrated into chromosome 19 (19q13.2).

Karyotype: This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %.
The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired.
There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.
Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.
Purified DNA: from this line is available as ATCC Catalog No. CRL-1573D™ (10 µg).

Biosafety Level: 2

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 5th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 2007. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions

These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a **37°C** water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the

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contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete growth medium and spin at approximately 125 xg for 5 to7 minutes.

4. Resuspend cell pellet with the recommended complete growth medium (see the specific batch information for the culture recommended dilution ratio) and dispense into a 25 cm^2 or a 75 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended ifusing the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached. The cells will re-attach to the flask over a period of several days in culture at 37°C.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm^2 flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Add 2.0 to 3.0 ml of 0.25% (w/v) Trypsin-0.53mM EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 10 minutes).

   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

3. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

4. Add appropriate aliquots of the cell suspension to new culture vessels. An inoculum of 2 X 10^3 to 6 X 10^3 viable cells/cm^2 is recommended.
   Subcultivation Ratio: 1:6 to 1:10 weekly

5. Incubate cultures at 37°C.

6. Subculture when cell concentration is between 6 and 7 X 10^4 cells/cm^2.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in **Culture Of Animal Cells: A Manual Of Basic Technique** by R. Ian Freshney, 5th edition, published by Wiley-Liss, N.Y., 2005.

Medium Renewal

Two to three times weekly

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020 (500ml) and ATCC Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO.
Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

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(additional references may be available in the catalog description at www.atcc.org)

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06/11

Cell Line Designation: CHO-K1
ATCC® Catalog No. CCL-61

Table of Contents:

Cell Line Description

Organism: *Cricetulus griseus* (hamster, Chinese)
Tissue: ovary
Gender: female
Morphology: epithelial-like
Growth properties: adherent
VirusSuscept: vesicular stomatitis (Indiana); Getah virus
VirusResist: poliovirus 2; modoc virus; Button Willow virus
Reverse Transcriptase: negative
Karyotype: Chromosome Frequency Distribution 50 Cells: 2n = 22. Stemline number is hypodiploid.
Depositors: T.T. Puck
Comments: The CHO-K1 cells were derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster by T.T. Puck in 1957.The cells require proline in the medium for growth.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions

These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium. and spin at approximately 125 xg for 5 to7 minutes.

4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio). and dispense into a 25 cm^2 or a 75 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with

phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension to new culture vessels.
Subcultivation Ratio: 1:4 to 1:8.

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in ***Culture of Animal Cells: A Manual of Basic Technique*** by R. Ian Freshney, 5th edition, published by Wiley - Liss, N.Y., 2005.

Medium Renewal

Once or twice between subculture.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004.
To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020.

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO.

Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

References

(additional references are available in the catalog at www.atcc.org)

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The viability of ATCC products is warranted for 30 days from the date of shipment. If you feel there is a problem with this product, contact Technical Services by phone at 800-638-6597 (U.S., Canada, and Puerto Rico) or 703-365-2700 (elsewhere) or by e-mail at tech@atcc.org.

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Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org.

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ATCC®

Product Information Sheet for ATCC CRL-1658™

Cell Line Designation: NIH/ 3T3
ATCC Catalog No. CRL-1658™

Table of Contents:

Cell Line Description

Organism: *Mus musculus* (mouse)
Strain: NIH/Swiss
Tissue: embryo
Morphology: fibroblast
Growth properties: adherent
VirusSuscept: murine sarcoma viruses; murine leukemia viruses
Depositors: S.A. Aaronson
Comments: The NIH/3T3, a continuous cell line of highly contact-inhibited cells was established from NIH Swiss mouse embryo cultures in the same manner as the original random bred 3T3 (ATCC CCL-92™) and the inbred BALB/c 3T3 (ATCC CCL-163™). The established NIH/3T3 line was subjected to more than 5 serial cycles of subcloning in order to develop a subclone with morphologic characteristics best suited for transformation assays. These cells are useful for DNA transfection and transformation studies.
Tested and found negative for ectromelia virus (mousepox).

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Use Restrictions

These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete growth medium and spin at approximately 125 xg for 5 to 7 minutes.

4. Resuspend cell pellet with the recommended complete growth medium (see the specific batch information for the culture recommended dilution ratio) and dispense into a 25 cm^2 or a 75 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product.

Handling Procedure For Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes

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handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Never allow the culture to become completely confluent. Subculture at 80% confluency or less.
Volumes used in this protocol are for 75 cm2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 10 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell supension to new culture vessels. Use 3-5 x 10(3) cells/cm^2 and subculture about every 3 days.

Note: In order to maintain this property of **high contact inhibition** it is necessary to transfer routinely at only high dilutions, otherwise variants tend to be selected having reduced contact inhibition. Such low density make culture vessels appear sparse and cell growth sensitive to sub-optimal temperature and media conditions.

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 10 in ***Culture of Animal Cells, a manual of Basic Technique*** by R. Ian Freshney, 3rd edition, published by Alan R. Liss, N.Y., 1994.

Medium Renewal

Two times per week.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium:

- bovine calf serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere. (Standard DMEM formulations contain 3.7 g/L sodium bicarbonate and a 10% CO_2 in air atmosphere is then recommended).
The calf serum initially employed and found to be satisfactory was from the Colorado Serum Co. Denver.

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO.
Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

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E-coli

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Office of Biohazard Containment and Safety
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October 20th, 2009

Ms. Shamila Survery / Mr. Michael Decosimo
Cedarlane Laboratories Ltd
4410 Paletta Court
Burlington, Ontario L7L 5R2

By Facsimile: (289) 288-0020

SUBJECT: Importation of *Escherichia coli* strains

Dear Ms. Survery / Mr. Decosimo:

Our office received your query about the importation of *Escherichia coli* from the American Type Culture Collection (ATCC) located in Manassas, Virginia, United States. The following *Escherichia coli* strains are consider to be level 1 animal pathogens:

- 5K
- 58
- 58-161
- 679
- 1532
- AB284
- AB311
- AB1157
- AB1206
- AG1
- B
- BB4
- BD792
- BL21
- BL21 (DE3)
- BM25.8
- C
- C-1a
- C-3000
- C25
- C41 (DE3)
- C43 (DE3)
- C600
- Cavalli Hfr
- CIE85
- DH1
- DH10 GOLD
- DH10B
- DH5
- DH5-alpha
- DP50
- DY145
- DY380
- E11
- EJ183
- EL250
- EMG2
- EPI 300
- EZ10
- FDA Seattle 1946
- Fusion-Blue
- H1443
- HF4714
- HB101
- HS(PFAMP)R
- Hfr3000
- Hfr3000 X74
- HMS174
- J52
- J53
- JC3272
- JC7661
- JC9387
- JF1504
- JF1508
- JF1509
- JJ055
- JM83
- JM101
- JM109
- K12
- KC8
- KA802
- KAM32
- KAM33
- KAM43
- LE450
- LE451
- LE452
- MB408
- MBX1928
- MC1061
- MC4100 (MuLac)
- MG1655
- MM294
- MS101
- NC-7
- Nissle 1917
- One Shot STBL3
- OP50
- P678
- PA309
- PK-5
- PMC103
- PR13
- Rri
- RV308
- S17-1λ -PIR
- SCS1
- SMR10
- SOLR
- SuperchargeEZ10
- SURE
- TOP10
- TG1
- U5/41
- W208
- W945
- W1485
- W3104
- W3110
- WA704
- WP2
- X1854
- X2160T
- X2541
- X2547T
- XL1-BLUE
- XL1-BLUE-MRF
- XLOLR
- Y10
- Y1090 (1090)
- YN2980
- W3110
- WG1
- WG439
- WG443
- WG445

The Office of Biohazard Containment and Safety (BCS) of the Canadian Food Inspection Agency (CFIA) only issues import permits for microorganisms that are pathogenic to animals, or parts of microorganisms that are pathogenic to animals. As the products listed above are not considered pathogenic to animals, the Office of BCS does not have any regulatory requirements for their importation.

Please note that other legislation may apply. You may wish to contact the Public Health Agency of Canada's (PHAC) Office of Laboratory Security at (613) 957-1779.

Note: Microorganisms pathogenic to animals and veterinary biologics require an import permit from the CFIA.

Sincerely,

Cinthia Labrie
Head, Animal Pathogen Importation Program
Office of Biohazard Containment & Safety

Canada

pGEM®-3Z Vector

Promega

Technical Bulletin No. 033

INSTRUCTIONS FOR USE OF PRODUCT P2151. *PLEASE DISCARD PREVIOUS VERSIONS.*
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I. Description

The pGEM®-3Z Vector[a] is intended for use as a standard cloning vector, as well as for the highly efficient synthesis of RNA in vitro. The vector carries the *lac*Z α-peptide and multiple cloning region arrangement from pUC18 (1). In addition, the vector contains both the SP6 and T7 RNA polymerase promoters flanking the multiple cloning region. This arrangement gives rise to a functional α-peptide that is capable of complementing the product of the *lac*ZΔM15 gene to produce functional β-galactosidase. Cells with the genotype, *lac*ZΔM15, and also containing the pGEM®-3Z Vector will be blue in color when plated on indicator media containing IPTG and X-Gal. However, when the *lac*Z α-peptide is disrupted by cloning into the pGEM®-3Z multiple cloning region, complementation does not occur and no β-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM®-3Z Vector constructs remain white.

The sequences of Promega vectors are available online at: **www.promega.com/vectors/** and are also available from the GenBank® database.

II. Product Components

Product	Size	Cat.#
pGEM®-3Z Vector	20µg	P2151

The pGEM®-3Z Vector is provided with a glycerol stock of bacterial strain JM109. The JM109 cells do not contain vector and are not competent.

Storage Conditions: Store the pGEM®-3Z Vector at –20°C and the glycerol stock of JM109 cells at –70°C.

AF91TB033 0404TB033

III. pGEM®-3Z Vector Multiple Cloning Region and Circle Map

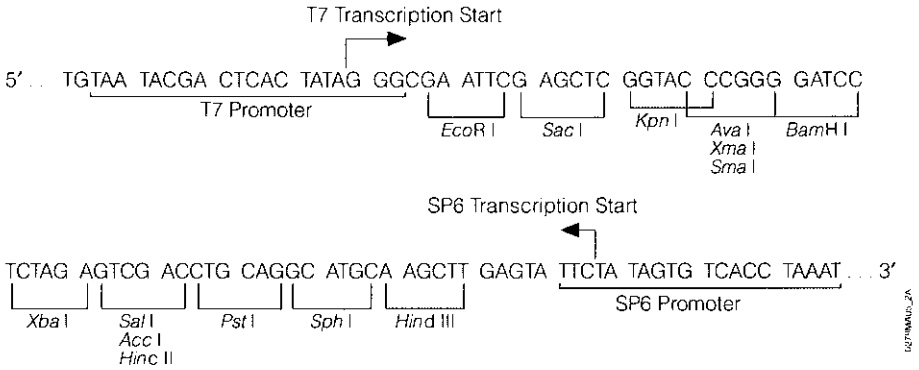

Figure 1. pGEM®-3Z Vector promoter and multiple cloning region sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

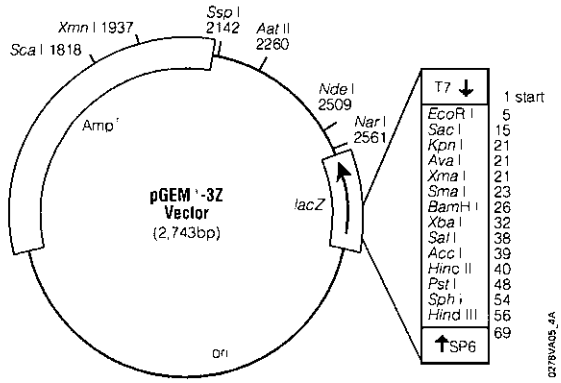

Figure 2. pGEM®-3Z Vector circle map and sequence reference points.

pGEM®-3Z Vector sequence reference points.

T7 RNA polymerase transcription initiation site	1
multiple cloning region	5–61
SP6 RNA polymerase promoter (–17 to +3)	67–86
SP6 RNA polymerase transcription initiation site	69
lac operon sequences	94–323; 2564–2724
binding site of pUC/M13 Reverse Sequencing Primer	104–125
lacZ start codon	108
lacZ operator	128–144
β-lactamase (Amp^r) coding region	1265–2125
binding site of pUC/M13 Forward Sequencing Primer	2677–2700
T7 RNA polymerase promoter (–17 to +3)	2727–3

Specialized applications of the pGEM®-3Z Vector.

- Blue/white screening for recombinants.
- Transcription in vitro from dual-opposed promoters. (For protocol information, please request Promega's *Riboprobe® in vitro Transcription Systems*[b,c] *Technical Manual*, #TM016.)

Note: The pGEM®-3Z and pGEM®-4Z Vectors are identical except for the orientation of the SP6 and T7 promoters.

Note: All Promega technical literature is available on the Internet at: **www.promega.com**

IV. pGEM®-3Z Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3′ end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. The vector sequence is available in the GenBank® database (GenBank®/EMBL Accession Number X65304) and on the Internet at: **www.promega.com/vectors/**

Table 1. Restriction Enzymes That Cut the pGEM®-3Z Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
***Aat* II**	1	2260
***Acc* I**	1	39
***Acc*65 I**	1	17
Acy I	3	1875, 2257, 2561
Afl III	1	445
***Alw*26 I**	4	1399, 2175, 2328, 2370
***Alw*44 I**	3	759, 2005, 2502
*Alw*N I	1	861
*Asp*H I	5	15, 763, 1924, 2009, 2506
***Ava* I**	1	21
***Ava* II**	2	1476, 1698
***Bam*H I**	1	26
***Ban* I**	4	17, 189, 1286, 2560
***Ban* II**	1	15
Bbe I	1	2564
***Bbu* I**	1	54
***Bgl* I**	2	1458, 2576
Bsa I	1	1399
*Bsa*O I	5	361, 785, 1708, 1857, 2604
*Bsa*H I	3	1875, 2257, 2561
*Bsa*J I	5	21, 22, 184, 605, 2679
***Bsp*1286 I**	5	15, 763, 1924, 2009, 2506
*Bsp*H I	3	1165, 2173, 2278
*Bsp*M I	1	51
*Bss*S I	3	618, 2002, 2309
***Bst*O I**	5	185, 473, 594, 607, 2680
*Cfr*10 I	1	1418
***Dra* I**	3	1204, 1223, 1915
Dra II	1	2314
Drd I	2	553, 2422
Eae I	3	284, 1726, 2713
Ear I	3	329, 2133, 2621
***Ecl*HK I**	1	1338
***Eco*ICR I**	1	13
***Eco*R I**	1	5

Enzyme	# of Sites	Location
Ehe I	1	2562
***Fok* I**	5	1304, 1485, 1772, 2415, 2659
Fsp I	2	1560, 2583
***Hae* II**	3	323, 693, 2564
Hga I	4	556, 1134, 1864, 2422
***Hinc* II**	1	40
Hind II	1	40
***Hind* III**	1	56
***Hsp*92 I**	3	1875, 2257, 2561
Kas I	1	2560
***Kpn* I**[d]	1	21
Mae I	4	33, 940, 1193, 1528
Mae II	5	1148, 1564, 1937, 2257, 2699
***Nar* I**	1	2561
***Nde* I**	1	2509
Nsp I	3	54, 449, 2366
Ple I	5	44, 339, 824, 1327, 2727
*Psp*A I	1	21
***Pst* I**	1	48
***Pvu* I**	2	1708, 2604
***Pvu* II**	2	269, 2633
***Rsa* I**	3	19, 1818, 2494
***Sac* I**	1	15
***Sal* I**	1	38
***Sca* I**	1	1818
***Sin* I**	2	1476, 1698
***Sma* I**	1	23
***Sph* I**	1	54
*Sse*8387 I	1	48
***Ssp* I**	1	2142
***Taq* I**	4	9, 39, 545, 1989
Tfi I	2	280, 420
***Vsp* I**	3	216, 275, 1510
***Xba* I**	1	32
***Xma* I**	1	21
***Xmn* I**	1	1937

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Table 2. Restriction Enzymes That Do Not Cut the pGEM®-3Z Vector.

***Acc*B7 I**	*Bpu*1102 I	***Bsu*36 I**	***Eco*47 III**	***Hpa* I**	***Not* I**
***Acc* III**	*Bsa*A I				
Afl II	*Bsa*B I	***Cla* I**	***Eco*52 I**	**I-*Ppo* I**	***Nru* I**
***Age* I**	***Bsa*M I**	***Csp* I**	*Eco*72 I	***Mlu* I**	***Nsi* I**
***Apa* I**	*Bsm* I				
Asc I	*Bsp*120 I	***Csp*45 I**	*Eco*81 I	***Nae* I**	*Pac* I
Avr II	*Bsr*G I				
***Bal* I**	***Bss*H II**	*Dra* III	*Eco*N I	***Nco* I**	*Pae*R7 I
*Bbr*P I	*Bst*1107 I				
Bbs I	***Bst*98 I**	*Dsa* I	***Eco*R V**	***Ngo*M IV**	*Pfl*M I
***Bcl* I**	***Bst*E II**	*Eag* I	*Fse* I	***Nhe* I**	*Pin*A I
***Bgl* II**	***Bst*X I**				
Blp I	***Bst*Z I**				

Table 3. Restriction Enzymes That Cut the pGEM®-3Z Vector 6 or More Times.

Aci I	***Cfo* I**	***Hinf* I**	*Mnl* I	*Nla* IV
***Alu* I**	***Dde* I**	***Hpa* II**	*Mse* I	***Sau*3A I**
Bbv I	***Dpn* I**	*Hph* I	***Msp* I**	*Sau*96 I
Bsr I	*Dpn* II	***Hsp*92 II**	***Msp*A1 I**	*Scr*F I
***Bsr*S I**	*Fnu*4H I	*Mae* III	***Nci* I**	*Sfa*N I
*Bst*71 I	***Hae* III**	***Mbo* I**	***Nde* II**	***Tru*9 I**
*Bst*U I	***Hha* I**	***Mbo* II**	*Nla* III	***Xho* II**

Note: The enzymes listed in boldface are available from Promega.

V. Related Products

Product	Size	Cat.#
pGEM®-4Z Vector[a]	20µg	P2161
pGEM®-3Zf(+) Vector[a]	20µg	P2271
pGEM®-3Zf(–) Vector[a]	20µg	P2261
pGEM®-5Zf(+) Vector[a]	20µg	P2241
pGEM®-5Zf(–) Vector[a]	20µg	P2351
pGEM®-7Zf(+) Vector[a]	20µg	P2251
pGEM®-7Zf(–) Vector[a]	20µg	P2371
pGEM®-9Zf(–) Vector[a]	20µg	P2391
pGEM®-11Zf(+) Vector[a]	20µg	P2411
pGEM®-11Zf(–) Vector[a]	20µg	P2421
pGEM®-13Zf(+) Vector[a]	20µg	P2541

All pGEM® Vectors are provided with a glycerol stock of bacterial strain JM109. The JM109 cells do not contain vector and are not competent.

Product	Size	Cat.#
pSP64 Poly(A) Vector	20µg	P1241
pSP72 Vector[a]	20µg	P2191
pSP73 Vector[a]	20µg	P2221

Sequencing Primers

Product	Size	Cat.#
SP6 Promoter Primer	2µg	Q5011
T7 Promoter Primer	2µg	Q5021
pUC/M13 Primer, Reverse (17mer)	2µg	Q5401
pUC/M13 Primer, Forward (17mer)	2µg	Q5391
pUC/M13 Primer, Forward (24mer)	2µg	Q5601
pUC/M13 Primer, Reverse (22mer)	2µg	Q5421

Riboprobe® in vitro Transcription Systems

Product	Cat.#
Riboprobe® System - SP6[b,c]	P1420
Riboprobe® System - T7[b,c]	P1440

For Laboratory Use.

RiboMAX™ Large Scale RNA Production Systems

Product	Cat.#
RiboMAX™ Large Scale RNA Production System - SP6[b,c,f]	P1280
RiboMAX™ Large Scale RNA Production System - T7[b,c,f,g]	P1300

For Laboratory Use.

VI. Reference

1. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–19.

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[a]U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

[b]U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803 and Japanese Pat. Nos. 3009458 and 3366596 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor.

[c]U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[d]NOTICE TO PURCHASER: LIMITED USE LICENSE
This product is sold under licensing arrangements between Promega Corporation and Invitrogen Corporation. The purchase price of this product includes limited, nontransferable rights under U.S. Pat. Nos. 5,082,784 and 5,192,675 owned by Invitrogen Corporation to use the product only for the internal research purposes of the purchaser. For information on purchasing a license to use the purchased product for purposes other than the internal research of the purchaser, contact the Director of Licensing, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008.

[e]U.S. Pat. No. 5,391,487 has been issued to Promega Corporation for Restriction Endonuclease *Sgf* I.

[f]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

[g]The RiboMAX™ Large Scale RNA Production System—T7 (Cat.# P1300) is covered by U.S. Pat. Nos. 5,256,555, 6,586,218, 6,586,219 and other pending patents and is sold under a license from Ambion, Inc. It is intended for research use only. Parties wishing to use this product for other applications should contact Ambion, Inc.

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All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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ISO 9001 Certified

pGEM®-4Z Vector

Promega

Technical Bulletin No. 036

INSTRUCTIONS FOR USE OF PRODUCT P2161. *PLEASE DISCARD PREVIOUS VERSIONS.*
All technical literature is available on the Internet at www.promega.com
Please visit the web site to verify that you are using the most current version of this Technical Bulletin.

I. Description

The pGEM®-4Z Vector[a] is intended for use as a standard cloning vector, as well as for the highly efficient synthesis of RNA in vitro. The vector carries the *lac*Z α-peptide and multiple cloning region arrangement from pUC18 (1). In addition, the vector contains both the SP6 and T7 RNA polymerase promoters flanking the multiple cloning region. This arrangement gives rise to a functional α-peptide that is capable of complementing the product of the *lac*ZΔM15 gene to produce functional β-galactosidase. Cells with the genotype, *lac*ZΔM15, and also containing the pGEM®-4Z Vector will be blue in color when plated on indicator media containing IPTG and X-Gal. However, when the *lac*Z α-peptide is disrupted by cloning into the pGEM®-4Z multiple cloning region, complementation does not occur and no β-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM®-4Z Vector constructs remain white.

The sequences of Promega vectors are available online at:
www.promega.com/vectors/ and are also available from the GenBank® database.

II. Product Components

Product	Size	Cat.#
pGEM®-4Z Vector	20µg	P2161

The pGEM®-4Z Vector is provided with a glycerol stock of bacterial strain JM109. The JM109 cells do not contain vector and are not competent.

Storage Conditions: Store the pGEM®-4Z Vector at –20°C and the glycerol stock of JM109 cells at –70°C.

