Modification Form for Permit BIO-RRI-0032

Permit Holder: Paula Foster

Approved Personnel
(Please stroke out any personnel to be removed)

Gabrielle Siegers

Laura GonzalezLara

Carmen Simedrea

Dean Percy

Christiane Mallet

Catherine Ramsay

Emeline Ribot

Marlama Henry

Mevan Perera

Yuhua Chen

Roja Rohani

Vasiliki Economopoulos

Jennifer Noad

Shruti Krishnamoorthy

Additional Personnel
(Please list additional personnel here)

Amanda Hamilton
Greg Dekaban

	Please stroke out any approved Biohazards to be removed below	**Write additional Biohazards for approval below. Give the full name - do not abbreviate.**
Approved Microorganisms		
Approved Primary and Established Cells	Human (established): THP-1, FaDu Luc2.11, MDA-MB-231(PA), MDA-MB-231BR. Rodent (established): B16F10, Glioma-261, Glioma-261 RFP, C4-2 Cell line, C4-2B Cell Line, PC-3/M Cell line (Human Prostate Tumour	Melanoma cell line A2058 (Human)
Approved Use of Human Source Material		Human Blood
Approved Genetic Modifications (Plasmids/Vectors)	pCMV-DSRed Express, pGL4.14. Androgen Independent cell line, clone of C4. Androgen Independent Cell line colony of C4	PC-3MGFPluc(pCCL-EGFP-minCMV-hPGK-luc lentivira vector transduction) pcDNA3.1(+) pEYFP-C1 pDsRed Monomer-Hyg-N1

Approved Use of Animals	Nu/Nu (nude) mice, C57B1/6 mice AUS#: 2007-041-03, CB17/SCID 2010-210, 2009-042	
Approved Biological Toxin(s)	Pertussis	

** PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
*** PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. 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.wph.uwo.ca.

Signature of Permit Holder: Paula Fr

Current Classification: 2 **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Dec 19, 2008

Date of Last Modification (if applicable): Sep 17, 2010

BioSafety Officer(s): Ronald Ness (Jan. 14, 2011)

Chair, Biohazards Subcommittee: _____ **Date:** _____

To Whom it May Concern,

Dr. Foster, of the Robarts Research Institute at The University of Western Ontario, would like to purchase the skin cancer melanoma cell line A2058. The cells will be cultured and labeled with super paramagnetic iron oxide particles or gadolinium to permit their detection by magnetic resonance imaging. If the cell labeling is effective then the cells will be it is injected/implanted into mice for a cancer research.
The information of the cell line is attached.

Please let me know if you have questions or comments.

Regards,

Yuhua chen.

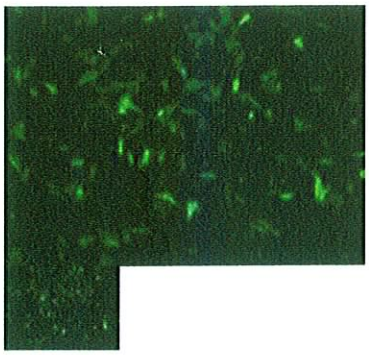
Mailing Address:
Dr. Paula Foster
Attn: Yuhua Chen/Cat Ramsay
P.O. Box 5015
100 Perth Dr
London ,ON
N6A 5K8
Phon# 519-663-5777 Ext: 24133
Fax# 519-931-5224
Email: Ychen@robarts.ca

Cell Information for

A2058 (ATCC)

Cell Type	**Skin/Hair Epith.** (Epithelial Cells, Cell Lines)
Description	Skin melanoma cells
Characteristics	Adherent
Species	Human
Supplier	American Type Culture Collection (ATCC)
Clone	CRL-11147
Tissue Origin	Dermal
Optimized Protocol	Nucleofector (PDF, 163 KB)

Images



GFP positive

1 images

Experiment Data

Experiments conducted with *Nucleofector*™ (1)

Experiment #1

Transient transfection

Substrate:	Plasmid (general)	**Reporter:**	maxGFP	**Vector:**	pmaxGFP; pmaxGFP **[1]**
Cell Amount:	1e6	**Culture Medium:**	DMEM with 4 mM L-glutamine adjusted to contain 1.5... **[2]**	**Cell Density:**	n/a

Program	Solution	Substrate Amount	Knockdown	Transfection Efficiency	Viable Cells	Cell Loss	Analysis Time	Analysis Method
X-001	C	2 µg		81%±2%	94%±1% **[3]**		24 h	FACS

A subcultivation ratio of 1:5 to 1:12 is recommended.Passage cells 2 days before nucleofection in a ratio of 1:5.

Footnotes

[1]: **Backbone:** pmaxGFP
Specific Name: pmaxGFP
Size: 3.5 kb
Promoter: CMV
Specific Sequences: none

[2]: DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L Na-bicarbonate and 4.5 g/L glucose [ATCC; Cat. No. 30-2002] + 10% FBS [ATCC; Cat. No. 30-2020]

[3]: % PI-negative (69.2% +/- 8 compared to non-nucleofected control)

ATCC

ATCC Advanced Catalog Search » **Product Details**

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Canada, China, Hong Kong, India, Israel, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Print this Page

Cell Biology

ATCC® Number: CRL-11147™ Order this Item **Price:** $329.00

Designations:	A2058
Depositors:	W Stetler-Stevenson
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Homo sapiens* (human)
Morphology:	epithelial
Source:	**Organ:** skin **Disease:** melanoma **Derived from metastatic site:** lymph node
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (technology from amaxa)
Receptors:	nerve growth factor (NGF), expressed laminin, expressed
Tumorigenic:	Yes
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 10,11 D13S317: 13,14 D16S539: 9,13 D5S818: 9,12 D7S820: 11 THO1: 7,9 TPOX: 8 vWA: 14,18
Age:	43 years adult
Gender:	male
Ethnicity:	Caucasian
Comments:	This cell line is highly invasive and provides a source of cellular invasion associated proteins (such as the 72000 dalton type IV collagenase. Tissue inhibitor of metalloproteinase-2 [TIMP-2], autocrine motility factor and the 67000 dalton laminin receptor.

Related Links

- NCBI Entrez Search
- Make a Deposit
- Frequently Asked Questions
- Material Transfer Agreement
- Technical Support
- Related Cell Culture Products

Propagation:

ATCC 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: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that 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.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:6 to 1:12 is recommended
Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002
recommended serum:ATCC 30-2020

References:

22590: Fabricant RN, et al. Nerve growth factor receptors on human melanoma cells in culture. Proc. Natl. Acad. Sci. USA 74: 565-569, 1977. PubMed: 265522
23263: Sherwin SA, et al. Human melanoma cells have both nerve growth factor and nerve growth factor-specific receptors on their cell surfaces. Proc. Natl. Acad. Sci. USA 76: 1288-1292, 1979. PubMed: 375235
23269: Todaro GJ, et al. Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc. Natl. Acad. Sci. USA 77: 5258-5262, 1980. PubMed: 6254071
23404: Stetler-Stevenson WG, et al. The activation of human type IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. J. Biol. Chem. 264: 1353-1356, 1989. PubMed: 2536363
23549: Stetler-Stevenson WG, et al. Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J. Biol. Chem. 264: 17374-17378, 1989. PubMed: 2793861

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To Whom it May Concern,

Dr. Gabrille Siegers who is Dr. Paula Foster's Postdoctoral. She is working with human blood for her research projects:

Gamma delta T cells (GDTc) constitute 2-5% of circulating lymphocytes in human blood. Since these cells elicit cytolytic responses against a variety of allogeneic and autologous tumors *in vitro* and *in vivo*, adoptive immunotherapy using GDTc is currently under investigation in clinical trials. Using primary human GDTc expanded via our established GDTc expansion protocol and our pre-clinical xenograft bioluminescent model of Ph+ leukemia, we aim to:

1) Determine mechanisms of cytotoxicity of GDTc against established cell lines derived from chronic myeloid leukemia and B cell chronic lymphocytic leukemia patients
2) label GDTc with clinically approved iron particles, optimizing for viability and function
3) track GDTc in healthy versus leukemia-bearing mice using novel cellular MRI technology developed in our lab

For point 1, we may also generate GDTc clones for use in these experiments. Both healthy donor and patient samples will be used in the context of this work, pending Research Ethics Board and Health Canada import approvals.

This work will be performed in Dr. Greg Dekaban's human cell culture facility Rm# 2222 in Robarts building 2nd floor . The Protocols of blood works are attached. Please let me know if you have questions or comments.

Regards,

Yuhua chen.

γδ T cell isolation and expansion protocol
Dec 2009

Materials

MACS buffer
AIMV medium + 5% heat inactivated hu serum
ConA
IL-2
IL-4
MACS antibody and beads
Ice
MACS column, magnet and holder
Eppis for counting
24 well plates (Falcon)

1. Isolate PBMCs from donors. Expected yield ~1.0 x 10^6 cells/ml
   COUNT:
2. Spin 5 min at 1800 rpm. Resuspend cells at 40µl/10^7 cells in cold MACS buffer (degassed) in a 50 ml conical.
   µl buffer used:
3. Remove 6µl for FACS analysis.
4. Add γδ TCR hapten antibody at 10µl/10^7 cells. Incubate in the fridge for 10 min.
   µl Ab used:
   incubation time:
5. Wash with 10 ml cold MACS buffer. Spin 5 min 1500 rpm 4 deg. Discard supernatant.
6. Resuspend cells at 30µl/10^7 cells in cold MACS buffer.
   µl buffer used:
7. Add anti-hapten-FITC antibody/beads at 20µl/10^7 cells. Incubate in the fridge for 15 min. µl Ab used:
   incubation time:
8. Wash with 10 ml cold MACS buffer. Spin 5 min 1500 rpm 4 deg.
9. Prime MACS column by inserting it into holder and carefully adding 500µl.
10. Discard supernatant and resuspend cell pellet in 500µl cold MACS buffer and then carefully add to column, by placing tip against the side of the column and allowing the cell suspension to run down the side.
11. Wash column with 3 x 500µl MACS buffer.
12. Add 3 ml medium to column. Remove column from magnetic holder and place into a fresh 15 ml conical. Insert plunger and press down to elute cells from the column.
13. Count cells, resuspend at 2.5 x 10^5 cells/ml and seed into 24-well plates (2ml/well). Use 1-2 ml for FACS analysis (wash in FACS buffer 1 x before antibody incubation).
14. Remove 75µl (15µl/sample) from MACS negative fraction for FACS analysis.