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III. pGEM®-4Z Vector Multiple Cloning Region and Circle Map

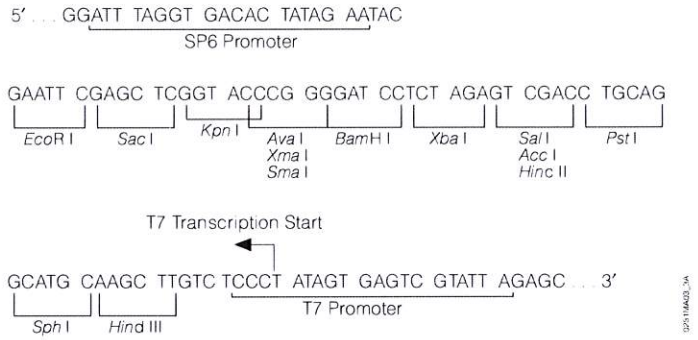

Figure 1. pGEM®-4Z Vector promoter and multiple cloning region sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

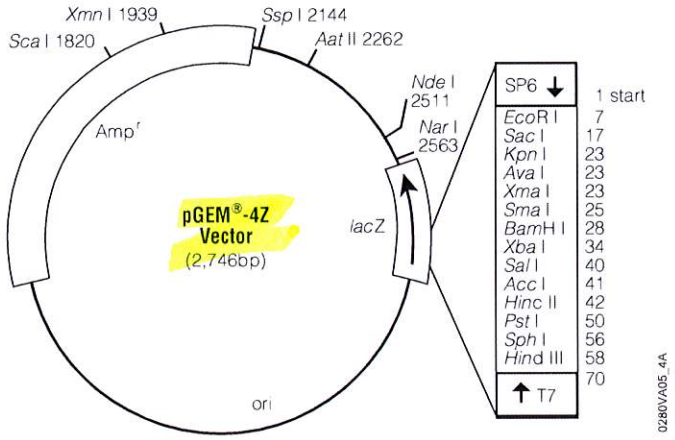

Figure 2. pGEM®-4Z Vector circle map and sequence reference points.

Note: The pGEM®-3Z and pGEM®-4Z Vectors are identical except for the orientation of the SP6 and T7 promoters.

pGEM®-4Z Vector sequence reference points.

SP6 RNA polymerase transcription initiation site	1
multiple cloning region	7–63
T7 RNA polymerase promoter (–17 to +3)	68–87
T7 RNA polymerase transcription initiation site	70
lac operon sequences	96–325; 2566–2726
binding site of pUC/M13 Reverse Sequencing Primer	106–122
lacZ start codon	110
lacZ operator	130–146
β-lactamase (Amp^r) coding region	1267–2127
binding site of pUC/M13 Forward Sequencing Primer	2686–2702
SP6 RNA polymerase promoter (–17 to +3)	2730–3

Specialized applications of the pGEM®-4Z Vector.

- Blue/white screening for recombinants.
- Transcription in vitro from dual-opposed promoters. (For protocol information, please request Promega's *Riboprobe® in vitro Transcription Systems*[b,c] *Technical Manual*, #TM016.)

Note: All Promega technical literature is available on the Internet at: **www.promega.com**

IV. pGEM®-4Z Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3′ end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. The vector sequence is available in the GenBank® database (GenBank®/EMBL Accession Number X65305) and on the Internet at: **www.promega.com/vectors/**

Table 1. Restriction Enzymes That Cut the pGEM®-4Z Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
***Aat* II**	1	2262
***Acc* I**	1	41
***Acc*65 I**	1	19
Acy I	3	1877, 2259, 2563
Afl III	1	447
***Alw*26 I**	5	69, 1401, 2177, 2330, 2372
***Alw*44 I**	3	761, 2007, 2504
*Alw*N I	1	863
*Asp*H I	5	17, 765, 1926, 2011, 2508
***Ava* I**	1	23
***Ava* II**	2	1478, 1700
***Bam*H I**	1	28
***Ban* I**	4	19, 191, 1288, 2562
***Ban* II**	1	17
Bbe I	1	2566
***Bbu* I**	1	56
***Bgl* I**	2	1460, 2578
Bsa I	1	1401
*Bsa*O I	5	363, 787, 1710, 1859, 2606
*Bsa*H I	3	1877, 2259, 2563
*Bsa*J I	5	23, 24, 186, 607, 2681
***Bsp*1286 I**	5	17, 765, 1926, 2011, 2508
*Bsp*H I	3	1167, 2175, 2280
*Bsp*M I	1	53
*Bss*S I	3	620, 2004, 2311
***Bst*O I**	5	187, 475, 596, 609, 2682
***Bst*X I**	1	2725
*Cfr*10 I	1	1420
***Dra* I**	3	1206, 1225, 1917
Dra II	1	2316
Drd I	2	555, 2424
Eae I	3	286, 1728, 2715
Ear I	3	331, 2135, 2623
***Ecl*HK I**	1	1340
***Eco*ICR I**	1	15
***Eco*R I**	1	7
Ehe I	1	2564
***Fok* I**	5	1306, 1487, 1774, 2417, 2661
Fsp I	2	1562, 2585
***Hae* II**	3	325, 695, 2566
Hga I	4	558, 1136, 1866, 2424
***Hinc* II**	1	42
Hind II	1	42
***Hind* III**	1	58
***Hsp*92 I**	3	1877, 2259, 2563
Kas I	1	2562
***Kpn* I**[(d)]	1	23
Mae I	4	35, 942, 1195, 1530
Mae II	5	1150, 1566, 1939, 2259, 2701
***Nar* I**	1	2563
***Nde* I**	1	2511
Nsp I	3	56, 451, 2368
Ple I	5	46, 85, 341, 826, 1329
*Psp*A I	1	23
***Pst* I**	1	50
***Pvu* I**	2	1710, 2606
***Pvu* II**	2	271, 2635
***Rsa* I**	3	21, 1820, 2496
***Sac* I**	1	17
***Sal* I**	1	40
***Sca* I**	1	1820
***Sin* I**	2	1478, 1700
***Sma* I**	1	25
***Sph* I**	1	56
*Sse*8387 I	1	50
***Ssp* I**	1	2144
***Taq* I**	4	11, 41, 547, 1991
Tfi I	2	282, 422
***Vsp* I**	3	218, 277, 1512
***Xba* I**	1	34
***Xma* I**	1	23
***Xmn* I**	1	1939

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Table 2. Restriction Enzymes That Do Not Cut the pGEM®-4Z Vector.

***Acc*B7 I**	*Bpu*1102 I	***Cla* I**	***Hpa* I**	*Pin*A I	***Spe* I**
***Acc* III**	*Bsa*A I	***Csp* I**	**I-*Ppo* I**	*Pme* I	*Spl* I
Afl II	*Bsa*B I	***Csp*45 I**	***Mlu* I**	*Pml* I	*Srf* I
***Age* I**	***Bsa*M I**	*Dra* III	***Nae* I**	*Ppu*10 I	***Stu* I**
***Apa* I**	*Bsm* I	*Dsa* I	***Nco* I**	*Ppu*M I	***Sty* I**
Asc I	*Bsp*120 I	*Eag* I	***Ngo*M IV**	*Psh*A I	*Swa* I
Avr II	*Bsr*G I	***Eco*47 III**	***Nhe* I**	*Psp*5 II	***Tth*111 I**
***Bal* I**	***Bss*H II**	***Eco*52 I**	***Not* I**	*Rsr* II	*Xcm* I
*Bbr*P I	*Bst*1107 I	*Eco*72 I	***Nru* I**	***Sac* II**	***Xho* I**
Bbs I	***Bst*98 I**	*Eco*81 I	***Nsi* I**	***Sfi* I**	
***Bcl* I**	***Bst*E II**	*Eco*N I	*Pac* I	***Sgf* I**(e)	
***Bgl* II**	***Bst*Z I**	***Eco*R V**	*Pae*R7 I	*Sgr*A I	
Blp I	***Bsu*36 I**	*Fse* I	*Pfl*M I	***Sna*B I**	

Table 3. Restriction Enzymes That Cut the pGEM®-4Z Vector 6 or More Times.

Aci I	***Cfo* I**	***Hinf* I**	*Mnl* I	*Nla* IV
***Alu* I**	***Dde* I**	***Hpa* II**	*Mse* I	***Sau*3A I**
Bbv I	***Dpn* I**	*Hph* I	***Msp* I**	*Sau*96 I
Bsr I	*Dpn* II	***Hsp*92 II**	***Msp*A1 I**	*Scr*F I
***Bsr*S I**	*Fnu*4H I	*Mae* III	***Nci* I**	*Sfa*N I
*Bst*71 I	***Hae* III**	***Mbo* I**	***Nde* II**	***Tru*9 I**
*Bst*U I	***Hha* I**	***Mbo* II**	*Nla* III	***Xho* II**

Note: The enzymes listed in boldface are available from Promega.

V. Related Products

Product	Size	Cat.#
pGEM®-3Z Vector[a]	20µg	P2151
pGEM®-3Zf(+) Vector[a]	20µg	P2271
pGEM®-3Zf(–) Vector[a]	20µg	P2261
pGEM®-5Zf(+) Vector[a]	20µg	P2241
pGEM®-5Zf(–) Vector[a]	20µg	P2351
pGEM®-7Zf(+) Vector[a]	20µg	P2251
pGEM®-7Zf(–) Vector[a]	20µg	P2371
pGEM®-9Zf(–) Vector[a]	20µg	P2391
pGEM®-11Zf(+) Vector[a]	20µg	P2411
pGEM®-11Zf(–) Vector[a]	20µg	P2421
pGEM®-13Zf(+) Vector[a]	20µg	P2541

All pGEM® Vectors are provided with a glycerol stock of bacterial strain JM109. The JM109 cells do not contain vector and are not competent.

Product	Size	Cat.#
pSP64 Poly(A) Vector	20µg	P1241
pSP72 Vector[a]	20µg	P2191
pSP73 Vector[a]	20µg	P2221

Sequencing Primers

Product	Size	Cat.#
SP6 Promoter Primer	2µg	Q5011
T7 Promoter Primer	2µg	Q5021
pUC/M13 Primer, Reverse (17mer)	2µg	Q5401
pUC/M13 Primer, Forward (17mer)	2µg	Q5391
pUC/M13 Primer, Forward (24mer)	2µg	Q5601
pUC/M13 Primer, Reverse (22mer)	2µg	Q5421

Riboprobe® in vitro Transcription Systems

Product	Cat.#
Riboprobe® System - SP6[b,c]	P1420
Riboprobe® System - T7[b,c]	P1440

For Laboratory Use.

RiboMAX™ Large Scale RNA Production Systems

Product	Cat.#
RiboMAX™ Large Scale RNA Production System - SP6[b,c,f]	P1280
RiboMAX™ Large Scale RNA Production System - T7[b,c,f,g]	P1300

For Laboratory Use.

VI. Reference

1. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–19.

Promega

[a]U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

[b]U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803 and Japanese Pat. Nos. 3009458 and 3366596 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor.

[c]U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[d]NOTICE TO PURCHASER: LIMITED USE LICENSE
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[e]U.S. Pat. No. 5,391,487 has been issued to Promega Corporation for Restriction Endonuclease *Sgf* I.

[f]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

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Internet www.promega.com
ISO 9001 Certified

pBluescript® II Phagemid Vectors

INSTRUCTION MANUAL

Catalog #212205, #212206, #212207 and #212208

Revision #083001m

For In Vitro Use Only

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This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Stratagene. Stratagene shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

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France	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 919 288	0800 919 287	0800 919 289
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 182 8232	0800 182 8231	0800 182 8234
Netherlands	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 023 0446	+31 (0)20 312 5700	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 563 080	0800 563 082	0800 563 081
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 917 3282	0800 917 3283	0800 917 3281

All Other Countries

Please contact your local distributor. A complete list of distributors is available at *www.stratagene.com*.

pBluescript® II Phagemid Vectors

CONTENTS

pBluescript® II Phagemid Vectors

MATERIALS PROVIDED

Material Provided	Catalog Number			
	#212205	#212206	#212207	#212208
pBluescript® II SK(+) phagemid, 1 µg/µl	20 µg	—	—	—
pBluescript® II SK(–) phagemid, 1 µg/µl	—	20 µg	—	—
pBluescript® II KS(+) phagemid, 1 µg/µl	—	—	20 µg	—
pBluescript® II KS(–) phagemid, 1 µg/µl	—	—	—	20 µg
XL1-Blue MRF′ host strain, glycerol stock, Catalog #200301	1 tube	1 tube	1 tube	1 tube

STORAGE CONDITIONS

Phagemids: –20°C
Bacterial Strains: –80°C

Revision #083001m

INTRODUCTION

The pBluescript® II phagemids (plasmids with a phage origin) are cloning vectors designed to simplify commonly used cloning and sequencing procedures, including the construction of nested deletions for DNA sequencing, generation of RNA transcripts *in vitro* and site-specific mutagenesis and gene mapping. The pBluescript II phagemids have an extensive polylinker with 21 unique restriction enzyme recognition sites. Flanking the polylinker are T7 and T3 RNA polymerase promoters that can be used to synthesize RNA *in vitro*.[1,2] The choice of promoter used to initiate transcription determines which strand of the insert cloned into the polylinker will be transcribed.

Circular maps and lists of features for the pBluescript II phagemids are shown in figures 1 and 2. The polylinker and T7 and T3 RNA polymerase promoter sequences are present in the N-terminal portion of a *lac*Z gene fragment. A total of 131 amino acids of β-galactosidase coding sequence is present in the pBluescript II phagemid, but the coding sequence is interrupted by the large polylinker. (There are 36 amino acids from the initiator Met sequence to the *Eco*R I site.) pBluescript II phagemids having no inserts in the polylinker will produce blue colonies in the appropriate strains of bacteria (i.e., strains containing *lac*Z*ΔM15* on an F′ episome, such as XL1-Blue MRF′, among others). pBluescript II phagemids that have inserts will produce white colonies using the same strain, because the inserts disrupt the coding region of the *lac*Z gene fragment.

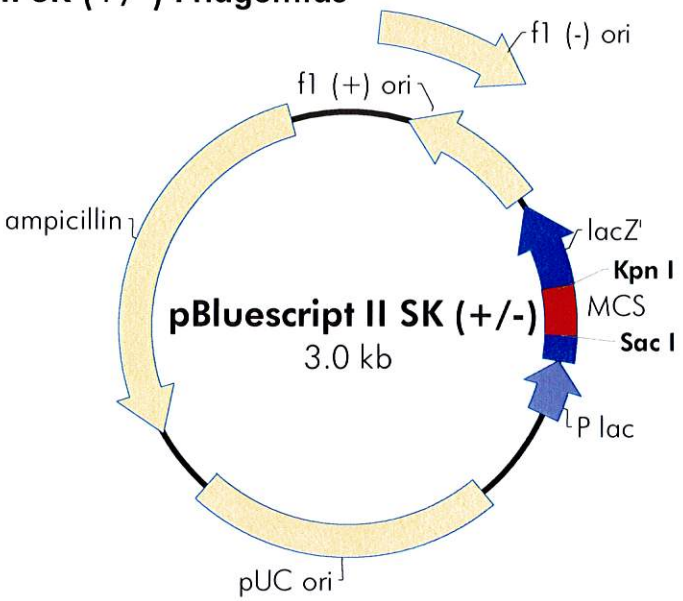
pBluescript II (+) and (–) are available with two polylinker orientations designated as either KS or SK using the following convention: (1) in the KS orientation, the *Kpn* I restriction site is nearest the *lac*Z promoter and the *Sac* I restriction site is farthest from the *lac*Z promoter; and (2) in the SK orientation, the *Sac* I site is the closest restriction site to the *lac*Z promoter and the *Kpn* I site is the farthest.

Flanking the T3 and T7 promoters are *Bss*H II sites. This rare six-base cutter will allow the insert plus the T phage RNA promoters to be excised and used for gene mapping.

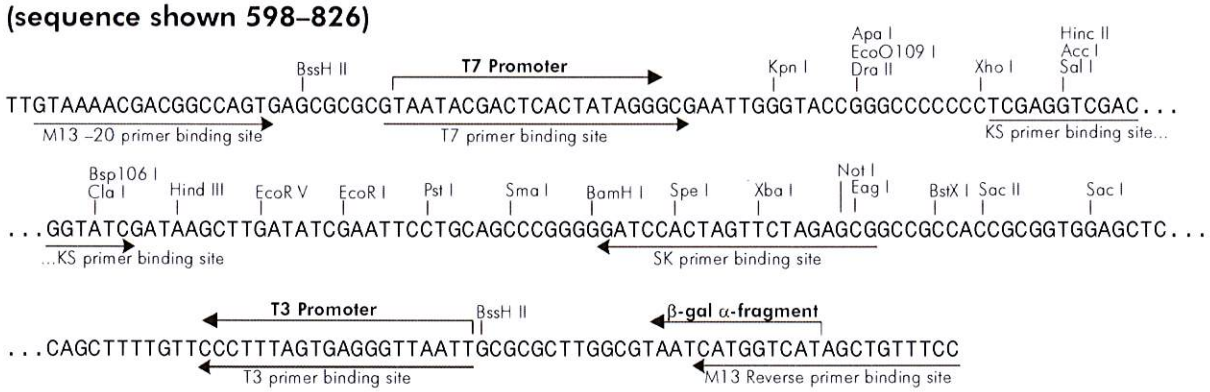
pBluescript II phagemids can be rescued as single-stranded (ss) DNA. pBluescript II phagemids contain a 454-bp filamentous f1 phage intergenic region (M13 related), which includes the 307-bp origin of replication. The (+) and (–) orientations of the f1 intergenic region allow the rescue of sense or antisense ssDNA by a helper phage. This ssDNA can be used for dideoxynucleotide sequencing (Sanger method) or site-specific mutagenesis.

Note *We have discovered that the use of excess amounts of* Eco*R I to digest pBluescript II results in* Eco*R I prime activity. This appears as cleavage at a non-EcoR I site at the 3′ end of the f1 intergenic region, causing confusion when interpreting results from an agarose gel. If a restriction pattern appears incorrect, check whether reducing the units of* Eco*R I restores a normal restriction pattern.*

pBluescript® II SK (+/–) Phagemids



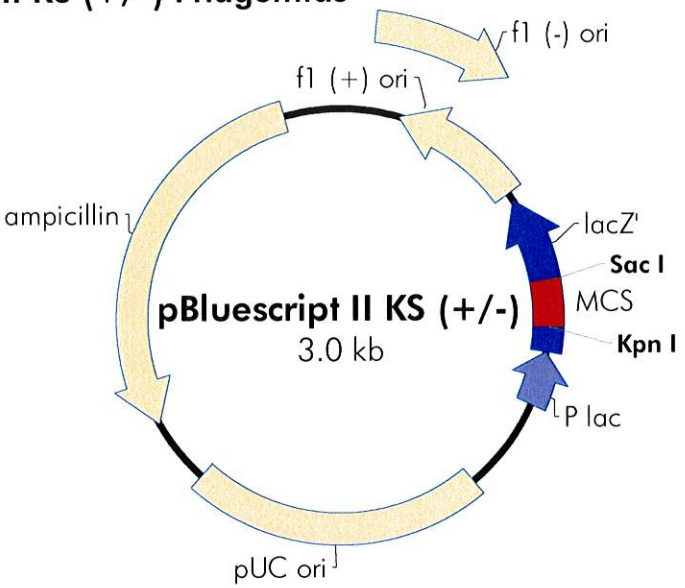
pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



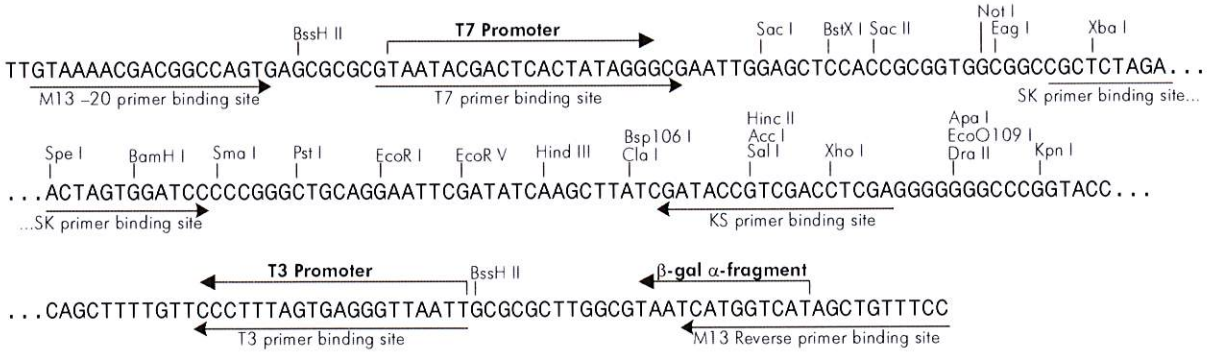
Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript SK (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBluescript SK (–) only]	21–327
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 1 The pBluescript® II SK (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.stratagene.com*. Genbank® #X52328 [SK(+)] and #X52330 [SK(–)].

pBluescript® II KS (+/–) Phagemids



pBluescript II KS (+/–) Multiple Cloning Site Region (sequence shown 598–826)



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lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 2 The pBluescript® II KS (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.stratagene.com*. Genbank® #X52327 [KS(+)] and #X52329 [KS(–)].

LIGATION INTO pBLUESCRIPT® II PHAGEMIDS

Stratagene suggests dephosphorylation of the digested pBluescript II phagemid with calf intestinal alkaline phosphatase (CIAP) prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the DNA on an agarose gel and removing the desired vector band through electroelution, leaving behind the small fragment that appears between the two restriction enzyme sites.

After gel purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer [5 mM Tris (pH 7.5), 0.1 mM EDTA] that will allow the concentration of the vector DNA to be the same as the concentration of the insert DNA(~0.1 µg/µl).

For ligation, the ideal ratio of insert to vector DNA is variable; however, a reasonable starting point is 2:1 (insert:vector), measured in available picomole ends. This is calculated as:

picomole ends/micrograms of DNA = $(2 \times 10^6) \div$ (number of base pairs × 660)

We suggest the following protocol, which includes three controls:

Component	1	2	3	4	5
Prepared vector (0.1 µg/µl)	1 µl	1 µl	1 µl	1 µl	0 µl
Prepared insert (0.1 µg/µl)	X µl	X µl	0 µl	0 µl	1 µl
10 mM rATP (pH 7.0)	1 µl	1 µl	1 µl	1 µl	1 µl
10× Ligase buffer	1 µl	1 µl	1 µl	1 µl	1 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.5 µl	0.5 µl	0 µl	0.5 µl
ddH_2O (to 10 µl)	X µl	X µl	X µl	X µl	X µl

1. Ligate for 2 hours at room temperature (22°C) or overnight at 4°C. When ligating blunt ends, incubate the ligation overnight at 12–14°C.

2. Transform 1–2 µl of the ligation mix into the appropriate competent bacteria. (See *Transformation with pBluescript II Phagemids.*) Plate on selective media.

3. Interpretation of test results:

   Reactions 1 and 2 vary the insert:vector ratio.
   Control 3 tests for the effectiveness of the CIAP treatment.
   Control 4 indicates if the vector was cleaved completely or if residual uncut vector remains.
   Control 5 verifies that the insert alone is not contaminated with any vector DNA.

4. Expected plating results:
   Plates 1 and 2 should have mostly white colonies, representing recombinants.
   Plate 3 should have low numbers of blue colonies if the CIAP treatment was effective.
   Plate 4 should have no colonies if the digest was complete.
   Plate 5 should have no colonies if the insert was pure.

TRANSFORMATION WITH pBLUESCRIPT II® PHAGEMIDS

Note *pBluescript II phagemids will replicate autonomously as plasmids. Therefore, colonies—**not plaques**—are obtained following transformation.*

Suggested Host Strain and Genotype

Stratagene recommends the host strain XL1-Blue MRF′ for propagation of pBluescript II phagemids and for transformation of recombinant phagemids. XL1-Blue MRF′ allows blue-white color selection and single-stranded DNA rescue, and is restriction-deficient aiding in the construction of libraries made from methylated DNA.[3]

XL1-Blue MRF′ Genotype: Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F′ *pro*AB *lac*I^{q}ZΔM15 Tn10 (Tet^r)]

Note *The XL1-Blue MRF′ is provided as a glycerol stock. Additional tubes of glycerol stock can be purchased from Stratagene (Catalog #200301); alternatively, XL1-Blue MRF′ is available from Stratagene as high-efficiency frozen competent cells (>1 × 10^9 colonies/μg of pUC 18, Catalog #200230).*

For the appropriate media and plates for growth of XL1-Blue MRF′, please refer to the following table:

Bacterial strain	Plates for bacterial streak	Media for glycerol stock
XL1-Blue MRF′	LB–tetracycline agar[a]	LB–tetracycline[a]

[a]12.5 μg/ml.

Streaking Cells from a –80°C Bacterial Glycerol Stock

Prepare the following from a frozen glycerol stock:

Note *Do not allow the contents of the vial to thaw. The vials can be stored at –20° or –80°C, but most strains remain viable longer if stored at –80°C.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.
2. Streak the splinters onto an LB plate containing the appropriate antibiotic.

   Restreak the cells fresh each week.

Preparation of a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of the appropriate liquid media with one or two colonies from a plate of freshly-streaked cells. Grow the cells to late log phase.
2. Add 4.5 ml of a sterile glycerol–liquid media solution (prepared by combining 5 ml of glycerol + 5 ml of liquid media) to the bacterial culture from step 1. Mix well.
3. Aliquot into sterile centrifuge tubes (1 ml/ tube). This preparation may be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

Blue-White Color Selection

The XL1-Blue MRF′ strain allows blue–white color selection for pBluescript II phagemids because of *lacZΔM15* complementation on the F′ episome. The color selection may be seen when plating on LB plates containing 100 µg/ml of ampicillin, 80 µg/ml of fresh X-gal, and 20 mM IPTG. Alternatively, plates for color selection can be prepared by spreading 100 µl of 40 mM IPTG and 100 µl of 2% X-gal on LB–ampicillin plates 30 minutes prior to plating your transformants. X-gal should be prepared in dimethyl formamide and IPTG in sterile, distilled H_2O (store stock solutions at –20°C until use). Colonies containing phagemids without inserts will be blue after incubation for 12–18 hours at 37°C. Colonies with phagemids containing inserts will remain white. Further enhancement of the blue color may be obtained by placing plates at 4°C for 2 hours following overnight growth at 37°C.

Occasionally, β-galactosidase fusion proteins are toxic to the host bacteria. If there is any suspicion that an insert might be toxic, the X-gal and IPTG may be left out of the ampicillin plates. Under these conditions there will be no color selection, but recombinants will express lower levels of the potentially toxic proteins.

Background White Colonies

Since the Δ*M15 lac* gene carried on the F′ episome is needed for the blue–white color assay, host bacteria that have lost the F′ episome will remain as white colonies on an X-gal/IPTG agar plate even if the pBluescript II phagemid does not contain an insert. XL1-Blue MRF′ is a *lac*⁻ AG1 derivative with Tn*10*, *lacI*^{q}, and *lacZΔM15* on the F′. Selection for bacteria containing the F′ in this strain is accomplished by plating on 12.5 µg/ml tetracycline instead of minimal media plates. XL1-Blue MRF′ transformants containing pBluescript II phagemids can be plated on tetracycline–ampicillin plates to select for colonies that contain both the F′ and the pBluescript II phagemid. This advantage further reduces the background of false positives.

For bacteria containing an F′ without a Tn*10* gene, growth on a minimal medium plate supplemented with 1 mM thiamine-HCl will maintain selection for the F′; however, colonies will grow more slowly. If there is any doubt about whether a white colony represents a pBluescript II recombinant or a colony lacking the F′, streak it onto a minimal medium plate.[4] A cell lacking an F′ will not grow; an F$^+$ will grow slowly since it carries the *proAB* genes on the F′ episome.

SCREENING COLONIES

Colonies containing pBluescript II phagemids may be screened for recombinants by double-stranded DNA, RNA, or oligonucleotide hybridization.[5] Colonies may also be screened by restriction mapping or by sequencing miniprep plasmid DNA. Antibodies may be used to screen colonies[6] since cDNA cloned into the appropriate reading frame of the *lacZ* gene will be expressed as fusion proteins.

When screening with antibodies, the bacteria produce fusion proteins containing several amino acids from the amino-terminus of the β-galactosidase protein (3.5 kDa to the *Eco*R I site). Some fusion proteins are toxic to *E. coli*. Therefore, it is best to initially plate transformants on nitrocellulose filters on top of ampicillin plates lacking IPTG. After 8–10 hours (when the colonies are 1 mm in diameter), transfer the filters to plates containing 5 mM IPTG for several hours. This will induce synthesis of the fusion proteins. When screening with antibodies, Stratagene's *pico*Blue™ immunoscreening kit is recommended. To synthesize large amounts of the fusion proteins in liquid culture, grow the cells to an OD_{600} = 0.7 in the absence of IPTG. Add IPTG to 5 mM and grow for another 2–3 hours. The β-galactosidase portion of the fusion protein is ~3.5 kDa from the Met amino acid to the *Eco*R I site in the polylinker.

Identification of recombinant clones within pBluescript II can be performed by colony hybridization. The following protocol minimizes problems associated with colony screening procedures. For the following protocol to be effective, the screening should be performed on **duplicate sets of filters**.

Fixing Replica Sets of Colonies to Nitrocellulose Filters

Use the following protocol to make multiple replica plates of transformants. Keep the original or master filter to pick colonies identified by the screening of the replica filters.

1. Place 100–mm Duralon–UV™ or nitrocellulose filters on 150–mm LB–ampicillin plates.

2. Spread ~1.0×10^6 cfu on the filters.

3. Incubate the plates at 37°C overnight or until colonies are 1.0 mm in diameter (~7–10 hours).

4. Make a replica of the library growing on the nitrocellulose filter:

   a. Place a piece of sterile Whatman® 3MM paper on a glass surface.

   b. Remove the filter from the agar and place it colony side up on the Whatman 3MM paper.

   c. Align a fresh filter, prewetted on an LB plate, over the master filter and cover with another piece of Whatman 3MM paper. Press in place with a glass plate.

   d. Mark the filters with a small needle to aid in realignment after hybridization.

   e. Separate the master and replica filters and place face up on LB agar plates containing ampicillin.

   f. Incubate both the master and replica filters for at least 4 hours at 37°C.

   g. Seal the master plate with Parafilm® and store at 4°C.

5. The replica filter is then prepared for hybridization:

   a. Place the replica filter colony side up for 30 seconds on the surface of Whatman 3MM paper prewetted with 0.5 M NaOH.

   b. Remove filter and place on another sheet of Whatman 3MM paper prewetted with 1 M Tris-HCl (pH 7.6) for 30 seconds.

   c. Remove the filter and place on a third piece of Whatman 3MM paper prewetted with 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl for 30 seconds.

   d. Immerse the filter in 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl and remove bacterial debris by rubbing the filter gently with a gloved hand.

e. Rinse the filter in 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl. Blot dry on paper towels.

f. Crosslink the DNA to the filters using the autocrosslink setting on the Stratalinker® UV crosslinker (120,000 μJ of UV energy). Alternatively, oven bake at 80°C for ~1.5–2 hours.

Prehybridization

Prehybridization Solution for Oligonucleotide Probe

6× SSC
20 mM NaH_2PO_4
0.4% sodium dodecyl sulfate* (SDS)
5× Denhardt's
Denatured, sonicated salmon sperm DNA (500 μg/ml)

OR

Prehybridization Solution for Double-Stranded Probe

2× Pipes buffer
50% Deionized formamide
0.5% SDS*
Denatured, sonicated salmon sperm DNA (100 μg/ml)

The amount of prehybridization solution to make is dependent on the number of filters used (generally 2–3 ml/membrane).

1. Preheat the prehybridization solution to ~50°C without the salmon sperm DNA. Preboil the salmon sperm DNA for ~10 minutes and add it to the warm prehybridization solution.

2. Wet each filter (quickly) in the prehybridization buffer in a tray, placing each filter on top of the next, until each is wet through. Add more prehybridization solution as necessary. (This helps wet the filters completely to allow more even hybridization later.)

3. Put the wet prehybridization filter "stack" in a heat-seal bag, add the remaining prehybridization buffer and heat seal.

4. Calculate the hybridization temperature (generally 42°C) and prehybridize for a minimum of 1 hour.

5. Prehybridize and hybridize a blank filter ("background") along with the rest and wash it to determine when and at what temperature the background counts disappear.

* For Stratagene's Duralon-UV™ membranes, increase the SDS concentration to 1% (w/v).

Hybridization

Labeling Oligonucleotide Probes

Label oligonucleotides with fresh [γ-^{32}P]ATP. High-specific-activity γ-label yields the best results.

a. Perform a polynucleotide kinase (PNK) labeling in 1× ligase buffer for 30 minutes at 37°C.

b. Incubate for 15 minutes at 65°C to inactivate the kinase.

c. Run the solution over a G-50 column or a NucTrap® probe purification column to get rid of the unincorporated counts.

Labeling Double-Stranded Probes

When using double-stranded probes, nick translate with fresh [α-^{32}P]dATP.

Stratagene offers the Prime-It® II random primer kit designed to produce high-specific-activity DNA probes in 2 minutes.

It is best to use ~1 × 10^6–5 × 10^6 counts/ml of hybridization solution. Keep the concentration of counts high and use ~1 × 10^7 counts/filter.

Hybridization Solution

Hybridization Solution for Oligonucleotide Probes

6× SSC
20 mM NaH_2PO_4
0.4% SDS*
Denatured, sonicated salmon sperm DNA (500 µg/ml)

1. Make the hybridization solution.

2. Boil the salmon sperm DNA and then add it to the prewarmed hybridization solution.

3. Pour out the prehybridization buffer from the filter bag. Add the hybridization solution and then the appropriate amount of labeled oligonucleotide.

* For Stratagene's Duralon-UV™ membranes, increase the SDS concentration to 1% (w/v).

4. Heat seal and hybridize at 5–10°C below T_m. Calculate T_m using the following formula:

Note *The first method below overestimates the T_m of hybrids involving longer nucleotides.*

OLIGONUCLEOTIDES SHORTER THAN 18 BASES

$$T_m = 2°C(A + T) + 4°C(G + C)$$

OLIGONUCLEOTIDES 14 BASES AND LONGER (UP TO 60–70 NUCLEOTIDES)

$$T_m = 81.5 - 16.6\ (\log_{10}[Na^+]) + 0.41(\%G + C) - (600/N),\ \text{where } N = \text{chain length}$$

Hybridization Solution for Double-Stranded Probes

2× Pipes buffer
50% Deionized formamide
0.5% SDS*
Denatured, sonicated salmon sperm DNA (100 µg/ml)

1. Prepare the hybridization solution.

2. Warm the solution, boil the appropriate amount of salmon sperm DNA with the probe for 4 minutes and then add it to the hybridization buffer.

3. Decant the prehybridization buffer and replace it with the hybridization solution and probe. Hybridize overnight at 42°C.

Washes

Oligonucleotide Probes

Use 6× SSC buffer and 0.1% (w/v) SDS. Wash the filters three times for 5 minutes each at room temperature. The final washing temperature depends on the GC ratio of the probe. It is best to stay several degrees below the melting temperature. A rough estimate of the melting temperature of an oligonucleotide probe can be determined by the following formula:

$$T_m = 4(G + C) + 2(T + A)$$

If the probe sequence is unknown, start with a room temperature wash and gradually increase the temperature until the background diminishes. DO NOT allow the membranes to completely dry out or the probe may be irreversibly bound.

Double-Stranded Probes

Use 0.1× SSC buffer and 0.1% (w/v) SDS. Wash the filters at 50–65°C with agitation.

* For Stratagene's Duralon-UV™ membranes, increase the SDS concentration to 1% (w/v).

Exposure to Film

After washing, remove the excess liquid by blotting on Whatman 3MM paper and place the filters between two sheets of plastic wrap in cassettes with intensifying screens. Leave overnight at –80°C. (By keeping the filters slightly moist between plastic wrap, you can wash again if the background is high.)

T3 AND T7 RNA TRANSCRIPTION

The RNA transcripts synthesized from inserts cloned into vectors containing either T3 or T7 polymerase promoters can be used for many purposes. Transcripts can be used for both Southern and Northern hybridization experiments and for either S1 or RNase A analysis. In addition, RNA transcripts can be used to produce protein by translation in vitro or translation in vivo after microinjection into *Xenopus* oocytes or tissue culture cells. Stratagene's mCAP™ RNA capping kit may be used with both T3 and T7 RNA polymerases to incorporate 5′-7MeGpppG-5′ cap analogs, increasing RNA stability by up to 95%.

The pBluescript II vectors have a *Bss*H II site outside each RNA promoter. This feature allows the excising of the insert with the promoters and subsequent mapping using phosphorylated T3 and/or T7 primers.

Handling RNA

Note *Wear gloves at all times to prevent RNase contamination.*

When working with RNA, caution must be used to eliminate RNase contamination from any source. The following general principles will help in the production of full-length transcripts:

1. Make all buffers, DTT, and rNTPs in highly pure water treated with diethylpyrocarbonate (DEPC) as follows:

   Add DEPC to water to a final concentration of 0.1%, heat to 37°C for 8 hours and autoclave. If DEPC scent remains after autoclaving, place the water in a 90°C water bath for at least 1 hour or until the scent is gone.

   Note *Do not treat Tris solutions with DEPC!! Instead, use water that has been treated with DEPC to make up all Tris solutions.*

   Stratagene's RNAMaxx™ high-yield transcription kit (Catalog #200339) may be used for transcription reactions performed with T7 RNA polymerase.

2. All tubes and pipet tips should be autoclaved and baked for several hours at 80°C. A common source of RNase contamination on gel electrophoresis equipment comes from DNA mini–preps which have been treated with RNase A. Thoroughly clean all gel tanks, gel combs, gel spacers and glassware, using soap and water. Followed with an ethanol rinse. Next, soak the equipment in 3% hydrogen peroxide for 10 minutes at room temperature and rinse with DEPC-treated water. Keep cleaned items covered and away from bare hands. Autoclave all glass plates and other appropriate materials on dry cycle prior to use.

3. Phagemid templates for transcription must be RNase-free. Cesium chloride preps are advisable, but minipreps may be used if care is taken to remove contaminating RNases. Generally the plasmid template is linearized with an enzyme that cleaves "downstream" of the RNA polymerase promoter and the insert in the multiple cloning site. It is strongly advised to purify the post-restriction digest DNA by adding 50 µg/ml proteinase K to the restriction buffer at 37°C for 30 minutes, followed by two phenol–chloroform [1:1 (v/v)] extractions and ethanol precipitation prior to the transcription reaction. Resuspend digested, proteinase K treated DNA at 1 mg/ml in a 10 mM Tris (pH 7.4) and 0.1 mM EDTA solution made with DEPC-treated water.

4. Working with RNA is simplified by using a ribonuclease inhibitor in transcription reactions. Stratagene's RNase Block Ribonuclease Inhibitor has been tested and adjusted to work optimally with Stratagene transcription kits.

Nonspecific Initiation with T7 and T3 RNA Polymerases

T7 and T3 RNA polymerases are highly specific for their respective promoters,[1] however, nonspecific initiation of RNA transcripts may occur at the ends of the DNA template. This is most prevalent with a 3′-protruding terminus. Nonspecific initiation may be reduced by increasing the NaCl concentration in the transcription buffers to 100 mM, although this will result in a decrease of the total transcription efficiency by ~50%. When possible, use restriction enzymes that leave blunt or 5′-protruding ends.

When the T7 or T3 polymerase enzymes are used in molar excess of the DNA template, there is a risk of polymerization from the wrong promoter. T7 polymerase can synthesize RNA inefficiently from a plasmid containing only a T3 promoter. Conversely, T3 polymerase can synthesize RNA inefficiently from a plasmid containing only a T7 promoter. Synthesis is extremely promoter specific when both promoters are present, provided that the enzyme is not in molar excess of the specific promoter. Do not use excessive amounts of the polymerases if promoter specificity is important to your experiment. Best results are obtained when the ratios stated in this manual are followed.

Nonradioactive Transcripts

Nonradioactive transcripts can be used for nucleotide sequencing, *in vitro* translation and injection into cells for *in vivo* translation. Set up the transcription reaction as described, but add 1 µl of 10 mM rUTP instead of radioactive rUTP. For larger amounts of RNA, scale up the reaction appropriately. Each molecule of DNA template yields 10–20 nonradioactive RNA molecules if the ribonucleotides are not a limiting factor.

DNase Treatment after Transcription

The DNA template will be present after the transcription reaction and can be removed with RNase-free DNase. After the transcription reaction, add 10 U of RNase-free DNase/µg of DNA template and incubate at 37°C for 15 minutes. Extract with phenol–chloroform [1:1 (v/v)], add 1/10 volume of 3 M sodium acetate at pH 5.2 and precipitate RNA with 2.5 volumes of 100% (v/v) ethanol.

High-Specific-Activity RNA Probes

Any vector containing T3 and T7 RNA promoters can be used to synthesize high specific activity, strand-specific RNA probes. The choice between T3 and T7 RNA polymerase will determine which strand will be used as the template. This is important because probes used for Northern or S1 analysis must complement the RNA targeted for detection.

The initiation of RNA transcription requires rGTP; the reaction has a K_m of ~180 µM. The elongation reaction has a K_m of 40 µM for each ribonucleotide. Therefore, radioactive rGTP should not be used to generate high specific-activity probes unless the concentration of rGTP exceeds 180 µM. This usually means supplementing the radioactive rGTP with cold rGTP. Adding 50 µCi of 500 Ci/mmol [^{32}P]rXTP to a 25-µl reaction only produces a rXTP concentration of 4 µM. To generate high specific-activity probes, we suggest using radioactive rATP, rCTP, or rUTP as the labeled nucleotide. However, any triphosphate present at just 4 µM will not produce many transcripts per template molecule because the reaction simply runs out of radioactive rXTP. To make large amounts of long, radioactive transcripts, the reactions must be supplemented with cold rXTP. It is therefore necessary to choose between full length, quantity and high-specific-activity when producing probes.

Transcription Reaction

Note *Stratagene's RNAMaxx high-yield transcription kit (Catalog #200339) may be used for transcription reactions performed with T7 RNA polymerase.*

1. In the order given, add
   5 µl of 5× transcription buffer§
   1 µg of restricted, proteinase K-treated DNA template
   1 µl of 10 mM rATP
   1 µl of 10 mM rCTP
   1 µl of 10 mM rGTP
   [1 µl of 1 mM rUTP is optional (see above)]
   1 µl of 0.75 M dithiothreitol (DTT)
   1 µl of RNase Block Ribonuclease Inhibitor (optional)
   5 µl of 400–800 Ci/mmol, 10 µCi/µl [α-^{32}P]rUTP
   10 U of T3 or T7 RNA polymerase*
   DEPC-treated water to a final volume of 25 µl

2. Incubate at 37°C for 30 minutes.

3. RNA transcripts may be purified away from the unincorporated nucleotides using Stratagene's NucTrap® probe purification column with a push-column beta-shield device.

Alternatively, an RNase-free G-50 column can be used. However, care must be taken that there are no ribonucleases present in the column that could degrade the probe.

Note *Do not use large excesses of T3 polymerase (10 U of polymerase/pmol of promoter is sufficient). T3 RNA polymerase may utilize the T7 promoter 1 in 20 times when the T3 enzyme concentration exceeds the T3 promoter concentration by 10-fold. However, T3 polymerase in the recommended concentrations will not make T7 transcripts in the presence of a T3 promoter. If any T7 hybridization should result from a T3 transcription, decrease the amount of T3 polymerase by a factor of 5 or 10.*

§ See *Preparation of Media and Reagents*.

* Use supplied RNA polymerase dilution buffer to dilute enzymes just before use.

Hybridization Conditions for RNA Probes in Southern Blots

Prehybridization

Prehybridize the membrane with 0.1–0.5 ml/cm^2 of the following solution for 2 hours at 42°C with constant agitation in a heat-sealable bag:

6× SSC
5× Denhardt's (see *Preparation of Media and Reagents*)
20 mM NaH_2PO_4
500 µg/ml of denatured, sonicated salmon sperm DNA

Hybridization

Pour off the prehybridization solution and add the probe to the bag with the minimum volume of the following hybridization solution:

6× SSC
20 mM NaH_2PO_4
0.4% SDS*
500 µg/ml denatured sonicated salmon sperm DNA

Incubate overnight at 42°C with constant agitation.

Washes

Wash in 2× SSC buffer and 0.1% (w/v) SDS twice for 15 minutes each at 55°C and twice in 0.1× SSC buffer and 0.1% (w/v) SDS for 15 minutes each at 55°C.

Hybridization Conditions for RNA Probes in Northern Blots

Prehybridization

Prehybridize the membrane with 0.1–0.5 ml/cm^2 of the following solution for ~1 hour at 42°C with constant agitation in a heat-sealable bag:

50% deionized formamide
10% dextran sulfate
1% SDS*
1 M NaCl
100 µg/ml of denatured sonicated salmon sperm DNA

Hybridization

Hybridize overnight with the riboprobe at the same temperature and in the prehybridization solution.

Washes

Wash in 2× SSC buffer and 0.1% (w/v) SDS twice for 15 minutes each at 42°C and twice in 0.1× SSC buffer and 0.1% (w/v) SDS for 15 minutes each at 42°C. If a high background is observed, the temperature may be increased or the NaCl concentration may be decreased for greater stringency.

* For Stratagene's Duralon-UV™ and Illuminator™ membranes, increase the SDS concentration to 1% (w/v).

RECOVERY OF SINGLE-STRANDED DNA FROM CELLS CONTAINING pBLUESCRIPT II® PHAGEMIDS

pBluescript II is a phagemid that can be secreted as single-stranded DNA in the presence of M13 helper phage. These phagemids contain the intergenic (IG) region of a filamentous f1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *E. coli* with the F^+ phenotype (containing an F′ episome), pBluescript II phagemids will be secreted as single-stranded f1 "packaged" phage when the bacteria has been infected by a helper phage. Since these filamentous helper phages (M13, fl) will not infect *E. coli* without an F′ episome coding for pili, **it is essential to use XL1-Blue MRF′ or a similar strain containing the F′ episome.**[7,8]

Stratagene offers helper phages that *preferentially* package pBluescript II phagemids. Typically, 30–50 pBluescript II molecules are packaged/helper phage DNA molecule. pBluescript II phagemids are offered with the IG region in either of two orientations: pBluescript II (+) is replicated such that the sense strand of the β-galactosidase gene is secreted within the phage particles; pBluescript II (–) is replicated such that the antisense strand of the β-galactosidase gene is secreted in the phage particles.

Yields of single-stranded (ss)DNA depend on the specific insert sequence. For most inserts, over 1 µg of ssDNA can be obtained from a 1.5-ml miniprep if grown in XL1-Blue MRF′. A faint single-strand helper phage band may appear on a gel at ~4 kb for R408 or at 6 kb for VCSM13. This DNA mixture can be sequenced with primers that are specific for pBluescript II and do not hybridize to the helper phage genome.

Site-specific mutagenesis is also possible using standard techniques. The advantages of using pBluescript II phagemids for either purpose are as follows: (1) pBluescript II phagemids do not replicate via the M13 cycle, lessening the tendency to delete DNA inserts, therefore it is unlikely that even 10-kb inserts will be deleted. (2) "Packaging" of pBluescript II phagemids containing inserts is efficient since the pBluescript II vector is significantly smaller than wild-type M13. (3) Oligonucleotide mutagenesis in pBluescript II vectors is advantageous because the mutagenized insert is located between the T3 and T7 promoters. The resultant mutant transcripts can be synthesized *in vitro* without further subcloning.