FACS Staining:

	preMACS	MACS+	MACS-
unstained	1	7	9
CD3 FITC 1:100	2	-	10
γδ TCR PE 1:10	3	-	11
αβ TCR PE 1:20	4	8	12
Vδ2 PE 1:100	5	-	
Vδ1 FITC 1:50	6		

Antibodies: 20-50 μl dilution/sample

Important notes:

50 ml conicals for isolation and antibody incubations
25-27 min primary antibody and 15 min secondary antibody incubations for MACS sorting
Con A 7-8 days (8 days better!)
remove all medium and replace with fresh at each feeding
spin down at 800rpm (sugg by Simone) ROOM TEMP
96 or 24 well-plates are better than 6-wells or 12-wells
12 and 6-wells are fine for passages beyond ConA and the first passage after ConA removal
expansion stops in T75 flasks

best seeding density 2.5-5 x 10E5 cells/ml
don't change med until med is apricot or yellowish
spin at RT

AIM-V medium (can probably use RPMI)
5% hu AB serum
10 ng/ml IL-2
10 ng/ml IL-4

first 6-8 days:
1μg/ml Concanavalin A

GDTc cloning protocol (P. Fisch)

Materials/Reagents:

96-well round bottom plates (tissue-culture treated), 200 microlitres/well
Culture medium: Iscove's, 2mM L-Gln, 100 IU/ml penicillin, 100μg/ml streptomycin, 500 U/ml rIL-2, 0.25μg/ml purified PHA (HA-15 or HA-16). Can also add 'AAG'.
Serum: better cloning success with NO heat inactivation, use at 10%.
Freezing medium: 50% serum, 40% fresh medium and 10% DMSO.
PHA: Do not add if splitting the clones between stimulations (if they grow too well) but do add it after thawing and after adding fresh feeders.

Protocol:

1) Isolate PBLs from 20 ml blood.

2) FACS sort (and count) $\gamma\delta$ T cells by positive selection directly into vial containing irradiated feeder cells* (1 x 10^6/ml) in medium, then mix well with fresh medium. The laser should be turned down as low as possible <100 mW.

3) Plate out at 1 cell/well (according to FACS count) into 96 well plates. Actual density will likely be 0.5 cell/well. Grow at 7% CO_2. Clones should come up after 9-12 days.**

4) Wait another 2-3 days, then pick the clones, transferring them from 1 well into 6 into fresh medium with fresh feeders. Use only the inner 60 wells, thus you have 10 clones/plate.

5) Once they have grown a bit, test them for desired characteristics (cytotoxicity, FACS...) and then select them carefully. Once selected, plate only 1 clone/plate.

6) Freeze 2-3 vials/clone as early as possible. Once confluent (medium can get slightly yellowish), harvest plate almost entirely and freeze down in cold freezing medium. Transfer the remaining cells to a fresh plate with fresh medium and feeders.

7) Passage the cells once per week-10 days. When plating, seed at less than 2 x 10^6/plate and allow to grow up to 10 x 10^6.

* Feeder cells can be allogeneic. Freeze aliquots of PBLs, thaw and then irradiate. For best results, use fresh feeders. Use 2 x 10^4 – 5 x 10^4 PBLs/well and 1 x 10^4 LCLs. LCLs are very important for establishing clones; less yield is achieved with PBLs alone. Irradiate PBLs at 5-10 Gey, LCLs at 30 Gey. If only using autologous PBLs (no LCLs), then use 3-5x10^4 per well.

** If none appear after 2 weeks, unlikely to get clones; after 3 weeks, can discard.

Keep the clones up to 4 weeks in culture after they begin to expand. You cannot say by vision if cells are clonal or polyclonal. As a matter of fact, gamma delta clones typically grow in a "flower-type" shape since the cells show active motility,particularly the acivated clones with high cytotoxic potential...They may however also grow as round colonies if they are longer in culture or if they tend "to be overgrown".

Thawing: Thaw 1 vial in fresh Iscoves Medium with 10% HS no cytokines. Centrifuge cells, then resuspend in complete medium with IL-2, irradiated feeders and PHA. Plate 60 wells on one plate. Paul never counted them after thawing. If it worked you should see growth with naked eyed within 1 to three days... If you have too many clones to thaw you can also plate on ½ plate (30 wells) and expand later once they grow. To improve conditions it helps taking fresh feeder cells and not frozen ones...