VCSM13 and R408 helper phage produce the largest amount of single-strand pBluescript II. R408 (single-strand size ~4 kb) is more stable and can be grown more easily. VCSM13 (single-strand size ~6 kb), is more efficient at single-stranded DNA rescue and yields more single-stranded phagemid; however it is more unstable and reverts to wild-type more frequently. This difficulty can be addressed by periodically propagating VCSM13 in the presence of kanamycin. VCSM13 (a derivative of M13KO7) has a kanamycin gene inserted into the intergenic region, while R408 has a deletion in that region. We suggest R408 for excision of pBluescript II from the Lambda ZAP vector and VCSM13 for single-stranded rescue.

Single-Stranded Rescue Protocol

1. Inoculate a single colony into 5 ml of 2× YT containing 100 µg/ml ampicillin and VCM13 or R408 helper phage at 10^7–10^8 pfu/ml (MOI ~10).

2. Grow the culture at 37°C with vigorous aeration for 16–24 hours, or until growth has reached saturation.

   Note *If using VCSM13, after 1–2 hours, add kanamycin to 70 µg/ml to select for infected cells.*

3. Centrifuge 1.5 ml of the cell culture for 5 minutes in a microcentrifuge.

4. Remove 1 ml of the supernatant to a fresh tube, then add 150 µl of a solution containing 20% PEG8000 and 2.5 M NaCl. Allow phage particles to precipitate on ice for 15 minutes.

   Note *For increased yield, perform the PEG precipitation overnight at 4°C.*

5. Centrifuge for 5 minutes in a microcentrifuge. (A pellet should be obvious.)

6. Remove supernatant. Centrifuge the PEG pellets a few seconds more to collect residual liquid, then remove and discard the residual liquid.

7. Resuspend the pellet in 400 µl of 0.3 M NaOAc (pH 6.0) and 1 mM EDTA by vortexing vigorously.

8. Extract with 1 volume phenol–chloroform and centrifuge for 1–2 minutes to separate phases.

9. Transfer the aqueous phase to a fresh tube and add 1 ml of ethanol. Centrifuge for 5 minutes.

10. Remove ethanol and dry the DNA pellet.

11. Dissolve the pellet in 25 µl of TE buffer.

12. Analyze 1–2 µl on an agarose gel.

SITE-DIRECTED MUTAGENESIS

Isolated single-stranded DNA (see *Recovery of Single–Stranded DNA from Cells Containing pBluescript II Phagemids*) can be used for site-directed oligonucleotide mutagenesis.[9] The following protocol is recommended:

1. Phosphorylation of the oligonucleotide with polynucleotide kinase:
   100 ng of oligonucleotide
   4 µl of 10× ligase buffer[§]
   4 µl of 10 mM rATP
   2 µl of polynucleotide kinase (10 U)
   Water to 40 µl final volume
   Incubate at 37°C for 30 minutes.

2. Synthesis of mutant DNA strand

   a. Anneal Oligonucleotide

      20 µl of oligonucleotide from the kinase reaction (50 ng)
      5 µl of salmon sperm DNA (1 µg template)
      Incubate at 65°C for 10 minutes, then at room temperature for 5 minutes.

   b. Primer Extension Reaction

      Add the following to the annealing reaction:
      4.0 µl of 10× ligase buffer[§]
      2.0 µl of 2.5 mM dNTPs (N = A, C, G and T in equal concentration)
      4.0 µl of 10 mM rATP
      1.0 µg of single-stranded DNA binding protein
      1.5 U of Klenow
      0.5 µl of T4 DNA ligase (2 U)
      Water to 40 µl final volume
      Incubate at room temperature for 3–4 hours.

3. Transform XL1-Blue MRF′ *E. coli* with 10 µl of synthesis reaction and plate onto nitrocellulose filters across three plates.

4. Screen as described in *Screening Colonies.* One percent mutants should be obtained.

[§] See *Preparation of Media and Reagents.*

EXONUCLEASE III/MUNG BEAN NUCLEASE DELETIONS

Stratagene's Exo III/Mung Bean Nuclease Deletion Kit (Catalog #200330) has been optimized to produce unidirectional deletions of predictable sizes. The technique takes advantage of the properties of exonuclease III and pBluescript II phagemids. Exonuclease III will not digest 3′-single-stranded overhangs ≥4 bases, but will digest 3′ ends from blunt ends or 5′ overhangs.[4] The polylinker in the pBluescript II phagemids has unique restriction sites on the outside edges of the polylinker with 3′ overhangs and internal sites with 5′-overhangs or blunt ended cleavage products. To create deletions in the insert but not in the vector DNA, simply double-digest the clone with a 3′-overhang-producing restriction enzyme and 5′-overhang or blunt end-producing restriction enzyme, creating a substrate for unidirectional exonuclease digestion by exonuclease III. Afterward, mung bean nuclease is used to digest the single-stranded DNA ends to allow blunt-end ligation of the deletion products. Taking advantage of the convenient restriction sites and the predictable progression of exonuclease III, nested deletion construction can be accomplished very quickly.

Stratagene's Exo III/Mung Bean Nuclease Deletion Kit provides the following buffers:

2× Exo III buffer[§]
10× mung bean buffer[§]
1× mung bean dilution buffer[§]

The 2× Exo III buffer and 10× mung bean buffer are used for the exonuclease III and mung bean nuclease digestions, respectively. The 10× mung bean buffer is also used to terminate the exonuclease III digestion. The 1× mung bean dilution buffer is used to dilute the mung bean nuclease to the appropriate concentration for the reaction. A fresh dilution of mung bean nuclease is necessary, because dilute concentrations of mung bean nuclease are not stable.

It is necessary to start the digestions with highly supercoiled DNA (>85%). Exonuclease III can initiate digestion from nicks in the DNA, producing high background and making it more difficult to interpret results. Restriction enzymes with any nicking activity will contribute to these problems. Therefore, use the highest quality restriction enzymes available.

Protocol Outline

1. Clone the inserts into internal restriction sites of pBluescript II phagemid (*Eco*R I or *Pst* I are best).

2. Perform cesium chloride banding and purification of the dsDNA.

[§] See *Preparation of Media and Reagents*.

3. Double-digest the clones to **COMPLETION** at a unique restriction site producing 3′-overhangs and a unique restriction site producing 5′ overhangs or blunt ends that lies between the insert and the 3′-overhang site chosen. **Check for completion of the first digest on an agarose gel.** Make sure that the 5′-overhang or blunt end-producing restriction site, where deletions will be initiated by Exo III, is between the 3′-overhang-producing restriction site and the insert. Ensure that the 3′-overhang is ≥4 nucleotides in length; shorter 3′-overhangs are susceptible to cleavage by Exo III. The 3′-overhang-producing digest can be replaced with a 5′-overhang-producing digest if the overhang is filled in with deoxythio-derivatives by Klenow fragment to block the end from Exo III digestion.[10] When protecting with deoxythioderivatives, Stratagene recommends the following protocol. If a 3'-overhang was produced in a double-digest, proceed directly to step 4.

Thioderivative Fill-In (for Dual 5′-Overhangs)

a. Select two unique, 5'-overhang-producing restriction sites on the same side of the insert within the polylinker. Digest ~20–30 µg of DNA in a 500-µl reaction with the restriction enzyme whose site is to be protected (i.e. the site farthest from the insert). Do not digest the DNA at the second site, to be used for unidirectional deletions, until step h.

b. Heat the restriction digest to 75°C for 15 minutes.

c. Add 2 µl of a 1 mM stock of thio-dNTP mix and 5 U of Klenow fragment.

d. Incubate at room temperature for 10 minutes.

e. Extract with phenol–chloroform [1:1 (v/v)].

f. Ethanol precipitate the DNA.

g. Verify the success of the fill-in reaction by incubating 1 µg of filled-in DNA with 20 U of exonuclease III for 15 minutes at 37°C. Run the products on an agarose gel to check for protection against deletion.

h. Proceed with the second 5′-overhang-producing restriction digestion.

i. Extract with phenol–chloroform [1:1 (v/v)].

j. Ethanol precipitate the DNA.

4. Treat the double-digested DNA with exonuclease III (as described in *Exo III Deletion Series and Mung Bean Nuclease Digestion*) so that a portion of the insert is made single stranded.

5. Digest the ssDNA with mung bean nuclease to create blunt ends.

6. Ligate the ends to recircularize.

7. Transform the DNA into competent *E. coli* cells.

To obtain unidirectional deletions, it is important that the DNA is completely digested, phenol–chloroform extracted and ethanol precipitated (as described in *Exo III Deletion Series and Mung Bean Nuclease Digestion*, below). When selecting sites to use for digestion prior to exonuclease treatment, select restriction sites as far apart as possible to increase the likelihood of obtaining a complete double-digestion. Stratagene has observed that the **overhang from *Sac* II digestion does not protect against exonuclease III digestion.**

Keep mung bean nuclease concentrated until just before use; store the mung bean nuclease on ice for only short periods of time. Check restriction enzymes for nicking activity before use (see *Troubleshooting*).

Exo III Deletion Series and Mung Bean Nuclease Digestion

The length of DNA converted from double stranded to single stranded by exonuclease III can be controlled by the reaction temperature and time of incubation:

At 37°C, ~400 bp are converted per minute
At 34°C, ~375 bp are converted per minute
At 30°C, ~230 bp are converted per minute
At 23°C, ~125 bp are converted per minute

When using the exonuclease III/mung bean nuclease system, it is possible to produce nested deletions of varying lengths simultaneously by setting up a single reaction for exonuclease III and removing aliquots at varying time points. Each aliquot is then treated with mung bean nuclease and is ligated separately. The following protocol has been optimized to obtain multiple nested deletions:

1. Prepare a stop solution for each exonuclease III time point. Dilute 20 µl of 10× mung bean buffer into 155 µl of water in a microcentrifuge tube for each time interval desired. Use this diluted mung bean buffer to terminate the exonuclease III deletions at the desired time points.

2. Start the reaction by adding 20 U of exonuclease III for each picomole of susceptible 3′ ends of DNA. Incubate reaction at the desired temperature (see the guidelines for conversion at different temperatures above) and remove 25-µl aliquots from the reaction mixture at the appropriate time intervals. Add the 25-µl aliquot directly to the tubes containing the 175-µl aliquots of diluted mung bean nuclease buffer prepared in step 1 above and place the tubes on dry ice.

The exonuclease reactions for all time points are started in a single tube, and aliquots are removed at each time point. For **each** time point, the reaction contains the following components:

5.0 µg of double-digested DNA (1 µg/µl)
12.5 µl of 2× Exo III buffer
2.5 µl of fresh 100 mM β-mercaptoethanol
X µl of exonuclease III (20 U/pmol end)
Water to 25 µl (total reaction volume per time point)

Multiply each component by the total number of time points to be taken. An example for an exonuclease III/mung bean nuclease deletion with five time points is as follows:

5.0 µl × 5 = 25 µl of double-digested DNA (1 µg/µl)
12.5 µl × 5 = 62.5 µl of 2× Exo III buffer
2.5 µl × 5 = 12.5 µl of fresh 100 mM β-mercaptoethanol
100.0 U × 5 = 500.0 U of exonuclease III
Water to 125 µl (total reaction volume for 5 time points)

3. When all aliquots have been removed, heat the tubes at 68°C for 15 minutes and then place the tubes on ice.

4. Dilute mung bean nuclease to 15 U/µl in 1× mung bean dilution buffer. Add 1 µl to each time point tube and incubate for 30 minutes at 30°C.

Optional *Steps 5–11 below are optional and are performed to completely remove residual mung bean nuclease from the DNA.*

5. Add the following components:
   10 µl of 1 M Tris-HCl (pH 9.5)
   20 µl of 8 M LiCl
   4 µl of 20% (w/v) SDS
   250 µl of buffer-equilibrated phenol–chloroform

6. Vortex and then spin for 1 minute in a microcentrifuge. Transfer the upper aqueous layer to a fresh tube and extract the upper layer with chloroform.

7. Add 25 µl of 3 M sodium acetate at pH 7.0 to the aqueous phase. Transfer RNA (tRNA) may be added to a final concentration of 10 ng/µl as a carrier for the precipitation.

8. Add 650 µl of cold ethanol. Chill on dry ice for 10 minutes and spin in a microcentrifuge for 20 minutes.

9. Drain off the supernatants and wash the pellets with 80% (v/v) ethanol.

10. Dry the pellet.

11. Redissolve the DNA pellet in 15 µl of TE buffer.

Ligation

12. Ligate the DNA deletions by adding the following:

1.0 µl (~3 µg) of exonuclease III/mung bean nuclease-treated DNA
2.0 µl of 10× ligase buffer
1.0 µl of 10 mM rATP (pH 7.0–7.5)
0.5 µl of T4 DNA ligase (2 U)
15.5 µl of water
20.0 µl total volume

Incubate at room temperature for 4 hours or at 4°C overnight.

13. Use 7 of the remaining 14 µl (20 of 200 µl if steps 5–11 were omitted) of the exonuclease/mung bean nuclease-treated DNA for gel electrophoresis analysis. The deletions can only be visualized after treatment with mung bean nuclease. Before treatment, there will be only a slight difference in mobility between the exonuclease-digested DNA and the full-length, linearized DNA.

14. Use 1 µl of the ligation reaction to transform 100 µl of *E. coli* competent cells (such as Stratagene's XL1-Blue MRF′ competent cells) and plate the cells on LB–ampicillin plates (100 µg/ml ampicillin).

Low-Melting-Temperature Agarose Enrichment Technique

To minimize screening of the deletions, run a portion of the deletion in low-melting-temperature (LMT) agarose, excise the band of interest and proceed with the ligation. Stratagene recommends keeping the agarose level below 0.5% in the ligation reaction.

1. Perform steps 1–4 from *Deletions*.

2. Add 10 µl of 3 M sodium acetate at pH 5.2 and 0.5 ml of cold ethanol. Chill on ice for 10 minutes and spin in a microcentrifuge for 20 minutes.

3. Dry the pellet.

4. Redissolve the DNA pellet in 15 µl of TE buffer.

5. Load 7 µl in a 1% low-melting-point agarose gel and separate by gel electrophoresis.

6. Excise deletion band. Heat agarose to 68°C for 30 minutes, then use 10 ng for ligation.

7. Ligate DNA deletions using the following conditions:
   1.0 µl of Exo/Mung-treated DNA
   2.0 µl of 10× ligase buffer[§]
   1.0 µl of 10 mM rATP (pH 7.0–7.5)
   0.5 µl of T4 DNA ligase (4 U/µl)
   15.5 µl of water
   (20.0 µl total reaction volume)
   Incubate at room temperature for 4 hours or 4°C overnight.

8. Use 1 µl of the ligation reaction to transform 100 µl of competent *E. coli* (e.g. Stratagene's XL1-Blue MRF′ competent cells) and plate on LB-ampicillin plates.

Quick Screen of the Transformants

1. Isolate three to four colonies from each time interval with sterile toothpicks and streak each as a single line onto LB–ampicillin plates (~12 streaks/plate).

2. Grow overnight at 37°C.

3. Scrape bacteria with sterile toothpick and resuspend in 40 µl of 1× STE buffer.[§]

4. Add 40 µl of phenol–chloroform and vortex.

5. Microcentrifuge for 1 minute.

6. Transfer supernatant to a microcentrifuge tube and add 1 µl of RNase A (1 mg/ml).

7. Incubate at room temperature for 2 minutes. Add loading buffer. Load 20 µl onto a 1% agarose gel and separate by gel electrophoresis to compare supercoiled Exo/Mung-deleted plasmids. *

[§] See *Preparation of Media and Reagents.*

* **WARNING: Samples cannot be restriction digested with this technique. Care must be taken when loading the gel since high sample viscosity may make it difficult to keep the sample in the wells. Make sure the wells are deeply immersed in running buffer while loading. If restriction digestion is desired, see *Plasmid Boiling Miniprep Protocol.***

PLASMID BOILING MINIPREP PROTOCOL

The following protocol yields high-quality dsDNA template simply and rapidly. (Caution: *Escherichia coli* strain HB101 and derivatives give low yields using this protocol.) This DNA is suitable for restriction enzyme digestion or for enzyme sequencing.[11]

1. Grow a 3-ml culture overnight in LB broth plus ampicillin (100 µg/ml) from a single colony.

2. Pellet 1.5 ml of the culture in a microcentrifuge at 4°C for 2 minutes. Remove the supernatant by aspiration.

3. Resuspend the pellet in 110 µl of STETL buffer (see *Preparation of Media and Reagents*).

4. Place the tube in a boiling water bath for 30 seconds.

5. Immediately spin the tube in a microcentrifug for 15 minutes at room temperature.

6. Remove and discard the pellet with a sterile toothpick. Save the supernatant. [RNase treatment (20 µg/ml) is optional at this stage.)]

7. Add 110 µl of isopropanol to the supernatant and immediately spin the tube in a microcentrifuge for 15 minutes.

8. Resuspend the pellet in 100 µl of TE buffer.

9. Extract twice with an equal volume of phenol–chloroform [1:1 (v/v)] and once with chloroform.

   Note *To purify the sample, StrataClean™ resin may be used in place of the phenol–chloroform extraction.*

10. Add an equal volume of 7.5 M ammonium acetate and precipitate with 2.5 volumes of ethanol. Incubate on ice 15 minutes and spin at 4°C for 20 minutes.

11. Rinse with 1 ml of 80% (v/v) ethanol and spin in a microcentrifuge for 1 minute.

12. Vacuum dry the pellets.

13. Resuspend the pellets in 15 µl of TE buffer.

14. Use 5 µl of this DNA (about 2.0 µg) for sequencing.

IMPROVED SEQUENCING PRIMERS AND PCR PRIMER SETS

The traditional primers designed for the pBluescript phagemid vector and its derivatives were used primarily for primer extension reactions at 37°C or less. The advent of PCR and cycle sequencing requires that these primers bind efficiently at higher temperatures. Stratagene has redesigned these primers for exceptional performance in high-temperature primer extension reactions. The new primers maintain nearly the same template positions, but now have higher melting temperatures.

Improved Sequencing Primers

Primer	Sequence	Catalog #
T3	5′ AATTAACCCTCACTAAAGGG 3′	300301
T7	5′ GTAATACGACTCACTATAGGGC 3′	300302
M13 (–20)	5′ GTAAAACGACGGCCAGT 3′	300303
M13 reverse	5′ GGAAACAGCTATGACCATG 3′	300304
SK	5′ CGCTCTAGAACTAGTGGATC 3′	300305
KS	5′ TCGAGGTCGACGGTATC 3′	300306

Improved PCR Primer Sets

Primer set	Catalog #
T3/T7	302001
M13 (–20)/Reverse	302003
SK/KS	302005

TROUBLESHOOTING

Restriction Digests of pBluescript II Phagemids

Observation	Suggestion
Digestion with *Eco*R I produces multiple bands	Using excess amounts of *Eco*R I to digest pBluescript II vectors results in *Eco*R I prime activity. This appears as cleavage at a non-*Eco*R I site at the 3′ end of the f1 intergenic region, causing confusion when interpreting results from an agarose gel. Test whether reducing the units of *Eco*R I restores a normal restriction pattern
Nae I fails to cleave the pBluescript II vector	Stratagene has observed that the *Nae* I site in the pBluescript II phagemids presents a more challenging substrate for digestion than the sites in pBR322. Use 16U *Nae* I enzyme per µg DNA and increase the digestion period (overight digestion may be necessary). Even under these more stringent conditions, *Nae* I may not produce complete cleavage.

Exo/Mung Deletion Protocol

Observation	Suggestion
Gel electrophoresis analysis reveals the same size band before and after deletion. The band is the same molecular weight as the linearized starting plasmid vector. A large number of colonies are obtained after ligation and transformation	Incomplete digestion with restriction endonuclease that leaves a 5′ overhang. Increase the units of the appropriate restriction enzyme.
Transformants are obtained only for short fragments	Incomplete digestion with restriction endonuclease that leaves a 3′ overhang. Increase the units of the appropriate enzyme.
Deletions are observed on gel electrophoresis, but they are less extensive than expected. Few transformants are obtained after ligation of deleted DNA	Mung bean nuclease digestion did not go to completion. Increase the units of mung bean nuclease to remove all ssDNA.
Gel electrophoresis analysis reveals a smear on the gel instead of discrete deletion bands	Exonuclease III can delete from a 5′ overhang, a blunt end, or any nick in the plasmid. a. The initial plasmid should be greater than 85% supercoiled. b. The restriction enzymes should be checked for nonspecific nicking activity by incubating a supercoiled plasmid that does not contain the restriction site with the restriction enzyme and checking for change in mobility on a 1% agarose gel. Change in mobility indicates nicking activity.

PREPARATION OF MEDIA AND REAGENTS

5× Transcription Buffer
200 mM Tris, pH 8.0
40 mM $MgCl_2$
10 mM spermidine
250 mM NaCl

10× Ligase Buffer
500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)
Note *rATP is added separately in the ligation reaction.*

2× Exo III Buffer
100 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$

10× Mung Bean Buffer
300 mM NaOAc (pH 5.0)
500 mM NaCl
10 mM $ZnCl_2$
50% (v/v) glycerol

1× Mung Bean Dilution Buffer
10 mM NaOAc (pH 5.0)
0.1 mM ZnOAc
1 mM cysteine
0.01% (v/v) Triton® X-100
50% (v/v) glycerol

20× SSC
175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of water
10.0 N NaOH
Adjust to pH 7.0 with a few drops of 10.0 N NaOH
Adjust volume to 1 liter with water

M9 Minimal Medium (per Liter)
750 ml of sterile deionized water (cooled to 50°C)
200 ml of 5× M9 salts
Sterile deionized water to 1 liter
20 ml of a 20% solution of the appropriate carbon source (e.g., 20% glucose)

50× Denhardt's Reagent (per 500 ml)
5 g of Ficoll
5 g of polyvinylpyrrolidone
5 g of BSA (Fraction V)
Add deionized H_2O to a final volume of 500 ml
Filter through a disposable filter
Dispense into aliquots and store at –20°C

LB Broth (per Liter)
10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

STETL Buffer
8.0% sucrose
0.5% Triton X-100
50.0 mM Tris (pH 8.0)
50.0 mM EDTA
0.5 mg/ml lysozyme
All components except lysozyme can be prepared and stored indefinitely at 4°C. The lysozyme is made as a 5 mg/ml stock and stored in small aliquots at –20°C. Do not reuse the lysozyme stock after thawing.

1× STE Buffer
100 mM NaCl
20 mM Tris-HCl (pH 7.5)
10 mM EDTA

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ENDNOTES

MSDS INFORMATION

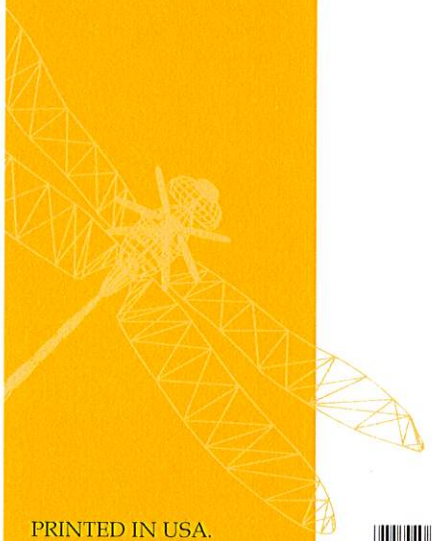
The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on Stratagene's website at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

Promega

Technical Manual

pGEM®-T and pGEM®-T Easy Vector Systems

INSTRUCTIONS FOR USE OF PRODUCTS A1360, A1380, A3600 AND A3610.



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Part# TM042

Promega

pGEM®-T and pGEM®-T Easy Vector Systems

All technical literature is available on the Internet at www.promega.com/tbs/
Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail techserv@promega.com.

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I. Description

The pGEM®-T and pGEM®-T Easy Vector Systems[a,b] are convenient systems for the cloning of PCR[c] products. The vectors are prepared by cutting the pGEM®-5Zf(+) and pGEM®-T Easy Vectors, respectively, with *Eco*R V and adding a 3′ terminal thymidine to both ends. These single 3′-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (1,2). As summarized in Table 1, these polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of the amplified fragments (3,4).

The high-copy-number pGEM®-T and pGEM®-T Easy Vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates. The multiple cloning region of the two vectors includes restriction sites conveniently arranged for use with the Erase-a-Base® System (Cat.# E5750) for generating nested sets of deletions.

Both the pGEM®-T and pGEM®-T Easy Vector contain multiple restriction sites within the multiple cloning region. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes *Eco*R I, *Bst*Z I and *Not* I, thus providing three single-enzyme digestions for release of the insert. The pGEM®-T Vector cloning region is flanked by recognition sites for the enzyme *Bst*Z I. Alternatively, a double-digestion may be used to release the insert from either vector.

The pGEM®-T and pGEM®-T Easy Vectors also contain the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA (ssDNA; see Section VIII). The ssDNA molecule exported corresponds to the bottom strand shown in Figure 1.

The pGEM®-T and pGEM®-T Easy Vector Systems include a 2X Rapid Ligation Buffer for ligation of PCR products. Reactions using this buffer may be incubated for 1 hour at room temperature. The incubation period may be extended to increase the number of colonies after transformation. Generally, an overnight incubation at 4°C will produce the maximum number of transformants.

Table 1. Comparison of PCR Product Properties for Thermostable DNA Polymerases.

	Thermostable DNA Polymerase						
Characteristic	*Taq*/ AmpliTaq®	*Tfl*	*Tth*	Vent® (*Tli*)	Deep Vent®	*Pfu*	*Pwo*
Resulting DNA ends	3′A	3′A	3′A	>95% Blunt	>95% Blunt	Blunt	Blunt
5′→3′ exonuclease activity	Yes	Yes	Yes	No	No	No	No
3′→5′ exonuclease activity	No	No	No	Yes	Yes	Yes	Yes

Specialized Applications of the pGEM®-T and pGEM®-T Easy Vectors:

- Cloning PCR products.
- Construction of unidirectional nested deletions with the Erase-a-Base® System.
- Production of ssDNA.
- Blue/white screening for recombinants.
- In vitro transcription from dual-opposed promoters. (For protocol information, please request the *Riboprobe® in vitro Transcription Systems Technical Manual* #TM016 (available at: www.promega.com/tbs/)

Citations Using the pGEM®-T and pGEM®-T Easy Vector Systems

- Perincheri, S. *et al.* (2005) Hereditary persistence of α-fetoprotein and H19 expression in liver of BALB/cJ mice is due to a retrovirus insertion in the *Zhx2* gene. *Proc. Natl. Acad. Sci. USA* **102**, 396-401.

  Notes: In this study, various mouse genomic DNA fragments were amplified and cloned into the pGEM®-T and pGEM®-T Easy Vectors. PCR fragments amplified with *Pfx* Platinum Polymerase were A-tailed with *Taq* DNA Polymerase and 0.2mM dATP at 70°C for 30 minutes before cloning into the pGEM®-T or pGEM®-T Easy Vector. The ORF containing the mouse *Zhx2* gene was amplified and cloned into the pGEM®-T Easy Vector and sequenced before insertion into a transthyretin expression vector to generate the transgene *TTR-Afr1*, which was then exised, purified, and used in complementation analysis to test whether *Zhx2* transgene expression restored repression of target genes.

- Shin, H-J. *et al.* (2005) STAT4 expression in human T cells is regulated by DNA methylation but not by promoter polymorphism. *J. Immunol.* **175**, 7143-50.

  Notes: To identify transctiptional start sites in the STAT4 promoter, total RNA was isolated from Jurkat T cells, cDNA synthesized, and 5′ RACE performed. Amplified PCR products were cloned into the pGEM®-T Easy Vector and sequenced.

- Regue, M. *et al.* (2005) A second outer-core region in *Klebsiella pneumoniae* lipopolysaccharide. *J. Bacteriol.* **187**, 4198-206.

  Notes: In this study, *Klebsiella pneuomoniae* strains with mutations in the LPS core biosynthetic gene cluster *waa* were constructed. The *waa* genes from *K. pneuomoniae* strains having two different core types were then amplified by PCR, cloned into the pGEM®-T Vector, and used in mutant complementation studies.

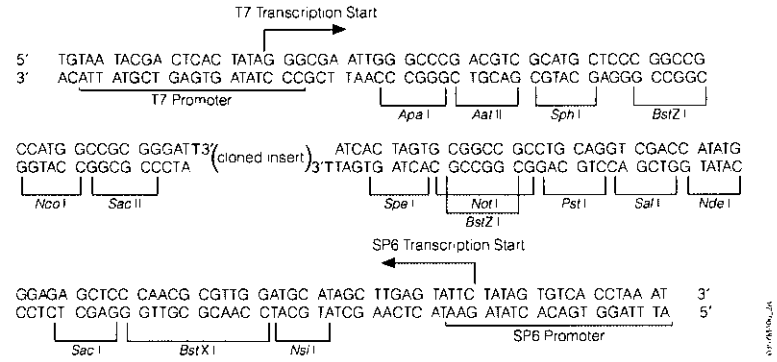
For additional peer-reviewed articles that cite use of the pGEM®-T Vectors, visit: www.promega.com/citations

Promega

II. pGEM®-T and pGEM®-T Easy Vector Multiple Cloning Sequences and Maps

II.A. Multiple Cloning Sequences

pGEM®-T Vector



pGEM®-T Easy Vector

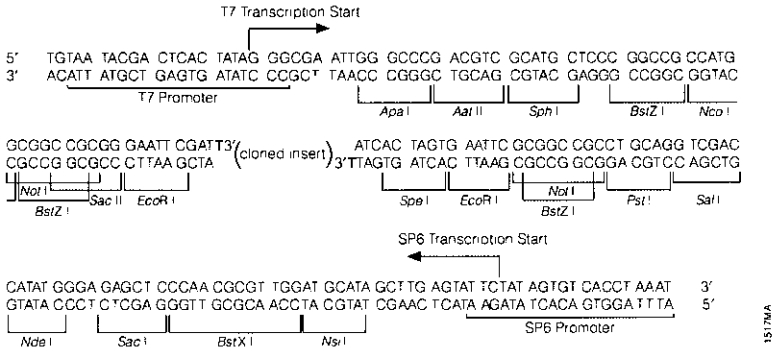


Figure 1. The promoter and multiple cloning sequence of the pGEM®-T and pGEM®-T Easy Vectors. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

II.B. pGEM®-T Vector Map and Sequence Reference Points

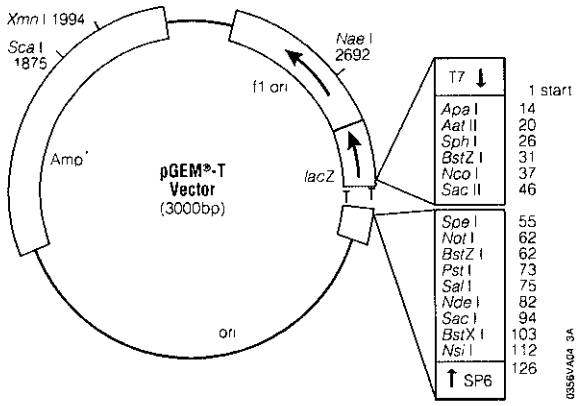


Figure 2. pGEM®-T Vector circle map and sequence reference points.

pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
*lac*Z start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

Note: Inserts can be sequenced using the SP6 Promoter Primer (Cat.# Q5011), T7 Promoter Primer (Cat.# Q5021), pUC/M13 Forward Primer (Cat.# Q5601), or pUC/M13 Reverse Primer (Cat.# Q5421).

Note: A single digest with *Bst*Z I (Cat.# R6881) will release inserts cloned into the pGEM®-T Vector. Double digests can also be used to release inserts.

II.C. pGEM®-T Easy Vector Map and Sequence Reference Points

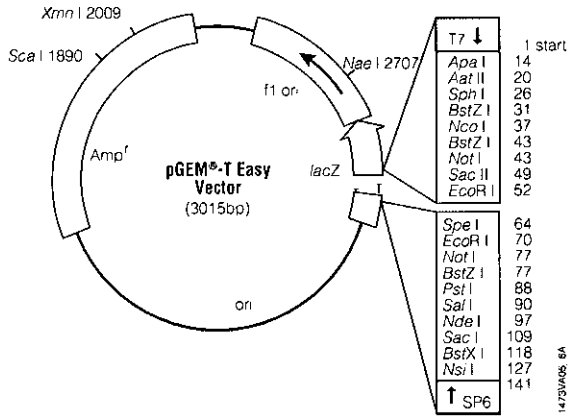


Figure 3. pGEM®-T Easy Vector circle map and sequence reference points.

pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Note: Inserts can be sequenced using the SP6 Promoter Primer (Cat.# Q5011), T7 Promoter Primer (Cat.# Q5021), pUC/M13 Forward Primer (Cat.# Q5601), or pUC/M13 Reverse Primer (Cat.# Q5421).

Note: A single digest with *Bst*Z I (Cat.# R6881) , *Eco*R I (Cat.# R6011) or *Not* I (Cat.# R6431) will release inserts cloned into the pGEM®-T Easy Vector. Double digests can also be used to release inserts.

III. Product Components and Storage Conditions

Product	Size	Cat.#
pGEM®-T Vector System I	20 reactions	A3600

For Laboratory Use. Includes:

- 1.2µg pGEM®-T Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase
- 1 Protocol

Product	Size	Cat.#
pGEM®-T Vector System II	20 reactions	A3610

For Laboratory Use. Includes:

- 1.2µg pGEM®-T Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase
- 1.2ml JM109 Competent Cells, High Efficiency (6 × 200µl)
- 1 Protocol

Product	Size	Cat.#
pGEM®-T Easy Vector System I	20 reactions	A1360

For Laboratory Use. Includes:

- 1.2µg pGEM®-T Easy Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase
- 1 Protocol

Product	Size	Cat.#
pGEM®-T Easy Vector System II	20 reactions	A1380

For Laboratory Use. Includes:

- 1.2µg pGEM®-T Easy Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase
- 1.2ml JM109 Competent Cells, High Efficiency (6 × 200µl)
- 1 Protocol

Storage Conditions: For Cat.# A3610, A1380, store the Competent Cells below -65°C. All other components can be stored at -15 to -25°C.

IV. Protocol for Ligations Using the pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tubes.
2. Set up ligation reactions as described below.

   Note: Use 0.5ml tubes known to have low DNA-binding capacity (e.g., VWR Cat.# 20170-310).

   ! Vortex the 2X Rapid Ligation Buffer vigorously before each use.

	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl*	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
deionized water to a final volume of	10µl	10µl	10µl

*Molar ratio of PCR product:vector may require optimization (see Section VI.C).

3. Mix the reactions by pipetting. Incubate the reactions 1 hour at room temperature.

   Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

Notes:

1. Use only Promega T4 DNA Ligase supplied with this system to perform pGEM®-T and pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.
2. 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.
3. It is important to vortex the 2X Rapid Ligation Buffer before each use.
4. Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.

V. Protocol for Transformations Using the pGEM®-T and pGEM®-T Easy Vector Ligation Reactions

Use high-efficiency competent cells (≥1 × 10^8cfu/µg DNA) for transformations. The ligation of fragments with a single-base overhang can be inefficient, so it is essential to use cells with a transformation efficiency of 1 × 10^8cfu/µg DNA (or higher) in order to obtain a reasonable number of colonies (see Section VI.E).

We recommend using JM109 High Efficiency Competent Cells (Cat.# L2001); these cells are provided with the pGEM®-T and pGEM®-T Easy Vector Systems II. Other host strains may be used, but they should be compatible with blue/white color screening and standard ampicillin selection.

JM109 cells should be maintained on M9 minimal medium plates supplemented with thiamine hydrochloride prior to the preparation of competent cells. This selects for the presence of the F′ episome, containing both the *pro*AB genes, which complement proline auxotrophy in a host with a (*pro*AB) deletion, and *lac*I^qZΔM15, required for blue/white screening. If you are using competent cells other than JM109 High Efficiency Competent Cells purchased from Promega, it is important that the appropriate transformation protocol be followed. Selection for transformants should be on LB/ampicillin/IPTG/X-Gal plates (see Section XI.C). For best results, do not use plates that are more than 1 month old.

The genotype of JM109 is *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rK–,mK+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^qZΔM15] (5).

Materials to Be Supplied by the User
(Solution compositions are provided in Section XI.C.)

- LB plates with ampicillin/IPTG/X-Gal
- SOC medium

1. Prepare two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency (see Section VI.E). Equilibrate the plates to room temperature prior to plating (Step 10).
2. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to a sterile 1.5ml microcentrifuge tube on ice (see Note 1). Set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells (see Section VI.E).
3. Remove tube(s) of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube.

Note: Avoid excessive pipetting, as the competent cells are extremely fragile.

4. Carefully transfer 50µl of cells into each tube prepared in Step 2 (100µl cells for determination of transformation efficiency).
5. Gently flick the tubes to mix and place them on ice for 20 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (Do not shake).
7. Immediately return the tubes to ice for 2 minutes.
8. Add 950µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).
9. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
10. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × *g* for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of two plates.
11. Incubate the plates overnight (16–24 hours) at 37°C. In our experience, if 100µl is plated approximately 100 colonies per plate are routinely seen when using competent cells that are 1 × 10^8cfu/µg DNA. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue color development. White colonies generally contain inserts; however, inserts may also be present in blue colonies. Please see Section VI.D for more information.

Notes:

1. In our experience, the use of larger (17 × 100mm) polypropylene tubes (e.g., Falcon Cat.# 2059) has been observed to increase transformation efficiency. Tubes from some manufacturers bind DNA, thereby decreasing the colony number, and should be avoided.
2. Colonies containing β-galactosidase activity may grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies may be smaller than the white colonies, which are approximately one millimeter in diameter.

VI. General Considerations

VI.A. PCR Product Purity

An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281). Exposure to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers. If smearing of the PCR product or inappropriate banding is observed on the gel, excise the bands to be cloned and purify the DNA with Wizard® SV Gel and PCR Clean-Up System. Even if distinct bands of the expected size are observed, primer-dimers should be removed by gel purification or by using the Wizard® SV Gel and PCR Clean-Up System. Use of crude PCR product may produce successful ligation in some cases; however, the number of white colonies containing the relevant insert may be reduced due to preferential incorporation of primer-dimers or other extraneous reaction products. Therefore, it may be necessary to screen numerous colonies in order to identify clones that contain the PCR product of interest.

VI.B. Blunt-Ended PCR Products

Thermostable DNA polymerases with proofreading activity, such as *Pfu* DNA Polymerase (Cat.# M7741), *Pwo* DNA polymerase and *Tli* DNA Polymerase (Cat.# M7101) generate blunt-ended fragments during PCR amplification. Nevertheless, PCR fragments generated using these polymerases can be modified using the A-tailing procedure outlined in Figure 4 and ligated into the pGEM®-T and pGEM®-T Easy Vectors (6). Using this method, only one insert will be ligated into the vector as opposed to multiple insertions that can occur with blunt-ended cloning. In addition, with T-vector cloning there is no need to dephosphorylate the vector, and there is a low background of religated vector.

Using this procedure with optimized insert:vector ratios, 55–95% recombinants were obtained when *Pfu* and *Tli* DNA Polymerases were used to generate the insert DNA (Table 2). It is critical that the PCR fragments are purified using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) or by direct isolation from a gel by other means. In the absence of purification, the proofreading activity of the *Pfu*, *Pwo* and *Tli* DNA Polymerases will degrade the PCR fragments, or remove the 3′-terminal deoxyadenosine added during tailing or the 3′-terminal deoxythymidine from the vector during the A-tailing reaction or ligation.

To optimize cloning efficiency, the amount of DNA in the A-tailing reaction and the ligation volumes must be adjusted depending on the molar yield of the purified PCR product. When molar concentrations are high due to small fragment size and/or good amplification, small volumes of the PCR fragment are needed for the A-tailing and ligation reactions. However, when molar concentration is low due to large fragment size and/or poor amplification,

large volumes of the PCR fragment are needed for the A-tailing and ligation reactions. We have successfully used 1–7µl of purified PCR fragment in A-tailing reactions to optimize the insert:vector ratio. (See Section VI.C for further discussion of optimizing the insert:vector ratio.) Recombinants were identified by blue/white screening, and 70–100% were shown to have the correct size insert by PCR. Few recombinants were observed in control reactions in which the PCR fragment was not tailed. These control results confirm that the majority of the pGEM®-T Easy Vector used contained 3′-terminal deoxythymidine and that, during the A-tailing, *Taq* DNA Polymerase added a 3′-terminal deoxyadenosine to a significant proportion of the PCR fragments.

Table 2. Comparison of A-Tailing Procedures Used With Different DNA Polymerases.

	% Recombinants[1]			
	1-Hour Ligation at 24°C (Standard)		16-Hour Ligation at 4°C (Alternative)	
Polymerase	**542bp**	**1.8kb**	**542bp**	**1.8kb**
Pfu DNA Polymerase	65–84%[2]	31–55%[3]	81–95%[2]	50–75%[3]
Tli DNA Polymerase	68–77%[4]	37–65%[5]	85–93%[4]	60–81%[5]

PCR fragments generated by *Pfu* and *Tli* DNA Polymerase were A-tailed and ligated into pGEM®-T Easy Vector for 1 hour at 24°C or for 16 hours at 4°C. Two microliters of ligation mix was transformed into JM109 Competent Cells and plated on LB/amp/IPTG/X-gal plates.

[1]% Recombinants = % white and/or pale blue colonies. PCR fragments were purified with the Wizard® PCR Preps DNA Purification System prior to A-tailing.

[2]Insert:vector ratios tested: 5:1, 3:1, 1:1. Volume of PCR amplification product used in A-tailing: 1–2µl.

[3]Insert:vector ratios tested: 3:1, 2:1, 1:1. Volume of PCR amplification product used in A-tailing: 3–7µl.

[4]Insert:vector ratios tested: 3:1, 2:1, 1:1. Volume of PCR amplification product used in A-tailing: 1–2µl.

[5]Insert:vector ratios tested: 2:1, 1:1. Volume of PCR amplification product used in A-tailing: 4–7µl.

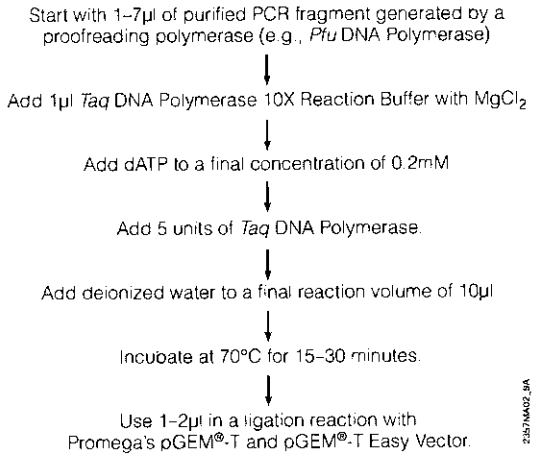


Figure 4. An A-tailing procedure for blunt-ended PCR fragments purified with the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) and used in T-vector cloning.

VI.C. Optimizing Insert:Vector Molar Ratios

The pGEM®-T and pGEM®-T Easy Vector Systems have been optimized using a 1:1 molar ratio of the Control Insert DNA to the vectors. However, ratios of 8:1 to 1:8 have been used successfully. If initial experiments with your PCR product are suboptimal, ratio optimization may be necessary. Ratios from 3:1 to 1:3 provide good initial parameters. The concentration of PCR product should be estimated by comparison to DNA mass standards on a gel or by using a fluorescent assay (7). The pGEM®-T and pGEM®-T Easy Vectors are approximately 3kb and are supplied at 50ng/µl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Example of insert:vector ratio calculation:

How much 0.5kb PCR product should be added to a ligation in which 50ng of 3.0kb vector will be used if a 3:1 insert:vector molar ratio is desired?

$$\frac{50\text{ng vector} \times 0.5\text{kb insert}}{3.0\text{kb vector}} \times \frac{3}{1} = 25\text{ng insert}$$

Using the same parameters for a 1:1 insert:vector molar ratio, 8.3ng of a 0.5kb insert would be required.

VI.D. Screening Transformants for Inserts

Successful cloning of an insert into the pGEM®-T and pGEM®-T Easy Vectors interrupts the coding sequence of β-galactosidase; recombinant clones can usually be identified by color screening on indicator plates. However, the characteristics of PCR products cloned into the pGEM®-T and pGEM®-T Easy Vectors can significantly affect the ratio of blue:white colonies obtained following transformation of competent cells. Clones that contain PCR products, in most cases, produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lacZ* gene. Such fragments are usually a multiple of 3 base pairs long (including the 3′-A overhangs), and do not contain in-frame stop codons. There have been reports of DNA fragments of up to 2kb that have been cloned in-frame and have produced blue colonies.

Even if your PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (e.g., deletions or point mutations) that may result in blue colonies when competent cells are transformed with the fragment inserted into the pGEM®-T or pGEM®-T Easy Vectors.

The Control Insert DNA supplied with the pGEM®-T and pGEM®-T Easy Systems is a 542bp fragment from pGEM®-*luc* Vector DNA (Cat.# E1541). This sequence has been mutated to contain multiple stop codons in all six reading frames, which ensures a low background of blue colonies for the control reaction. Results obtained with the Control Insert DNA may not be representative of those achieved with your PCR product.

VI.E. Experimental Controls

Promega strongly recommends performing the controls described below. These are necessary to accurately assess the performance of the pGEM®-T and pGEM®-T Easy Vector Systems.

Positive Control

Set up a ligation reaction with the Control Insert DNA as described in Section IV and use it for transformations as described in Section V. This control will allow you to determine whether the ligation is proceeding efficiently. Typically, approximately 100 colonies should be observed, 10–40% of which are blue, when competent cells that have a transformation efficiency of 1×10^8cfu/µg DNA are transformed. Greater than 60% of the colonies should be white. The Control Insert DNA is designed to produce white colonies; however, other insert DNA may not yield white colonies (see Section VI.D). Background blue colonies from the positive control ligation reaction arise from non-T-tailed or undigested pGEM®-T or pGEM®-T Easy Vector. These blue colonies are a useful internal transformation control; if no colonies are obtained, the transformation has failed. If blue colonies are obtained, but no whites, the ligation reaction may have failed. If <50% white colonies are seen in the positive control reaction, then the ligation conditions were probably suboptimal.

The concentration of the Control Insert DNA is such that 2µl (4ng/µl) can be used in a 10µl ligation reaction to achieve a 1:1 molar ratio with 50ng of the pGEM®-T or pGEM®-T Easy Vectors.