Public Health Agency of Canada | Agence de santé publique du Canada

Centre for Emergency Preparedness and Response | Centre de mesures et d'interventions d'urgence

Protected when Completed
Protéger une fois rempli

Application for permit to Import human pathogen(s)

Demande de permis d'importation d'un (d')agent(s) anthropopathogène(s)

Under the authority of the Human Pathogens Importation Regulations.

Sous le régime du Règlement sur l'importation des agents anthropopathogènes.

For Office use only À l'usage du bureau seulement
Permit no. - N° de permis

Forward copy to:
Office of Laboratory Security
100 Colonnade Road, Loc.: 6201A
OTTAWA ON K1A 0K9
Telephone: (613) 957-1779 Facsimile: (613) 941-0596

Envoyer la copie au :
Bureau de la sécurité des laboratoires
100, chemin Colonnade, Loc. : 6201A
OTTAWA ON K1A 0K9
Téléphone : (613) 957-1779 Télécopieur : (613) 941-0596

1. Applicant - Name, address and postal code / Demandeur - Nom, adresse, et code postal

Gabrielle M. Siegers, Foster Lab, Robarts Research Institute, PO Box 5015, 100 Perth Dr. Rm 3296, London, ON, N6A 5K8

Facsimile / Télécopieur: 519-931-5224

Telephone no. / N° de téléphone: 519-663-5777 ext 24086

2. Supplier - Name and address / Fournisseur - Nom et adresse

Anna Kreutzman, Hematology Research Unit, Biomedicum Helsinki, C428b, Department of Medicine, Helsinki University Central Hospital, P.O. Box 700, FI-00029 HUCH, Finland

3. Description of material comprising human pathogen (Including name of material, country of origin and human or animal source)
Description de la matière comprenant un agent anthropopathogène (notamment dénomination, pays d'origine et source humaine ou animale)

blood samples from human chronic myeloid leukemia patients in Finland

4. Mode of transportation / Mode de transport

courier (air/road)

5. Canadian port(s) of entry / Point(s) d'entrée au Canada (Note: Not more than one entry is permissible in the case of a human pathogen that belongs to risk group 3 or 4. / Remarque : Une seule entrée est permise dans le cas d'un agent anthropopathogène des groupes de risque 3 ou 4.)

various

6. Quantity of material to be imported and in the case of a human pathogen belonging to risk group 2, any intervals at which, or period during which, the pathogens are to be imported.
Quantité de la matière à importer - Dans le cas d'un agent anthropopathogène du groupe de risque 2, toute intervalle ou période d'importation.

Approximately 600 ml human blood (60-90 ml/shipment at 4-6 week intervals) will be imported in a one-year period.

7. Description of applicant's facilities and equipment for handling material (Note: Appropriate containment is required: see the LABORATORY BIOSAFETY GUIDELINES as amended from time to time, established by Health Canada and the Medical Research Council of Canada).
Description des installations et de l'équipement du demandeur utilisés pour la manutention de la matière (Remarque : Confinement adéquat exigé : voir les LIGNES DIRECTRICES EN MATIÈRE DE BIOSÉCURITÉ EN LABORATOIRE, avec leurs modifications successives, établies par Santé Canada et le Conseil de recherche médicales du Canada).

Our laboratory meets Containment Level 2 requirements as per the Laboratory Biosafety Guidelines 3rd edition.

Additional information attached / Renseignements complémentaires ci-joint: ☐ Yes / Oui ☒ No / Non

8. Address of location where the human pathogen is to be used / Adresse du lieu où sera utilisé l'agent anthropopathogène

Robarts Research Institute,100 Perth Dr., London, ON, N6A 5K8

9. Method of treatment of material for the purposes of decontamination, sterilization and waste disposal
Méthode de traitement de la matière aux fins de décontamination, de stérilisation et de l'élimination des déchets

Liquid waste will be aspirated into a designated flask and treated with bleach prior to disposal.

10. Work objectives, proposed plan of work and additional pertinent information / Objectifs des travaux, plan de travail proposé et autres renseignements utiles

Gamma delta T cells (GDTc) constitute 2-5% of circulating lymphocytes in human blood. Since these cells elicit cytolytic responses against a variety of tumors in vitro and in vivo, immunotherapy using GDTc is currently under investigation in clinical trials. Using our GDTc isolation and expansion protocol we aim to: 1) assess whether GDTc can be expanded from the blood of chronic myeloid leukemia (CML) patients; 2) determine clonality of expanded GDTc; 3) assess functional capacity of expanded GDTc; 4) determine mechanisms of cytotoxicity of GDTc against CML cell lines and autologous tumours; and 5) assess iron oxide labeling of expanded GDTc for single cell imaging via MRI.