Background Control

Set up a ligation reaction with 50ng of pGEM®-T or pGEM®-T Easy Vector and no insert as described in Section IV and use it for transformations as described in Section V. This control allows determination of the number of background blue colonies resulting from non-T-tailed or undigested pGEM®-T or pGEM®-T Easy Vector alone. If the recommendations in Section V are followed, 10–30 blue colonies will typically be observed if the transformation efficiency of the competent cells is 1×10^8cfu/µg DNA. (Under these conditions, cells that have an efficiency of 1×10^7cfu/µg DNA would yield 1–3 blue colonies and cells with a transformation efficiency of 1×10^9cfu/µg DNA would yield 100–300 blue colonies). Compare the number of blue colonies obtained with this background control to the number of blue colonies obtained in the standard reaction using the PCR product. If ligation of the PCR product yields dramatically more blue colonies than the background control reaction, then recombinants are probably among these blue colonies (see Section VI.D).

Transformation Control

Check the transformation efficiency of the competent cells by transforming them with an uncut plasmid (not pGEM®-T or pGEM®-T Easy since these vectors are linearized) and calculating cfu/µg DNA. If the transformation efficiency is lower than 1×10^8cfu/µg DNA, prepare fresh cells. (Competent cells are available from Promega. See Section XI.D.) If you are not using JM109 High Efficiency Competent Cells (provided with pGEM®-T and pGEM®-T Easy Vector Systems II; Cat.# A3610 and A1380, respectively), be sure the cells are compatible with blue/white screening and standard ampicillin selection and have a transformation efficiency of at least 1×10^8cfu/µg DNA.

Example of Transformation Efficiency Calculation

After 100µl competent cells are transformed with 0.1ng uncut plasmid DNA, the transformation reaction is added to 900µl of SOC medium (0.1ng DNA/ml). From that volume, a 1:10 dilution with SOC medium (0.01ng DNA/ml) is made and 100µl plated on two plates (0.001ng DNA/100µl). If 200 colonies are obtained (average of two plates), what is the transformation efficiency?

$$\frac{200\text{cfu}}{0.001\text{ng}} = 2 \times 10^5\text{cfu/ng} = 2 \times 10^8\text{cfu/µg DNA}$$

VII. Isolation of Recombinant Plasmid DNA

Standard plasmid miniprep procedures may be used to isolate the recombinant plasmid DNA. The DNA Purification Chapter of the Promega *Protocols and Applications Guide* provides an overview of plasmid DNA purification methods (8). A convenient and reliable method is the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330).

VIII. Generation of Single-Stranded DNA from the pGEM®-T and pGEM®-T Easy Vectors

For induction of ssDNA production, bacterial cells containing either the pGEM®-T or pGEM®-T Easy Vector are infected with an appropriate helper phage (e.g., R408 Helper Phage, Cat.# P2291). The plasmid then enters the f1 replication mode, and the resulting ssDNA is exported as an encapsulated virus-like particle. The ssDNA is purified from the supernatant by simple precipitation and extraction procedures (9). For further information, please contact your local Promega Branch Office or Distributor. In the U.S., contact Technical Services at 1-800-356-9526.

IX. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptom	Causes and Comments
No colonies	A problem has occurred with the transformation reaction or the cells have lost competence. Background undigested vector and religated non-T-tailed vector should yield 10–30 blue colonies independent of the presence of insert DNA. Check the background control (Section VI.E).
	Use high-efficiency competent cells (≥1 × 10^8cfu/µg DNA). Test the efficiency by transforming the cells with an uncut plasmid that allows for antibiotic selection, such as the pGEM®-5Zf(+) Vector. If the guidelines in Section V.A are followed, cells at 1 × 10^8cfu/µg DNA typically yield 100 colonies. Therefore, you would not see any colonies from cells that are <1 × 10^7cfu/µg DNA (Section VI.E).
Less than 10% white colonies with Control Insert DNA	Improper dilution of the 2X Rapid Ligation Buffer. The T4 DNA ligase buffer is provided at a concentration of 2X. Use 5µl in a 10µl reaction.
	If the total number of colonies is high, but there are few/no white colonies, competent cells may be high efficiency (≥1 × 10^9cfu/µg) but there may be a ligation problem. Approximately 1,000 colonies can be obtained from the positive control ligation using cells that are 10^9cfu/µg DNA, with 70–90% white colonies. If ligation is suboptimal or fails, the total number of colonies will be high (up to 300 cells at 1 × 10^9cfu/µg), but the amount of white colonies will be low or zero. See comments below on ligation failure.

IX. Troubleshooting (continued)

Symptoms	Causes and Comments
Less than 10% white colonies with Control Insert DNA (continued)	Ligation reaction has failed. Ligase buffer may have low activity. The 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles by making single-use aliquots of the buffer. Use a fresh vial of buffer. To test the activity of the ligase and buffer, set up a ligation with ~20ng of DNA markers (e.g., Lambda DNA/*Hin*d III Markers, Cat.# G1711). Compare ligated and nonligated DNA on a gel and check that the fragments have been religated into high-molecular-weight material.
	T-overhangs have been removed, allowing blunt-ended ligation of vector and giving rise to more blue than white colonies. Avoid introduction of nucleases, which may degrade the T-overhangs. Use only the T4 DNA Ligase provided with the system, which has been tested for minimal exonuclease activity.
Less than 60% white colonies with Control Insert DNA	Improper dilution of the Rapid Ligation Buffer. The Rapid Ligation Buffer is provided at a 2X concentration. Use 5µl in a 10µl reaction.
	T-overhangs have been removed, allowing blunt-ended ligation of vector and giving rise to more blue than white colonies. Avoid introduction of nucleases, which may degrade the T-overhangs. Use only the T4 DNA Ligase provided with the system, which has been tested for minimal exonuclease activity.
	Ligation temperature is too high. Higher temperatures (>28°C) give rise to increased background and fewer recombinants.
Low number or no white colonies containing PCR product	Improper dilution of the Rapid Ligation Buffer. The Rapid Ligation Buffer is provided at a 2X concentration. Use 5µl in a 10µl reaction.
	Ligation incubation is not long enough. Optimal results are seen with an overnight ligation.
	Failed ligation due to an inhibitory component in the PCR product. Mix some of the PCR product with the positive control ligation to determine whether an inhibitor is present. If an inhibitor is indicated, repurify the PCR fragment.

IX. Troubleshooting (continued)

Symptoms	Causes and Comments
Low number or no white colonies containing PCR product (continued)	PCR product is not ligating because there are no 3′-A overhangs. As summarized in Table 1, not all thermostable DNA polymerases create a 3′-A overhang (3,4). Blunt-ended fragments may be subsequently A-tailed by treatment with an appropriate polymerase and dATP (10-12).
	PCR product cannot be ligated due to pyrimidine dimers formed from UV over-exposure. This is a common problem with gel-purified DNA. There is no way to fix this; the DNA must be remade. Exposure to shortwave UV should be limited as much as possible. Use a glass plate between the gel and UV source to decrease UV overexposure. If possible, only visualize the PCR product using a longwave UV source.
	The PCR fragment is inserted, but it is not disrupting the *lacZ* gene. If there are a higher number of blue colonies resulting from the PCR fragment ligation than with the background control, some of these blue colonies may contain insert. Screen blue and pale blue colonies (see Section VI.D).
	Insert:vector ratio is not optimal. Check the integrity and quantity of your PCR fragment by gel analysis. Optimize the insert:vector ratio (see Section VI.C).
	There may be primer-dimers present in PCR fragment preparation. Primer-dimers will ligate into the pGEM®-T or pGEM®-T Easy Vector but may not be seen after restriction digestion and gel analysis because of their small size. The vector will appear to contain no insert. More blue colonies may be seen with the ligation than on the background control plates. The PCR fragment should be gel-purified.
	Multiple PCR products may have been generated and cloned into the pGEM®-T or pGEM®-T Easy Vector. Gel-purify the PCR fragment of interest.

IX. Troubleshooting (continued)

Symptom	Causes and Comments
Low number or no white colonies with PCR product (continued)	DNA has rearranged. Check a number of clones to see whether the rearrangement is random. If so, the clone of interest should be present and can be identified by screening several clones. If the same rearrangement is found in all of the clones, use of a repair-deficient bacterial strain (e.g., SURE® cells) may reduce recombination events.
PCR product ligation reaction produces white colonies only (no blue colonies)	Ampicillin is inactive, allowing ampicillin-sensitive cells to grow. Check that ampicillin plates are made properly and used within one month. Test ampicillin activity by streaking plates, with and without ampicillin, using an ampicillin-sensitive clone.
	The bacterial strain (e.g., JM109) has lost its F′ episome. Check the background control. If these colonies are not blue, the cells may have lost the F′ episome (assuming *lacI*^{q}ZΔM15 is located on the F′ in the transformed strain and appropriate plates were used). Be sure that the cells are prepared properly for use with this system (see Section V).
	Plates are incompatible with blue/white screening. Check the background control. If these colonies are not blue, check that the plates have ampicillin/IPTG/X-Gal and are fresh. If there is any question about the quality of the plates, repeat plating with fresh plates.
Not enough clones contain the PCR product of interest	Insufficient A-tailing of the PCR fragment. After purification of the PCR fragment, set up an A-tailing reaction (10–12). Clean up the sample and proceed with the protocol.
	Insert:vector ratio is not optimal. Check the integrity and quality of your PCR fragment by gel analysis. Optimize the insert:vector ratio (see Section VI.C).
	Multiple PCR products are generated and cloned into the pGEM®-T or pGEM®-T Easy Vector. Gel purify the PCR fragment of interest.

X. References

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XI. Appendix

XI.A. pGEM®-T Vector Restriction Enzyme Sites

The pGEM®-T Vector is derived from the circular pGEM®-5Zf(+) Vector (GenBank® Accession No. X65308). The pGEM®-5Zf(+) Vector sequence is available on the Internet at: **www.promega.com/vectors/**

The following restriction enzyme tables are based on those of the circular pGEM®-5Zf(+) Vector. The pGEM®-T Vector has been created by linearizing the pGEM®-5Zf(+) Vector with *Eco*R V at base 51 and adding a T to both 3′-ends. This site will not be recovered upon ligation of the vector and insert. The tables below were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3′-end of the cut DNA (the base to the left of the cut site). Please contact your local Promega Branch Office or Distributor if you identify a discrepancy. In the U.S., contact Technical Services at 1-800-356-9526.

Table 3. Restriction Enzymes That Cut the pGEM®-T Vector 1- 5 Times.

Enzyme	# of Sites	Location
***Aat* II**	1	20
***Acc* I**	1	76
Acy I	2	17, 1932
Afl III	2	99, 502
***Alw*26 I**	2	1456, 2232
***Alw*44 I**	2	816, 2062
*Alw*N I	1	918
***Apa* I**	1	14
*Asp*H I	4	94, 820, 1981, 2066
***Ava* II**	2	1533, 1755
***Ban* I**	3	246, 1343, 2626
***Ban* II**	3	14, 94, 2664
***Bbu* I**	1	26
***Bgl* I**	3	39, 1515, 2833
Bsa I	1	1456
*Bsa*A I	1	2589
*Bsa*H I	2	17, 1932
*Bsa*J I	5	37, 43, 241, 662, 2936
*Bsp*120 I	1	10
*Bsp*H I	2	1222, 2230
*Bsp*M I	1	62
*Bss*S I	2	675, 2059
***Bst*O I**	5	242, 530, 651, 664, 2937
***Bst*X I**	1	103
***Bst*Z I**	2	31, 62
*Cfr*10 I	2	1475, 2690
***Dde* I**	4	777, 1186, 1352, 1892
***Dra* I**	3	1261, 1280, 1972
Dra III	1	2589
Drd I	2	610, 2544
Dsa I	2	37, 43
Eag I	2	31, 62
Ear I	3	386, 2190, 2878
***Ecl*HK I**	1	1395
*Eco*52 I	2	31, 62
***Eco*ICR I**	1	92
***Eco*R V**	1	51*
***Fok* I**	5	119, 1361, 1542, 1829, 2919
Fsp I	2	1617, 2840
***Hae* II**	4	380, 750, 2740, 2748
Hga I	4	613, 1191, 1921, 2806
***Hinc* II**	1	77
Hind II	1	77
***Hsp*92 I**	2	17, 1932
Mae I	5	56, 997, 1250, 1585, 2740
***Mlu* I**	1	99
***Nae* I**	1	2692
***Nci* I**	4	30, 882, 1578, 1929
***Nco* I**	1	37
***Nde* I**	1	82
***Ngo*M IV**	1	2690
***Not* I**	1	62
***Nsi* I**	1	112
Nsp I	2	26, 506
*Ppu*10 I	1	108
***Pst* I**	1	73
***Pvu* I**	2	1765, 2861
***Pvu* II**	2	326, 2890
***Rsa* I**	1	1875
***Sac* I**	1	94
***Sac* II**	1	46
***Sal* I**	1	75
***Sca* I**	1	1875
***Sfi* I**	1	39
***Sin* I**	2	1533, 1755
***Spe* I**	1	55
***Sph* I**	1	26
*Sse*8387 I	1	73
***Ssp* I**	2	2199, 2381
***Sty* I**	1	37
***Taq* I**	4	76, 602, 2046, 2622
Tfi I	2	337, 477
***Vsp* I**	3	273, 332, 1567
***Xmn* I**	1	1994

*The pGEM®-T Vector has been created by linearizing the pGEM®-5Zf(+) Vector with *Eco*R V at base 51 and adding a T to both 3′-ends. This site will not be recovered upon ligation of the vector and insert.

Note: The enzymes listed in boldface type are available from Promega.

Table 4. Restriction Enzymes That Do Not Cut the pGEM®-T Vector.

***Acc*B7 I**	*Bbs* I	***Bst*E II**	*Fse* I	*Pin*A I	*Spl* I
***Acc* III**	***Bcl* I**	***Bsu*36 I**	***Hin*d III**	*Pme* I	*Srf* I
***Acc*65 I**	***Bgl* II**	***Cla* I**	***Hpa* I**	*Pml* I	***Stu* I**
Afl II	*Blp* I	***Csp* I**	**I-*Ppo* I**	*Ppu*M I	*Swa* I
***Age* I**	*Bpu*1102 I	***Csp*45 I**	*Kas* I	*Psh*A I	***Tth*111 I**
Asc I	*Bsa*B I	*Dra* II	***Kpn* I**	*Psp*5 II	***Xba* I**
***Ava* I**	***Bsa*M I**	***Eco*47 III**	***Nar* I**	*Psp*A I	*Xcm* I
Avr II	*Bsm* I	*Eco*72 I	***Nhe* I**	*Rsr* II	***Xho* I**
***Bal* I**	*Bsr*G I	*Eco*81 I	***Nru* I**	***Sgf* I**	***Xma* I**
***Bam*H I**	***Bss*H II**	*Eco*N I	*Pac* I	*Sgr*A I	
Bbe I	*Bst*1107 I	***Eco*R I**	*Pae*R7 I	***Sma* I**	
*Bbr*P I	***Bst*98 I**	*Ehe* I	*Pfl*M I	***Sna*B I**	

Table 5. Restriction Enzymes that Cut the pGEM®-T Vector 6 or More Times.

Aci I	*Bst*71 I	***Hae* III**	*Mae* III	***Nde* II**	*Sfa*N I
***Alu* I**	*Bst*U I	***Hha* I**	***Mbo* I**	*Nla* III	***Tru*9 I**
Bbv I	***Cfo* I**	***Hinf* I**	***Mbo* II**	*Nla* IV	***Xho* II**
*Bsa*O I	***Dpn* I**	***Hpa* II**	*Mnl* I	*Ple* I	
***Bsp*1286 I**	*Dpn* II	*Hph* I	*Mse* I	***Sau*3A I**	
Bsr I	*Eae* I	***Hsp*92 II**	***Msp* I**	*Sau*96 I	
***Bsr*S I**	*Fnu*4H I	*Mae* II	***Msp*A1 I**	*Scr*F I	

Note: The enzymes listed in boldface type are available from Promega.

XI.B. pGEM®-T Easy Vector Restriction Enzyme Sites

The sequence of the pGEM®-T Easy Vector is available on the Internet at:
www.promega.com/vectors/
The pGEM®-T Easy Vector has been linearized at base 60 with *Eco*R V and a T added to both 3′-ends. This site will not be recovered upon ligation of the vector and insert. The tables below were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3′-end of the cut DNA (the base to the left of the cut site). Please contact your local Promega Branch Office or Distributor if you identify a discrepancy. In the U.S., contact Technical Services at 1-800-356-9526.

Table 6. Restriction Enzymes that Cut the pGEM®-T Easy Vector 1–5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
***Aat* II**	1	20	***Fok* I**	5	134, 1376, 1557, 1844, 2931
***Acc* I**	1	91	*Fsp* I	2	1632, 2855
Acy I	2	17, 1947	***Hae* II**	4	395, 765, 2755, 2763
Afl III	2	114, 517	*Hga* I	4	628, 1206, 1936, 2821
***Alw*26 I**	2	1471, 2247	***Hinc* II**	1	92
***Alw*44 I**	2	831, 2077	*Hind* II	1	92
*Alw*N I	1	933	***Hsp*92 I**	2	17, 1947
***Apa* I**	1	14	*Mae* I	5	65, 1012, 1265, 1600, 2755
*Asp*H I	4	109, 835, 1996, 2081	***Mlu* I**	1	114
***Ava* II**	2	1548, 1770	***Nae* I**	1	2707
***Ban* I**	3	261, 1358, 2641	***Nci* I**	4	30, 897, 1593, 1944
***Ban* II**	3	14, 109, 2679	***Nco* I**	1	37
***Bbu* I**	1	26	***Nde* I**	1	97
***Bgl* I**	4	39, 42, 1530, 2848	***Ngo*M IV**	1	2705
Bsa I	1	1471	***Not* I**	2	43, 77
*Bsa*A I	1	2604	***Nsi* I**	1	127
*Bsa*H I	2	17, 1947	*Nsp* I	2	26, 521
*Bsa*J I	5	37, 46, 256, 677, 2951	*Ppu*10 I	1	123
*Bsp*120 I	1	10	***Pst* I**	1	88
*Bsp*H I	2	1237, 2245	***Pvu* I**	2	1780, 2876
*Bsp*M I	1	77	***Pvu* II**	2	341, 2905
*Bss*S I	2	690, 2074	***Rsa* I**	1	1890
***Bst*O I**	5	257, 545, 666, 679, 2952	***Sac* I**	1	109
***Bst*X I**	1	118	***Sac* II**	1	49
***Bst*Z I**	3	31, 43, 77	***Sal* I**	1	90
*Cfr*10 I	2	1490, 2705	***Sca* I**	1	1890
***Dde* I**	4	792, 1201, 1367, 1907	***Sin* I**	2	1548, 1770
***Dra* I**	3	1276, 1295, 1987	***Spe* I**	1	64
Dra III	1	2604	***Sph* I**	1	26
Drd I	2	625, 2559	*Sse*8387 I	1	88
Dsa I	2	37, 46	***Ssp* I**	2	2214, 2396
Eag I	3	31, 43, 77	***Sty* I**	1	37
Ear I	3	401, 2205, 2893	***Taq* I**	5	56, 91, 617, 2061, 2637
***Ecl*HK I**	1	1410	*Tfi* I	2	352, 492
*Eco*52 I	3	31, 43, 77	***Vsp* I**	3	288, 347, 1582
***Eco*ICR I**	1	107	***Xmn* I**	1	2009
***Eco*R I**	2	52, 70			
***Eco*R V**	1	60*			

*The pGEM®-T Easy Vector has been linearized at base 60 with *Eco*R V and a T added to both 3′-ends. This site will not be recovered upon ligation of the vector and insert.

Note: The enzymes listed in boldface type are available from Promega.

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Table 7. Restriction Enzymes That Do Not Cut the pGEM®-T Easy Vector.

*Acc*B7 I	*Bbs* I	***Bst*E II**	***Hind* III**	*Pme* I	*Spl* I
Acc III	***Bcl* I**	***Bsu*36 I**	***Hpa* I**	*Pml* I	*Srf* I
*Acc*65 I	***Bgl* II**	***Cla* I**	**I-*Ppo* I**	*Ppu*M I	***Stu* I**
Afl II	*Blp* I	***Csp* I**	*Kas* I	*Psh*A I	*Swa* I
***Age* I**	*Bpu*1102 I	***Csp*45 I**	***Kpn* I**	*Psp*5 II	***Tth*111 I**
Asc I	*Bsa*B I	*Dra* II	***Nar* I**	*Psp*A I	***Xba* I**
***Ava* I**	***Bsa*M I**	***Eco*47 III**	***Nhe* I**	*Rsr* II	*Xcm* I
Avr II	*Bsm* I	*Eco*72 I	***Nru* I**	***Sfi* I**	***Xho* I**
***Bal* I**	*Bsr*G I	*Eco*81 I	*Pac* I	***Sgf* I**	***Xma* I**
***Bam*H I**	***Bss*H II**	*Eco*N I	*Pae*R7 I	*Sgr*A I	
Bbe I	*Bst*1107 I	*Ehe* I	*Pfl*M I	***Sma* I**	
*Bbr*P I	***Bst*98 I**	*Fse* I	*Pin*A I	***Sna*B I**	

Table 8. Restriction Enzymes that Cut the pGEM®-T Easy Vector 6 or More Times.

Aci I	*Bst*71 I	***Hae* III**	*Mae* III	***Nde* II**	*Sfa*N I
***Alu* I**	*Bst*U I	***Hha* I**	***Mbo* I**	*Nla* III	***Tru*9 I**
Bbv I	***Cfo* I**	***Hinf* I**	***Mbo* II**	*Nla* IV	***Xho* II**
*Bsa*O I	***Dpn* I**	***Hpa* II**	*Mnl* I	*Ple* I	
***Bsp*1286 I**	*Dpn* II	*Hph* I	*Mse* I	***Sau*3A I**	
Bsr I	*Eae* I	***Hsp*92 II**	***Msp* I**	*Sau*96 I	
***Bsr*S I**	*Fnu*4H I	*Mae* II	***Msp*A1 I**	*Scr*F I	

Note: The enzymes listed in boldface type are available from Promega.

XI.C. Composition of Buffers and Solutions

IPTG stock solution (0.1M)

1.2g IPTG

Add water to 50ml final volume. Filter-sterilize and store at 4°C.

X-Gal (2ml)

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Dissolve in 2ml N,N´-dimethyl-formamide. Cover with aluminum foil and store at -20°C.

LB medium (per liter)

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Adjust pH to 7.0 with NaOH.

LB plates with ampicillin

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml. Pour 30-35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal may be spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

SOC medium (100ml)

2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml 2M Mg^{2+} stock, filter-sterilized
1ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring to 100ml with sterile, distilled water. The final pH should be 7.0.

2M Mg^{2+} stock

20.33g $MgCl_2 \cdot 6H_2O$
24.65g $MgSO_4 \cdot 7H_2O$

Add distilled water to 100ml. Filter sterilize.

2X Rapid Ligation Buffer, T4 DNA Ligase (provided)

60mM Tris-HCl (pH 7.8)
20mM $MgCl_2$
20mM DTT
2mM ATP
10% polyethylene glycol (MW8000, ACS Grade)

Store in single-use aliquots at -20°C. Avoid multiple freeze-thaw cycles.

TYP broth (per liter)

16g Bacto®-tryptone
16g Bacto®-yeast extract
5g NaCl
2.5g K_2HPO_4

XI.D. Related Products

PCR Cloning Systems

Product	Size	Cat.#
pTARGET™ Mammalian Expression Vector System	20 reactions	A1410

Direct mammalian expression from a T-Vector.

Amplification Products

Please request our Amplification Products Brochure #BR139 or visit our Web site at **www.promega.com/applications/pcr/** to see a complete listing of our amplification products.