11. **Applicant**
I undertake that the material comprising the human pathogen will, in the event of its importation, be used in accordance with such terms and conditions as may be specified in the permit, and I certify that the facilities in which the material will, in that event, be manipulated and stored meet the following containment level;

Demandeur
Je m'engage à ce que la matière comprenant l'agent anthropopathogène, dans l'eventualité de son importation, soit utilisée conformément aux conditions du permis d'importation et j'atteste que les installations dans lesquelles cette matière sera manipulée et entreposée satisfont aux exigences du niveau de confinement suivant;

Containment level (Check one block only) / Niveau de confinement (Ne cocher qu'une seule case)

☐ 1 ☒ 2 ☐ 3 ☐ 4

Signature of applicant - Signature du demandeur: Gabrielle M Siegers

Date: 12 Jan 2011

***Note:**
Physical containment levels and mechanical systems, operational protocols and laboratory waste disposal facilities are subject to verification as may be required by the Director.

***Remarque :**
Les niveaux de confinement physique ainsi que les systèmes mécaniques, les protocoles opérationnels et les installations d'élimination des déchets de laboratoire sont soumis à une vérification si le Directeur l'exige.

Canada

To Whom it May Concern,

I am writing for modifying PC-3M cell line. The PC-3M cell line has been registered as a biohazard. The PC-3M cells are transducted using pCCL-EGFP-minCMV-hPGK-luc lentiviral vector. Dr. Paula Foster would like to use this modified cell line PC-3MGFPluc for cancer research.
These cells will be cultured and labeled with super paramagnetic iron oxide particles or gadolinium to permit their detection by magnetic resonance imaging. In addition, if the cell labeling is effective then the cells will be injected/implanted into mice for an animal cancer model and MRI.
The works of cells transduction are performed in Dr. Greg Dekaban's Lab.
The protocol of cells transduction and pCCL-EGFP-minCMV-hPGK-luc information are attached.
Please let me know if you have questions or comments.

Regards,

Yuhua chen.

Mailing Address:
Dr. Paula Foster
Attn: Yuhua Chen
Rm# 3296
P.O. Box 5015
100 Perth Dr
London ,ON
N6A 5K8
Phon# 519-663-5777 Ext: 24133
Fax# 519-931-5224
Email: Ychen@robarts.ca

Spin Infection

** This was done using the pCCL-EGFP-minCMV-hPGK-Luc lentiviral vector

*** Media used: PC3M cells used RPMI 1640 with 10% FBS, L-glutamine, Pen-Strep, 1x HEPES, and non-essential amino acids.

PC3M cells

1. Make sure the cells are growing nicely in log phase prior to transduction.
2. 2 days prior to transduction, seed a T25 flask approximately 1:4 from an 80% confluent flask.
3. On the day of transduction, lift the cells and count.
4. To each well of a 24 well plate, add 20 000 cells, 8ug/mL of protamine sulfate (dissolved in PBS), the lentivirus suspension, and top off to a final volume of 1mL.

1. The amount of lentivirus suspension to use depends on the multiplicity of infection you intend to go with. I used an MOI of 5 and got adequate levels of transductants.

1. The MOI refers to the number of transducing units per every cell. So an MOI of 5 with 20 000 cells meant 100 000TU

5. Spin down in the Beckman GS-15 centrifuge using the plate rotor

2. 2h30mins, 1000 x g, at room temperature, no brakes

1. I didn't try it with brakes

3. You'll need prewarmed media after this, so get some ready in the last hour of spinning.

6. Remove media and replace with a fresh 1mL of prewarmed media (per well)
7. Incubate at 37C, 5% CO2 for at least 48 hours to allow for integration and expression
8. Keep track of cell growth; if necessary, move to larger tissue culture ware

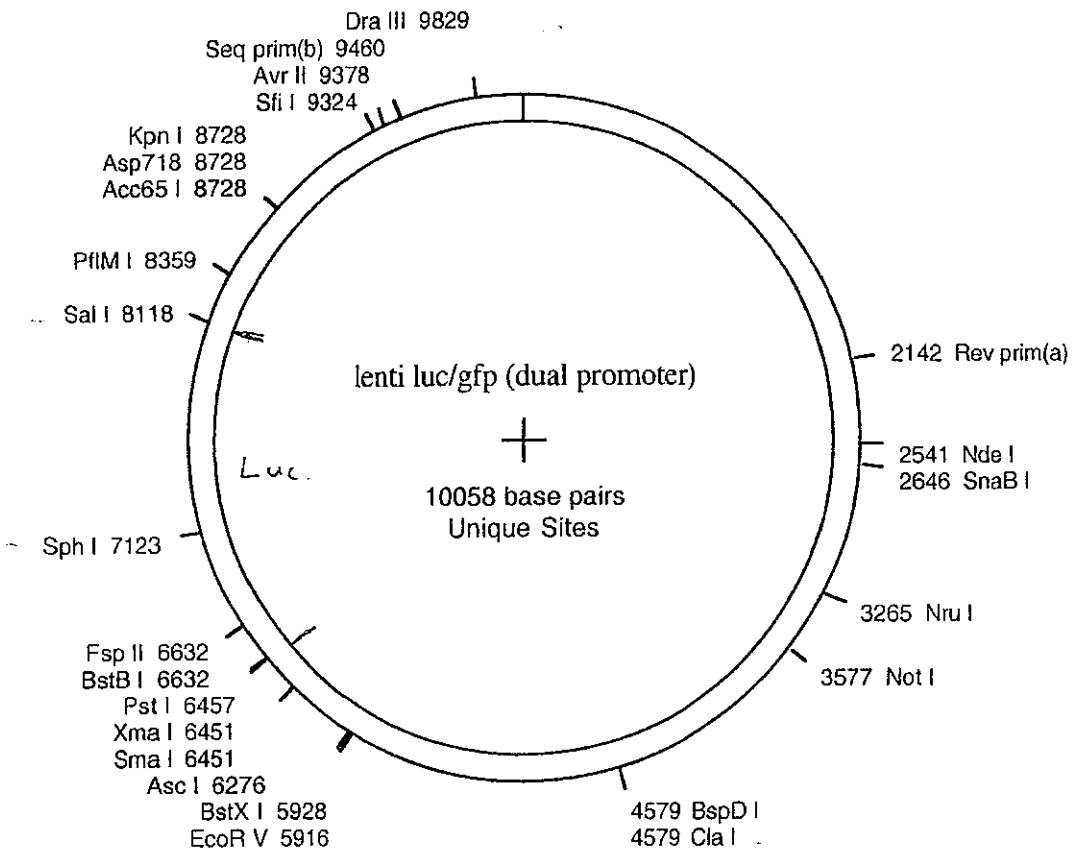
1. I didn't want to throw out any cells, so I lifted them with trypsin, and replated everything into a 6 well plate. Once that was confluent, it was moved to a T25, and lastly to a T75 as confluency dictated.

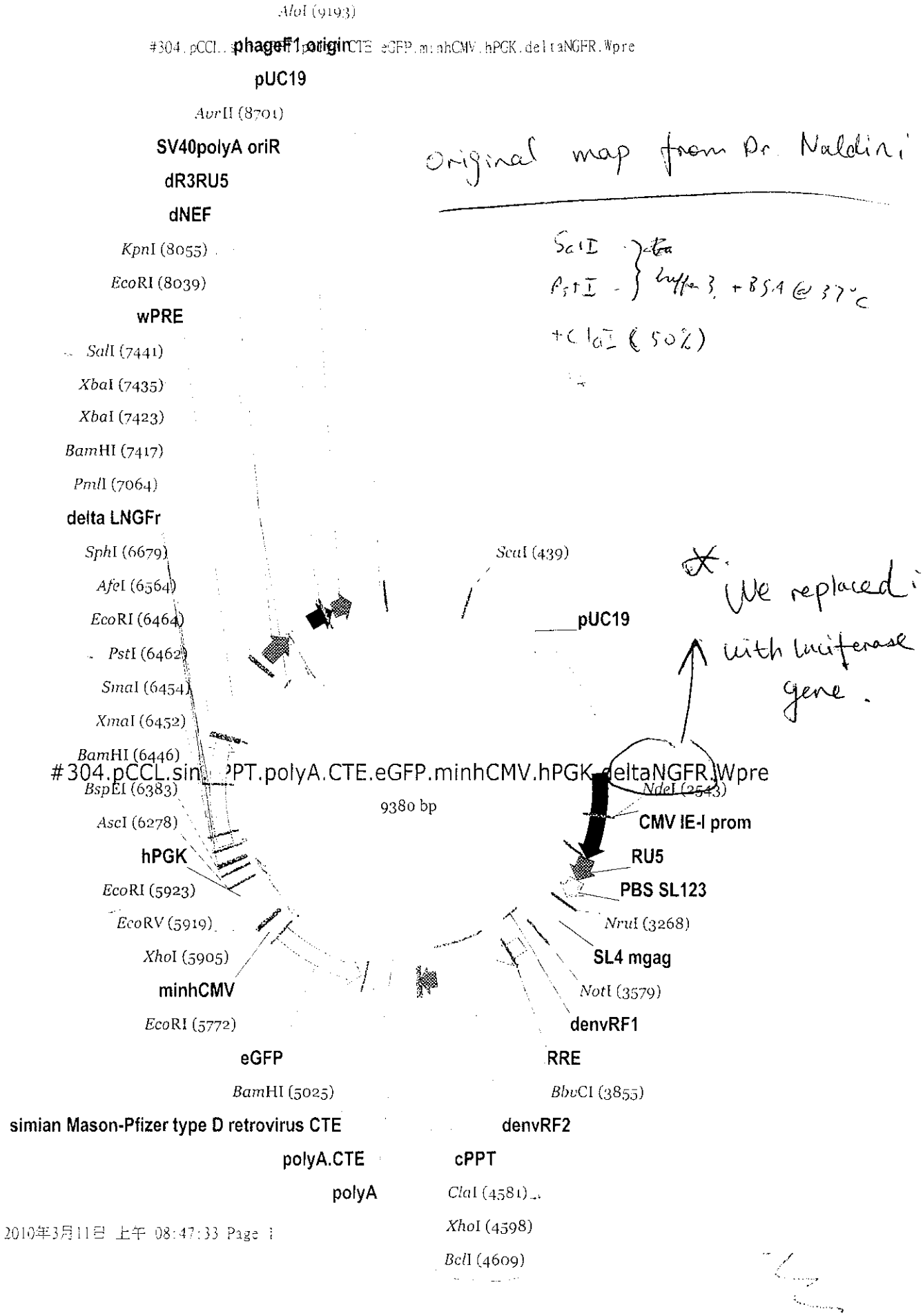
DNA Strider™ 1.2.1 ### Friday, March 31, 2006 10:07:19 AM