RT-PCR Systems

Product	Size	Cat.#
Access RT-PCR System	20 reactions	A1260
	100 reactions	A1250
	500 reactions	A1280
AccessQuick™ RT-PCR System	20 reactions	A1701
	100 reactions	A1702
	500 reactions	A1703
ImProm-II™ Reverse Transcription System	100 reactions	A3800

For Laboratory Use.

PCR Purification Systems

Product	Size	Cat.#
Wizard® SV Gel and PCR Clean-Up System	50 preps	A9281
	250 preps	A9282
Wizard® SV 96 PCR Clean-Up System	1 × 96 preps	A9340
	4 × 96 preps	A9341
	8 × 96 preps	A9342
Wizard® MagneSil® PCR Clean-Up System	4 × 96 preps	A1930
	8 × 96 preps	A1931
	100 × 96 preps	A1935

For Laboratory Use.

dNTPs

Product	Size	Cat.#
PCR Nucleotide Mix (10mM each)	200µl	C1141
	1,000µl	C1145
dATP, dCTP, dGTP, dTTP, each at 100mM	10µmol of each	U1330
dATP, dCTP, dGTP, dTTP, each at 100mM	40µmol of each	U1240
dATP, dCTP, dGTP, dTTP, each at 100mM	200µmol of each	U1410

For Laboratory Use.

Sequencing Primers

Product	Size	Cat.#
SP6 Promoter Primer	2µg	Q5011
T7 Promoter Primer	2µg	Q5021
pUC/M13 Primer, Forward (24mer)	2µg	Q5601
pUC/M13 Primer, Reverse (22mer)	2µg	Q5421

Competent Cells

Product	Size	Cat.#
JM109 Competent Cells, >10^8cfu/µg	5 × 200µl	L2001

For Laboratory Use.

Accessory Products

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

For Laboratory Use.

Promega

[a]Licensed under one or more of U.S. Pat. Nos. 5,487,993 and 5,827,657 and European Pat. No. 0 550 693.

[b]Licensed under U.S. Pat. No. 5,075,430.

[c]The PCR process is covered by patents issued and applicable in certain countries*. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

*In Europe, effective March 28, 2006, European Pat. Nos. 201,184 and 200,362, will expire. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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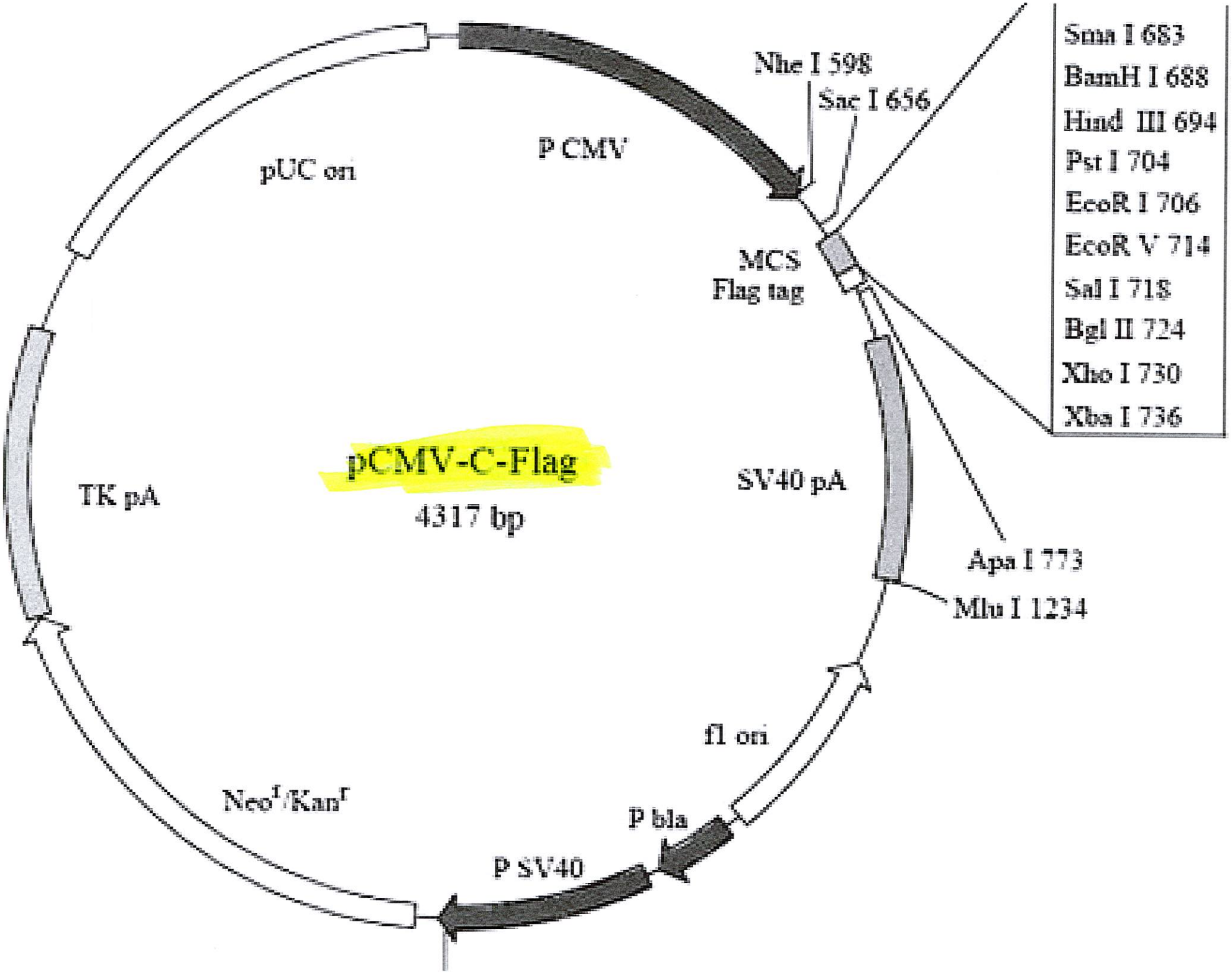
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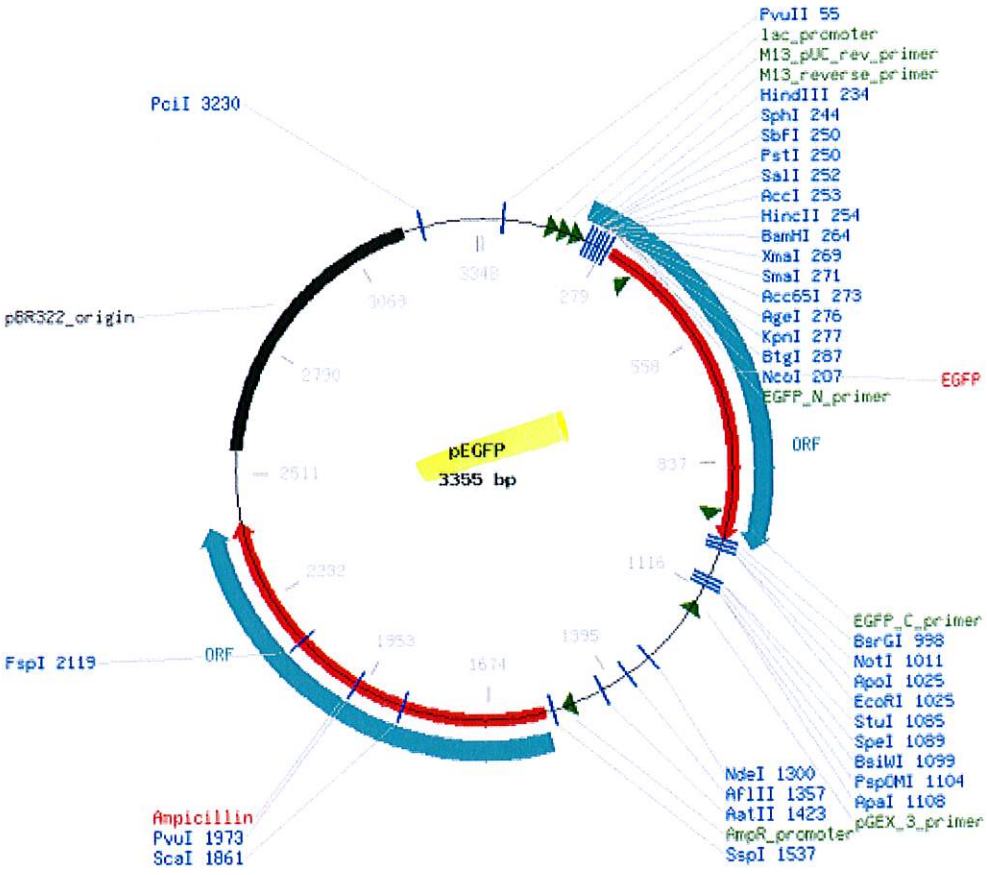
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Acc65I 273
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BtgI 287
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EGFP
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EcoRI 1025
StuI 1085
SpeI 1089
BsiWI 1099
PspOMI 1104
ApaI 1108
pGEX_3_primer
NdeI 1300
AflII 1357
AatII 1423
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Ampicillin
PvuI 1973
ScaI 1861
FspI 2119
ORF
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PciI 3230
1
279
558
837
1116
1395
1674
1953
2232
2511
2790
3069
3348

Invitrogen™
life technologies

Instruction Manual

pcDNA™3.1 Directional TOPO® Expression Kit

Five-minute, directional TOPO® Cloning of blunt-end PCR products into a mammalian expression vector

Catalog nos. K4900-01, K4900-40

Version D
5 January 2004
25-0396

Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the directional TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Step	Action
Design PCR Primers	• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with any epitope tags, if desired.
Amplify Your Gene of Interest	1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity of your PCR product.
Perform the TOPO® Cloning Reaction	1. Set up the following TOPO® Cloning reaction. **For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.** **Note:** If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in the TOPO® Cloning reaction. Fresh PCR product — 0.5 to 4 µl Salt Solution — 1 µl Sterile water — add to a final volume of 5 µl TOPO® vector — 1 µl Total volume — 6 µl 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot® TOP10 chemically competent *E. coli*, below.
Transform TOP10 Chemically Competent *E. coli*	1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µl of room temperature S.O.C. medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 50-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kits to perform the control reaction. See the protocol on pages 22-23 for instructions.

Important Information

Shipping/Storage

The pcDNA™3.1 Directional TOPO® Expression Kit is shipped on dry ice. Each kit contains a box of pcDNA™3.1D/V5-His TOPO® reagents (Box 1) and a box of One Shot® TOP10 chemically competent *E. coli* (Box 2). **Store Box 1 at -20°C and Box 2 at -80°C.**

Types of Kits

This manual is supplied with the following kits.

Kit	Amount	Catalog no.
pcDNA™3.1 Directional TOPO® Expression Kit	20 reactions	K4900-01
	40 reactions	K4900-40

TOPO® Reagents

pcDNA™3.1D/V5-His TOPO® reagents (Box 1) are listed below. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pcDNA™3.1D/V5-His-TOPO®	15-20 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP; 12.5 mM dGTP; 12.5 mM dTTP in water (pH 8)	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	--	1 ml
T7 Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
BGH Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Expression Plasmid (pcDNA™3.1D/V5-His/*lacZ*)	0.5 µg/µl in TE Buffer, pH 8	10 µl

continued on next page

Important Information, continued

Sequencing Primers

The table below provides the sequence and pmoles of the T7 sequencing primer and the BGH Reverse sequencing primer.

Primer	Sequence	Amount
T7	5′-TAATACGACTCACTATAGGG-3′	328 pmoles
BGH Reverse	5′-TAGAAGGCACAGTCGAGG-3′	358 pmoles

One Shot® Reagents

The table below describes the items included in the One Shot® TOP10 chemically competent *E. coli* cell kit (Box 2). **Store at -80°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10 Cells

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Accessory Products

Introduction

The products listed in this section may be used with the pcDNA™3.1 Directional TOPO® Expression Kit. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 30).

Additional Products

Many of the reagents supplied in the pcDNA™3.1 Directional TOPO® Expression Kit and other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Amount	Catalog no.
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Ampicillin	20 ml	11593-019
Lipofectamine™ 2000 Reagent	1.5 ml	11668-019
	0.75 ml	11668-027
Geneticin® Selective Antibiotic	1 g	11811-023
	5 g	11811-031
	20 ml (50 mg/ml)	10131-035
	100 ml (50 mg/ml)	10131-027
Phosphate Buffered Saline, pH 7.4	500 ml	10010-023
β-Gal Antiserum	50 µl	R901-25
β-Gal Assay Kit	100 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01

continued on next page

Accessory Products, continued

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using Anti-V5 or Anti-His(C-term) Antibodies available from Invitrogen. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods. Fluorescein isothiocyanate (FITC)-conjugated antibodies allow one-step detection in immunofluorescence experiments.

The amount of antibody supplied is sufficient for 25 Western blots or 25 immunostaining reactions (FITC-conjugated antibodies only).

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991) GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25
Anti-His(C-term)-FITC Antibody		R933-25

Purification of Recombinant Proteins

If your gene of interest in is frame with the C-terminal polyhistidine (6xHis) tag, you may use Invitrogen's ProBond™ or Ni-NTA Purification System to purify your recombinant fusion protein. See the table below for ordering information.

Product	Amount	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
	150 ml	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The pcDNA™3.1 Directional TOPO® Expression Kit provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a plasmid vector. Blunt-end PCR products clone directionally at greater than 90% efficiency, minimizing screening. No ligase, post-PCR procedures, or restriction enzymes are required. Once cloned, analyzed, and transfected, the gene of interest can be expressed directly in mammalian cell lines.

Features of pcDNA™3.1D/V5-His-TOPO®

pcDNA™3.1D/V5-His-TOPO® is a 5.5 kb expression vector designed to facilitate rapid directional cloning of blunt-end PCR products for expression in mammalian cells. The vector allows high-level expression, detection, and purification of heterologous proteins in most mammalian cells. The vector contains the following features:

- Human cytomegalovirus (CMV) immediate early enhancer/promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
- TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see next page for more information)
- C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag for detection and purification of recombinant protein
- Neomycin resistance gene for selection of stable cell lines using Geneticin® (Southern and Berg, 1982)

The control plasmid, pcDNA™3.1D/V5-His/*lacZ*, is included for use as a positive control for transfection and expression in the mammalian cell line of choice.

Tag-On-Demand™ System

The pcDNA™3.1D/V5-His-TOPO® vector is compatible with the Tag-On-Demand™ System which allows expression of both native and C-terminally-tagged recombinant protein from the same expression construct.

The System is based on stop suppression technology originally developed by RajBhandary and colleagues (Capone *et al.*, 1985) and consists of a recombinant adenovirus expressing a $tRNA^{ser}$ suppressor. When an expression vector encoding a gene of interest with the TAG (amber stop) codon is transfected into mammalian cells and the $tRNA^{ser}$ suppressor supernatant is present, the stop codon will be translated as serine, allowing translation to continue and resulting in production of a C-terminally-tagged fusion protein.

For more information, refer to the Tag-On-Demand™ Suppressor Supernatant manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).

How Directional TOPO® Cloning Works

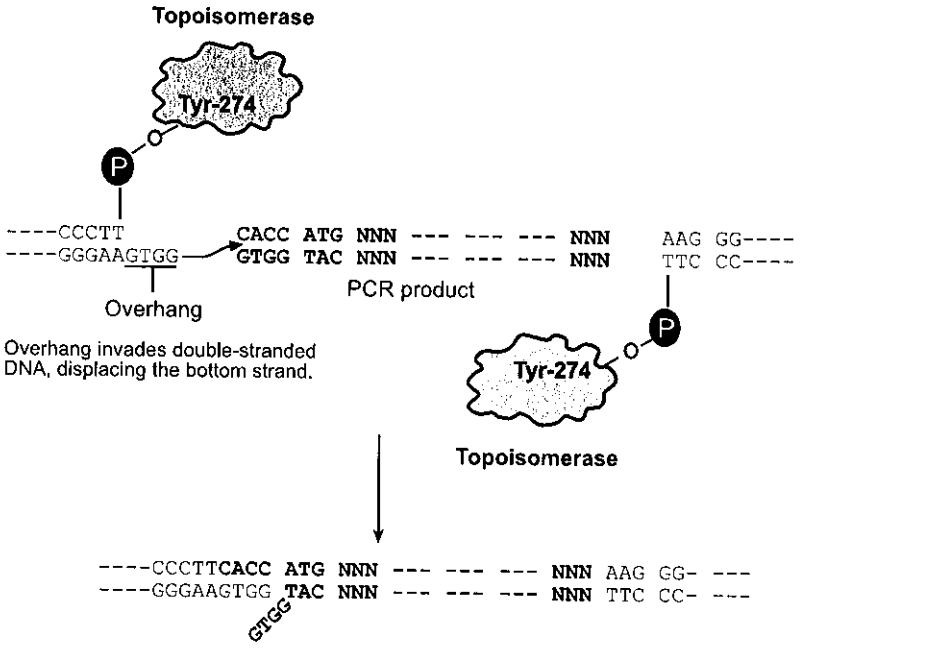
How Topoisomerase Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3' single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5' end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

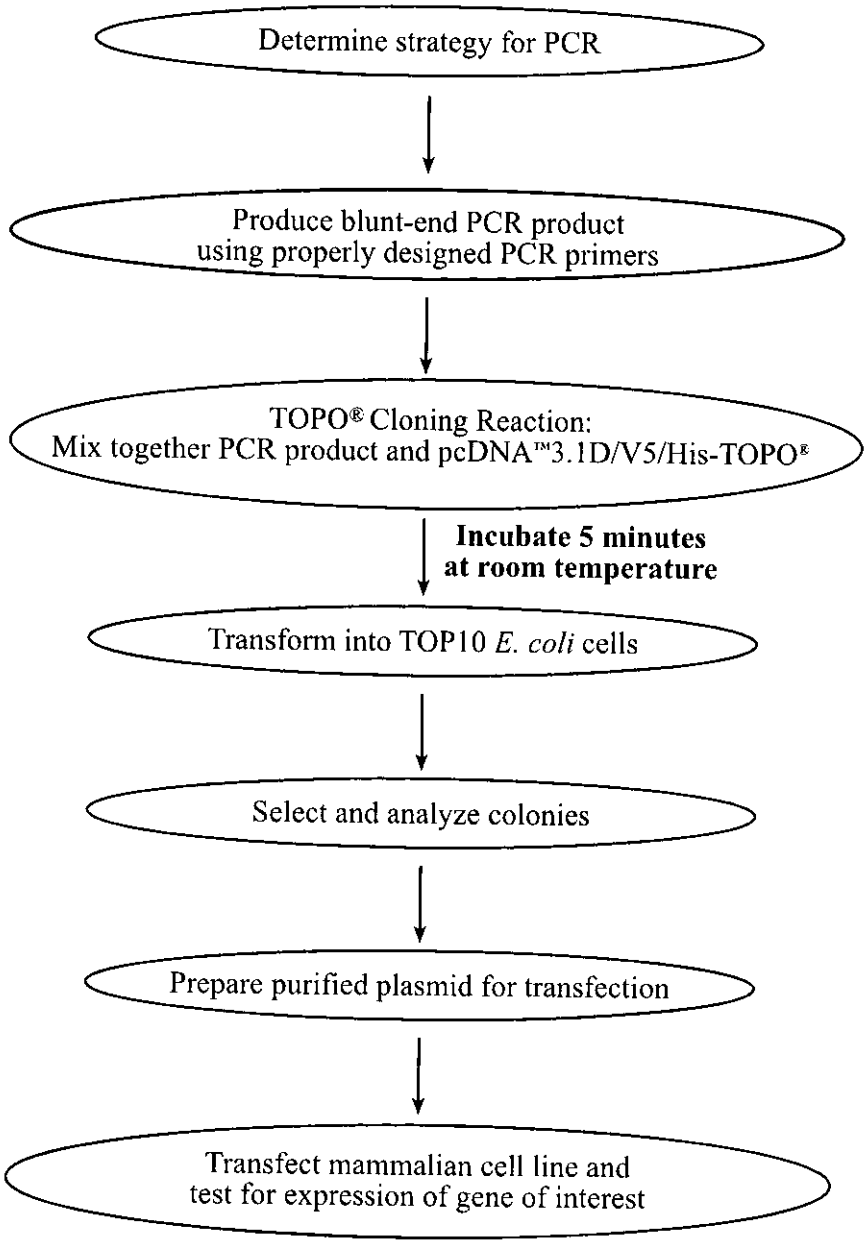
In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5' end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Experimental Outline

Experimental Outline

The flow chart below outlines the experimental steps necessary to clone and express your blunt-end PCR product.



Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with the C-terminal V5 epitope and 6xHis tag

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to page 6 for a diagram of the TOPO® Cloning site.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in pcDNA™3.1D/V5-His-TOPO®.
- Make sure your sequence of interest includes a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)**NNATG**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** below).

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then the ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

continued on next page

Designing PCR Primers, continued

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to page 6 for a diagram of the TOPO® Cloning site.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 below).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with the C-terminal V5 epitope and 6xHis tag, design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you **do not** wish to fuse your PCR product in frame with the C-terminal V5 epitope and 6xHis tag, include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3′

One possibility is to design the reverse PCR primer to start with the codon just up-stream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

Another possibility is to design the reverse primer so that it hybridizes just down-stream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine (see below).

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

continued on next page

Designing PCR Primers, continued

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

...GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TGA-3'

- To fuse the ORF in frame with a C-terminal tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5'-TGC AGT CGT CGA GTG CTC CGA CTT-3'

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.
- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5'-TCA TGC AGT CGT CGA GTG CTC CGA CTT-3'

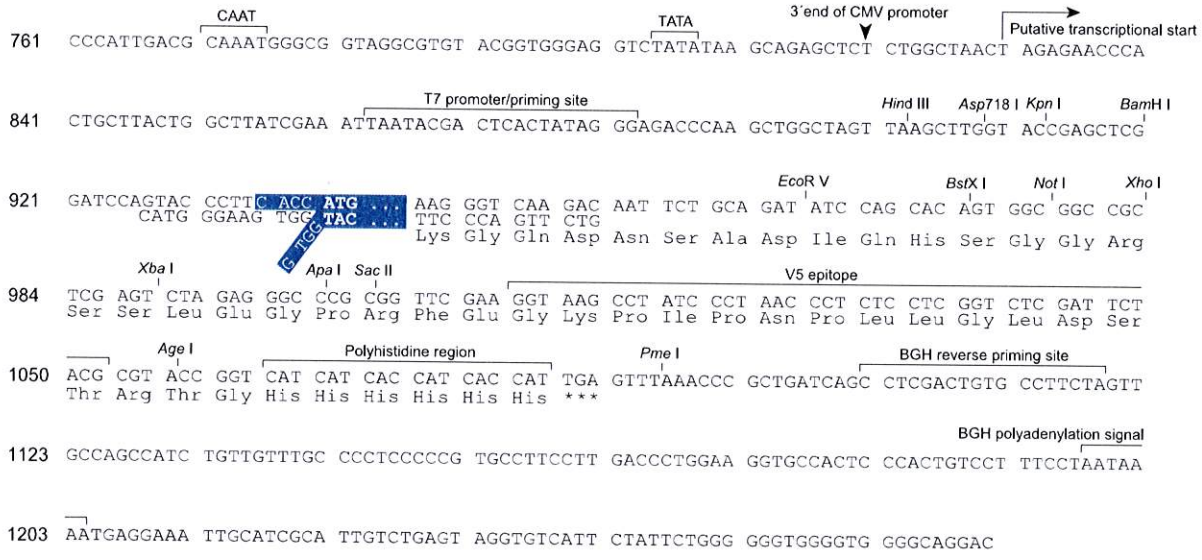
Important

- pcDNA™3.1D/V5-His-TOPO® vector accepts blunt-end PCR products.
- Do not add 5' phosphates to your primers for PCR. This will prevent ligation into the pcDNA™3.1D/V5-His-TOPO® vector.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

TOPO® Cloning Site

Use the diagram below to help you design PCR primers to clone your PCR product into pcDNA™3.1D/V5-His-TOPO®. **The complete sequence of pcDNA™3.1D/V5-His-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).**

Note: If you are using the pcDNA™3.1D/V5-His-TOPO® vector in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page 1).