lenti luc/gfp (dual promoter) -> Graphic Map

DNA sequence 10058 b.p. caggtggcactt ... tttacaatttcc circular

#304.pCCL.sin.cPPT.polyA.CTE.eGFP.minhCMV.hPGK.luc.Wpre
PGKpromoter= 5928-6443 luc= 6464-8117 (Pst1/Sal1)
minhCMVpromoter=5786-5894 (reverse) eGFP=5051-5767 (reverse)





Original map from Dr. Naldini

ScaI } delta
PstI } buffer 3 + BSA @ 37°C
+ ClaI (50%)

* We replaced it with luciferase gene.

2010年3月11日 上午 08:47:33 Page 1

To Whom it May Concern,

I am writing to modify the biosafety protocol for Dr. Paula Foster's lab. The proposed work involves the MDA-MB-231BR eGFP cell line. This cell line has already been registered as a biohazard in Dr Foster's lab. The MDA-MB-231BR eGFP cells will be transfected with the mammalian expression vector pDsRed Monomer-Hyg-N1 (Clontech). Under Dr. Paula Foster's supervision, I would like to use this modified cell line for cell localization and cancer research.

These cells will be cultured and incubated with iron to permit their detection by magnetic resonance imaging. In addition, if the cell labeling is effective then the cells will be injected/implanted into mice for an animal cancer model and MRI.

The cell transfection will be performed in Dr. Greg Dekaban's Lab. Future work may be performed with several other mammalian expression vectors including pcDNA3.1 (Invitrogen) and pEYFP (Clontech). I'd also like to have these vectors added to the biosafety modification.

The protocol of cell transfection and original vector information for all 3 vectors are attached. Any alteration in transfection protocol required for each of these vectors has been noted in the attached protocol.

Please let me know if you have questions or comments.

Regards,

Amanda Hamilton

Mailing Address:
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100 Perth Dr
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Mammalian cell transfection*

*I've used this technique previously for MDA-MB-231BR eGFP cells at Stanford University in Stanford, CA, USA.

** The media used for the below cell line is DMEM with 10% FBS.

*** This will be repeated using the pDsRed Monomer-Hyg-N1 vector. The procedure for other vectors only differs in the selection agent used, please see step 7.

**MDA-MB-231BR eGFP cells

1. Seed 1.25 x 10^5 cells/well in a 6 well plate. For each vector used seed triplicate wells.
2. Allow cells to adhere overnight at 37C, 5% CO_2
3. The following day exchange media with 500 uL of fresh DMEM
4. In separate sterile Eppendorf tubes combine 500ng of plasmid, 100uL of OptiMEM media and 2 uL of Lipofectamine 2000. Make one tube for each well of cells to be transfected. Incubate at room temperature for 30 min.
5. Add full contents of each tube to a separate cell-seeded well. Let incubate overnight at 37C, 5% CO_2
6. After 24h split cells 1:10. Let incubate overnight at 37C, 5% CO_2
7. Add mammalian selection agent to each well. ***For pDsRed Monomer-Hyg-N1 use 125 ug/mL Hygromycin, for pcDNA3.1 or pEYFP use 500ug/mL Geneticin/Neomyocin respectively.
8. Grow cells under selection for 2-3 weeks, changing media every 3-4 days.
9. Once distinct drug-resistant colonies form, pick individual colonies into individual 24 well plate wells. Continue to grow under selection under a large clonal population is formed.
10. 4. To each well of a 24 well plate, add 20 000 cells, 8ug/mL of protamine sulfate (dissolved in PBS), the lentivirus suspension, and top off to a final volume of 1mL.
11. Keep track of cell growth; if necessary, move to larger tissue culture ware. Cells can be stored longterm in liquid nitrogen.

pcDNA3.1(+)
pcDNA3.1(-)

Catalog nos. V790-20 and V795-20, respectively

Version I
081401
28-0104

Invitrogen™
life technologies

www.invitrogen.com
tech_service@invitrogen.com

Table of Contents

Important Information

Contents

pcDNA3.1 is supplied as follows:

Catalog no.	Contents
V790-20	20 µg pcDNA3.1(+), lyophilized in TE, pH 8.0 20 µg pcDNA3.1/CAT, lyophilized in TE, pH 8.0
V795-20	20 µg pcDNA3.1(-), lyophilized in TE, pH 8.0 20 µg pcDNA3.1/CAT, lyophilized in TE, pH 8.0

Shipping/Storage

Lyophilized plasmids are shipped at room temperature and should be stored at -20°C.