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Needed

You should have the following materials on hand before beginning.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers to produce the PCR product

Producing Blunt-End PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at -20°C for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 24-25).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Performing the TOPO® Cloning Reaction

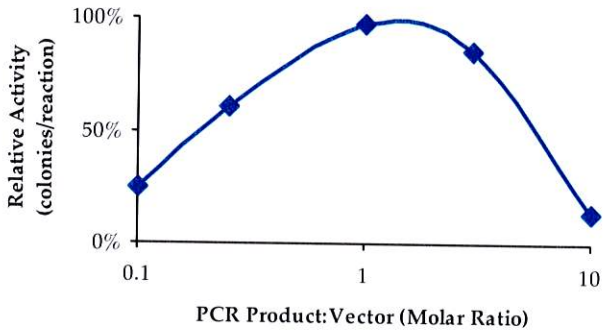
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into pcDNA™3.1D/V5-His-TOPO® and transform the recombinant vector into TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read the this section and the section entitled **Transforming One Shot® TOP10 Competent Cells** (pages 10-12) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 22-23 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pcDNA™3.1D/V5-His-TOPO® vector, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Performing the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page ix for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli*. **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at -20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pcDNA™3.1D/V5-His-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page ix for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Needed

You should have the following materials on hand before beginning:

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50-100 µg/ml ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing 50-100 µg/ml ampicillin at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 9 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 13). Refer to the **Troubleshooting** section on page 20 if you have problems obtaining transformants.

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 9 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the ampicillin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 13). Refer to the **Troubleshooting** section on page 20 if you have problems obtaining transformants.

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Transforming One Shot® TOP10 Competent Cells, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

- If you experience arcing during transformation, try one of the following suggestions:
- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation and in frame with the C-terminal V5 epitope and 6xHis tag. The T7 and BGH Reverse primers are included in the kit to help you sequence your insert (see the diagram on page6 for the location of the priming sites).

Important

If you download the sequence for pcDNA™3.1D/V5-His-TOPO® from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the T7 Promoter primer or the TK polyA Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants, the correct insert, or inserts in the correct orientation, refer to the **Troubleshooting** section (see page 20). We also recommend that you perform the control reactions described on pages 22-23. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50-100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Transfecting Cells

Introduction

Once you have the desired construct, you are ready to transfect the plasmid into the mammalian cells of choice. We recommend that you include the positive control vector pcDNA™3.1D/V5-His/*lac*Z, supplied with the kit, in your experiments to help you evaluate your results.

Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen. For more information about Lipofectamine™ 2000 and the other transfection reagents available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 30).

Positive Control

pcDNA™3.1D/V5-His/*lac*Z is provided as a positive control vector for mammalian transfection and expression and may be used to optimize transfection conditions for your cell line. This vector allows expression of a β-galactosidase fusion protein that may be detected by Western blot or functional assay.

Detecting Recombinant Fusion Proteins

Introduction

You may express you gene of interest in either transiently transfected cells or stable cell lines (see page 19 for guidelines to create stable cell lines). You may use a functional assay or a Western blot analysis to detect your recombinant protein (see below).

Preparing Cell Lysates

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols are suitable. To lyse cells:

1. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (see page ix for ordering information).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see the **Appendix**, page 26 for a recipe). Other cell lysis buffers are suitable. Vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.

   Note: You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration.

   Note: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer (see the **Appendix**, page 26 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 30).

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Detecting Recombinant Fusion Proteins, continued

Detecting Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page x for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 30).

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine region will add approximately 3.6 kDa to your protein.

Assay for β-galactosidase Activity

If you use the expression control plasmid, you may assay for β-galactosidase expression by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). Invitrogen offers the β-Gal Antiserum, the β-Gal Assay Kit, and the β-Gal Staining Kit (see page ix for ordering information) for fast and easy detection of β-galactosidase expression.

Purifying Recombinant Fusion Proteins

Introduction

You will need 5 x 10^6 to 1 x 10^7 transfected cells for purification of your protein on a 2 ml ProBond™ column (or other metal-chelating column). If you are using ProBond™ to purify your protein, refer to the protocol below to prepare cells for lysis. If you are using another metal-chelating resin, refer to the manufacturer's instructions to prepare the cells.

Preparing Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™. You will need 5 x 10^6 to 1 x 10^7 stably transfected cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are approximately 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1500 x g for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 x g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -70°C until needed.

Lysing Cells

If you are using ProBond™ resin, refer to the ProBond™ Purification System manual for details about sample preparation for chromotography.

If you are using other metal-chelating resin, refer to the manufacturer's instructions for recommendations on sample preparation.

Creating Stable Cell Lines

Introduction

The pcDNA™3.1D/V5-His-TOPO® vector contains the neomycin resistance gene to allow selection of stable cell lines using Geneticin®. If you wish to create stable cell lines, transfect your pcDNA™3.1D/V5-His-TOPO® construct into the mammalian cell line of choice and select for foci using Geneticin®. General information and guidelines are provided below.

Geneticin®

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phospho-transferase gene (APH), derived from Tn5, results in detoxification of Geneticin® Selective Antibiotic (Southern and Berg, 1982).

Determining Geneticin® Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Geneticin® required to kill your untransfected host cell line. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Let cells attach overnight before adding selective medium.
2. Prepare a set of 7 plates.
3. Prepare Geneticin® in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
4. Add the following concentrations of antibiotic to each plate: 0, 50, 125, 250, 500, 750, and 1000 µg/ml Geneticin®.
5. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
6. Count the number of viable cells at regular intervals to determine the appropriate concentration of Geneticin® that prevents growth within 1-3 weeks.

Geneticin® Selection Guidelines

Once you have determined the appropriate Geneticin® concentration to use for selection, you can generate a stable cell line expressing your pcDNA™3.1D/V5-His-TOPO® construct.

1. Prepare Geneticin® in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
2. Use the predetermined concentration of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 4 weeks of growth in selective medium.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions in parallel with your samples (see pages 22-23).

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR product too dilute	• Concentrate the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Long PCR product	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.

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Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence CACC at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Large number of incorrect inserts cloned	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	One Shot® transformation protocol not followed correctly	Follow the One Shot® transformation protocol provided on page 11.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you TOPO® Clone to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50-100 µg/ml ampicillin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Thermostable polymerase (1-2.5 units/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the a pcDNA™3.1D/V5-His-TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pcDNA™3.1D/V5-His-TOPO®	1 µl	1 µl
Final volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 cells (see page 11).
4. Spread 50-200 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pcDNA™3.1D/V5-His-TOPO®	*Xba* I	Correct orientation: 4727, 5537 Reverse orientation: 167, 6097 Empty vector: 5514

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation. Relatively few colonies should be produced in the vector-only reaction.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 11. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.

Note

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 7).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 9.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 9). Be sure to make the gel slice as small as possible for best results.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 9.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the method on page 11.

Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Recipes

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (100 µg/ml ampicillin) if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (100 µg/ml of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 µM leupeptin, or 0.1 µM aprotinin before use.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

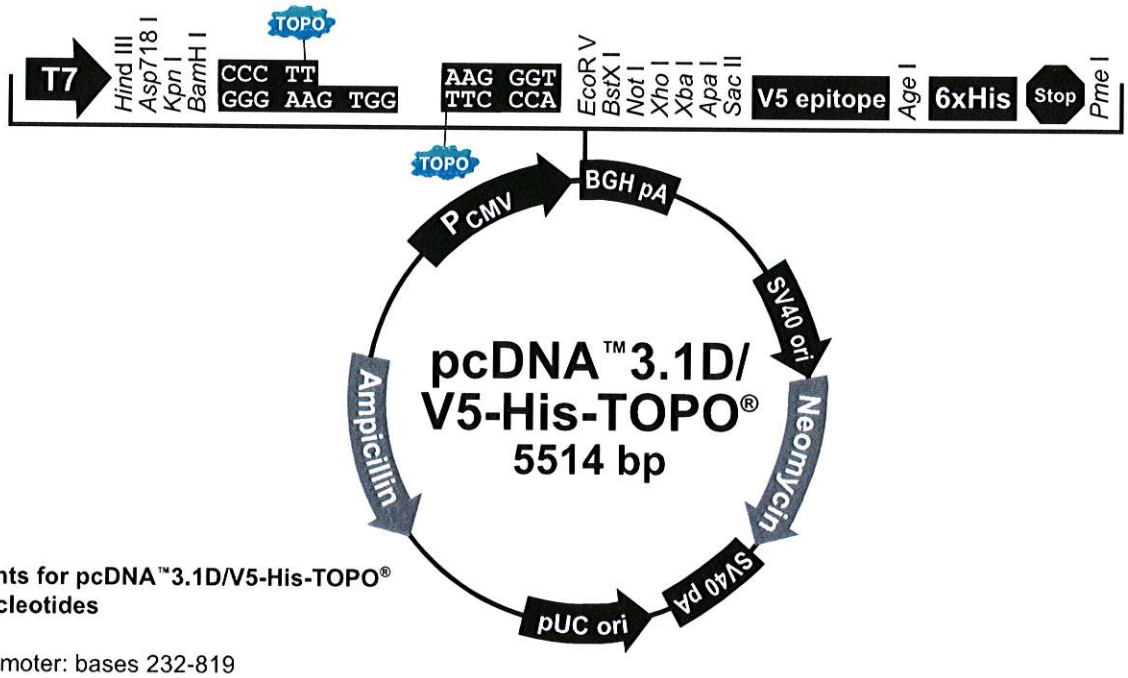
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Map and Features of pcDNA™3.1D/V5-His-TOPO®

Map

The map below shows the elements of the pcDNA™3.1D/V5-His-TOPO® vector. **The complete nucleotide sequence is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).**



Comments for pcDNA™3.1D/V5-His-TOPO®
5514 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
TOPO® recognition site 1: bases 930-934
Overhang sequence (complementary strand): bases 935-938
TOPO® recognition site 2: bases 939-943
V5 epitope: bases 1011-1052
Polyhistidine (6xHis) tag: bases 1062-1079
BGH reverse priming site: bases 1102-1119
BGH polyadenylation signal: bases 1108-1332
SV40 early promoter and origin: bases 1833-2142
Neomycin resistance gene: bases 2217-3011
SV40 early polyadenylation signal: bases 3189-3319
pUC origin: bases 3700-4373 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4518-5378 (complementary strand)
bla promoter: bases 5379-5477 (complementary strand)

continued on next page

Map and Features of pcDNA™3.1D/V5-His-TOPO®, continued

Features

pcDNA™3.1D/V5-His-TOPO® contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
TOPO® Cloning site (directional)	Allows directional cloning of your PCR product in frame with the V5 epitope and polyhistidine C-terminal tag, if desired
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 antibodies (Southern *et al.*, 1991)
C-terminal polyhistidine tag	Allows purification of your recombinant protein on metal-chelating resin such as ProBond™ Allows detection of your recombinant protein with the Anti-His (C-term) antibodies (Lindner *et al.*, 1997)
BGH reverse priming site	Allows sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin resistance gene
Ampicillin resistance gene (β-lactamase)	Allows selection of vector in *E. coli*

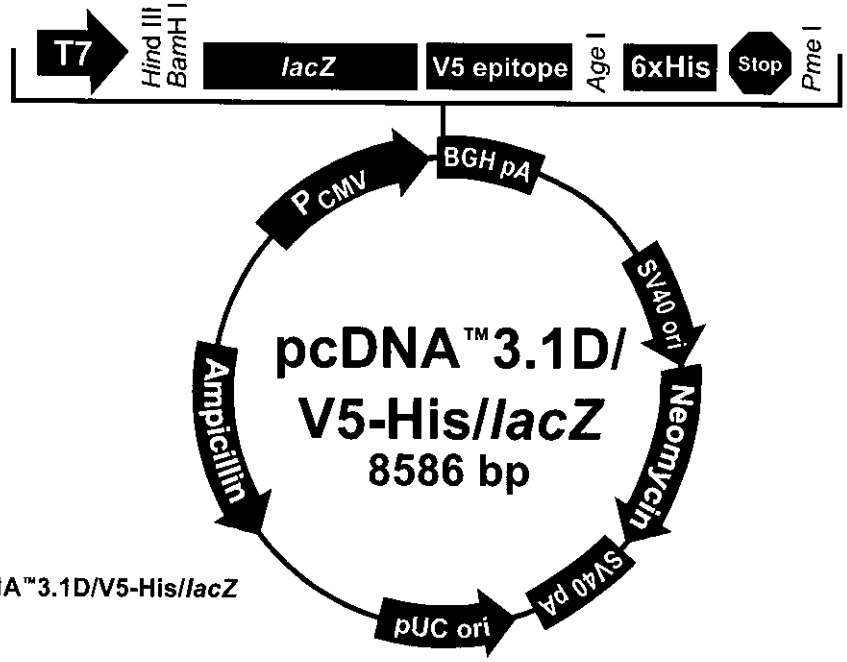
Map of pcDNA™3.1D/V5-His/*lacZ*

Description

pcDNA™3.1D/V5-His/*lacZ* is a 8586 bp control vector containing the gene for β–galactosidase. The *lacZ* gene was amplified and directionally TOPO® Cloned into pcDNA™3.1D/V5-His-TOPO® such that it is in frame with the C-terminal peptide. The size of the β-galactosidase fusion protein is approximately 120 kDa.

Map of Control Vector

The figure below summarizes the features of the pcDNA™3.1D/V5-His/*lacZ* vector. **The complete nucleotide sequence for pcDNA™3.1D/V5-His/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).**



Comments for pcDNA™3.1D/V5-His/*lacZ*
8586 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
LacZ ORF: bases 939-3995
V5 epitope: bases 4083-4124
Polyhistidine (6xHis) tag: bases 4134-4151
BGH reverse priming site: bases 4174-4191
BGH polyadenylation signal: bases 4180-4404
SV40 early promoter and origin: bases 4905-5214
Neomycin resistance gene: bases 5289-6083
SV40 early polyadenylation signal: bases 6261-6391
pUC origin: bases 6772-7445 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 7590-8450 (complementary strand)
bla promoter: bases 8451-8549 (complementary strand)

Technical Service

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- Obtain citations for Invitrogen products
- Request catalog and product literature

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pcDNA™3.1 Directional TOPO® Expression Kit.

Vectors

The pcDNA™3.1/V5-His (parental vector of pcDNA™3.1D/V5-His-TOPO®) and pcDNA™3.1D/V5-His/*lac*Z plasmids are qualified by restriction digest. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

TOPO® Cloning Efficiency

The pcDNA™3.1 Directional TOPO® vector is lot-qualified using the control reagents included in the kit. Under conditions described on pages 22-23, a 750 bp control PCR product is amplified using a forward primer containing CACC at its 5′ end and a reverse primer. The PCR product is TOPO® Cloned into the pcDNA™3.1D/V5-His-TOPO® vector and transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency. Forty transformants are characterized by restriction digest. Of the transformants characterized, greater than 90% should be in the correct orientation.

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® TOP10 Chemically Competent *E. coli*

1. One Shot® TOP10 chemically competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1 x 10^9 cfu/µg plasmid DNA.
2. To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
3. Untransformed cells are plated on LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

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Introduction

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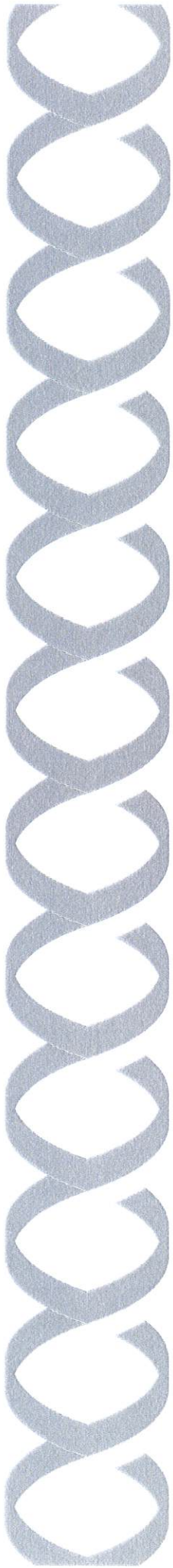


pSUPER RNAi System™

VECTOR: pSUPER.neo+GFP
CATALOG#: VEC-PBS-0005/0006

Length: 5429 bp

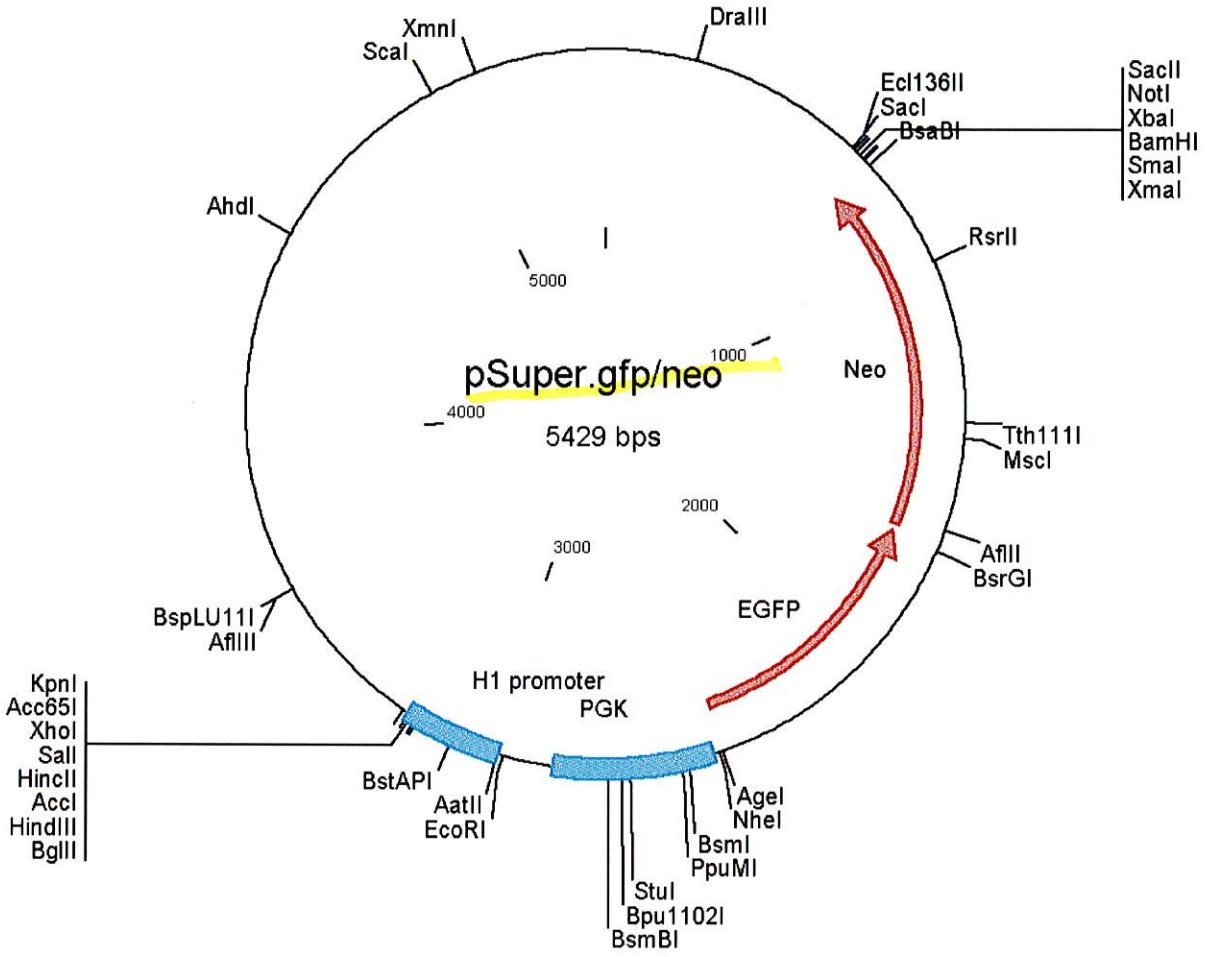
Key Sites

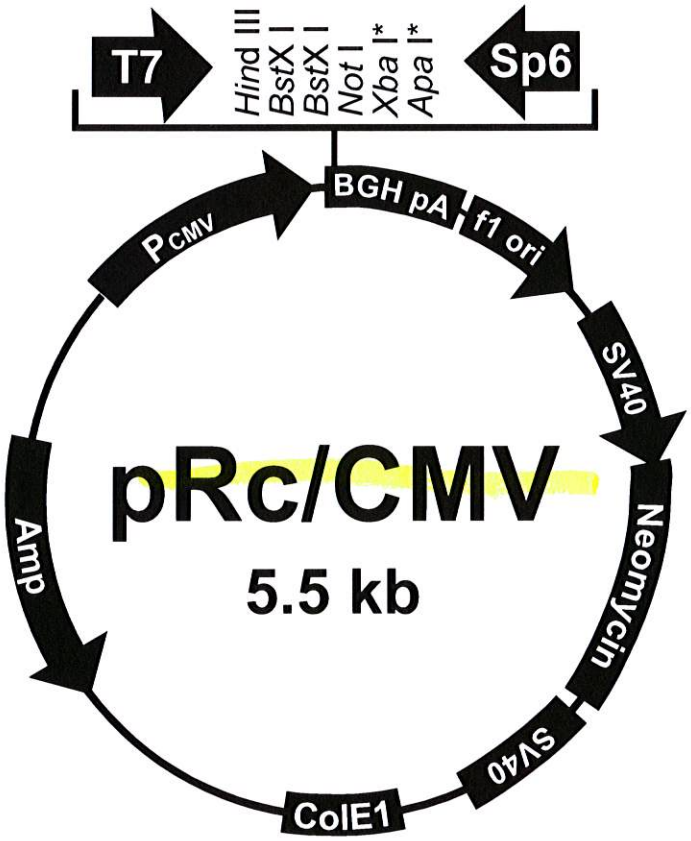
BglII: 3181
HindIII: 3187
EcoRI: 2960
SalI: 3202
XhoI: 3208

Vector Features

f1(+) origin: 135-441
PGK promoter: 2840-2442
Neo ORF: 1684-715
EGFP ORF: 2424-1691
H1 promoter: 2965-3213
Ampicillin resistance ORF: 5301-4444

T7 primer binding site (AATACGACTCACTATAG): 627-643
T3 primer binding site (CTTTAGTGAGGGTTAAT): 3242-3258
M13(-20) primer binding site (GTAAAACGACGGCCAGT): 600-616
M13 reverse primer binding site (CATGGTCATAGCTGTT): 3276-3291





Comments for pRc/CMV:
5542 nucleotides

CMV promoter: bases 209-864
T7 promoter: bases 865-883
Polylinker: bases 890-995
Sp6 promoter: bases 1008-1017
BGH poly A signal: bases 1019-1250
f1 origin: bases 1306-1828
SV40 Promoter: bases 1890-2215
SV40 origin of replication: bases 2084-2169
Neo ORF: bases 2251-3045
SV40 poly A: bases 3219-3348
ColE1 origin: bases 3728-4400
Ampicillin resistance gene: bases 5406-4546 (complementary strand)

* There is an ATG upstream of the *Xba* I site.

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