Product Qualification

Each of the pcDNA3.1 vectors is qualified by restriction enzyme digestion with specific restriction enzymes as listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel. The table below lists the restriction enzymes and the expected fragments.

Vector	Restriction Enzyme	Expected Fragments (bp)
pcDNA3.1(+)	*Nhe* I	5428
	Pst I	1356, 4072
	Sac I	109, 5319
pcDNA3.1(-)	*Nhe* I	5427
	Pst I	1363, 4064
	Sac I	169, 5258
pcDNA3.1/CAT	*Nhe* I	6217
	Pst I	2145, 4072
	Sac I	109, 6008

Purchaser Notification

Introduction

Use of pcDNA3.1 is covered under a number of different licenses as described below.

CMV Promoter

Use of the CMV promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned and licensed by the University of Iowa Research Foundation and may be used **for research purposes only**. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation. Inquiries for commercial use should be directed to:

Brenda Akins
University of Iowa Research Foundation (UIRF)
214 Technology Innovation Center
Iowa City, IA 52242
Phone: 319-335-4549

BGH Polyadenylation Signal

The bovine growth hormone (BGH) polyadenylation sequence is licensed under U.S. Patent No. 5,122,458 for research purposes only. "Research purposes" means uses directed to the identification of useful recombinant proteins and the investigation of the recombinant expression of proteins, which uses shall in no event include any of the following:

a. any use in humans of a CLAIMED DNA or CLAIMED CELL;

b. any use in human of protein or other substance expressed or made at any stage of its production with the use of a CLAIMED DNA or a CLAIMED CELL;

c. any use in which a CLAIMED DNA or CLAIMED CELL would be sold or transferred to another party other than Invitrogen, its AFFILIATE, or its SUBLICENSEE;

d. any use in connection with the expression or production of a product intended for sale or commercial use; or

e. any use for drug screening or drug development.

Inquiries for commercial use should be directed to:

Bennett Cohen, Ph.D.
Research Corporation Technologies
101 North Wilmot Road, Suite 600
Tucson, AZ 85711-3335
Tel: 1-520-748-4400
Fax: 1-520-748-0025

Methods

Overview

Introduction

pcDNA3.1(+) and pcDNA3.1(-) are 5.4 kb vectors derived from pcDNA3 and designed for high-level stable and transient expression in mammalian hosts. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells
- Multiple cloning sites in the forward (+) and reverse (-) orientations to facilitate cloning
- Neomycin resistance gene for selection of stable cell lines
- Episomal replication in cells lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-1, COS-7)

The control plasmid, pcDNA3.1/CAT, is included for use as a positive control for transfection and expression in the cell line of choice.

Experimental Outline

Use the following outline to clone and express your gene of interest in pcDNA3.1.

1. Consult the multiple cloning sites described on pages 3-4 to design a strategy to clone your gene into pcDNA3.1.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on LB plates containing 50 to 100 µg/ml ampicillin.
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and use sequencing to confirm that your gene is cloned in the proper orientation.
5. Transfect your construct into the mammalian cell line of interest using your own method of choice. Generate a stable cell line, if desired.
6. Test for expression of your recombinant gene by western blot analysis or functional assay.

Cloning into pcDNA3.1

Introduction

Diagrams are provided on pages 3-4 to help you design a cloning strategy for ligating your gene of interest into pcDNA3.1. General considerations for cloning and transformation are listed below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, please refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

Many *E. coli* strains are suitable for the propagation of this vector including TOP10F′, DH5α™-T1^R, and TOP10. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A).

For your convenience, TOP10F′ is available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
One Shot® TOP10F′ (chemically competent cells)	21 x 50 µl	C3030-03
Electrocomp™ TOP10F′	5 x 80 µl	C665-55
Ultracomp™ TOP10F′ (chemically competent cells)	5 x 300 µl	C665-03

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of pcDNA3.1

To propagate and maintain pcDNA3.1, we recommend resuspending the vector in 20 µl sterile water to make a 1 µg/µl stock solution. Store the stock solution at -20°C.

Use this stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10F′, DH5α™-T1^R, TOP10, or equivalent. Select transformants on LB plates containing 50 to 100 µg/ml ampicillin. Be sure to prepare a glycerol stock of your plasmid-containing *E. coli* strain for long-term storage (see page 5).

Cloning Considerations

pcDNA3.1(+) and pcDNA3.1(-) are nonfusion vectors. Your insert must contain a Kozak translation initiation sequence and an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Please note that other sequences are possible (see references above), 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 shown underlined.

(G/A)NN<u>ATG</u>**G**

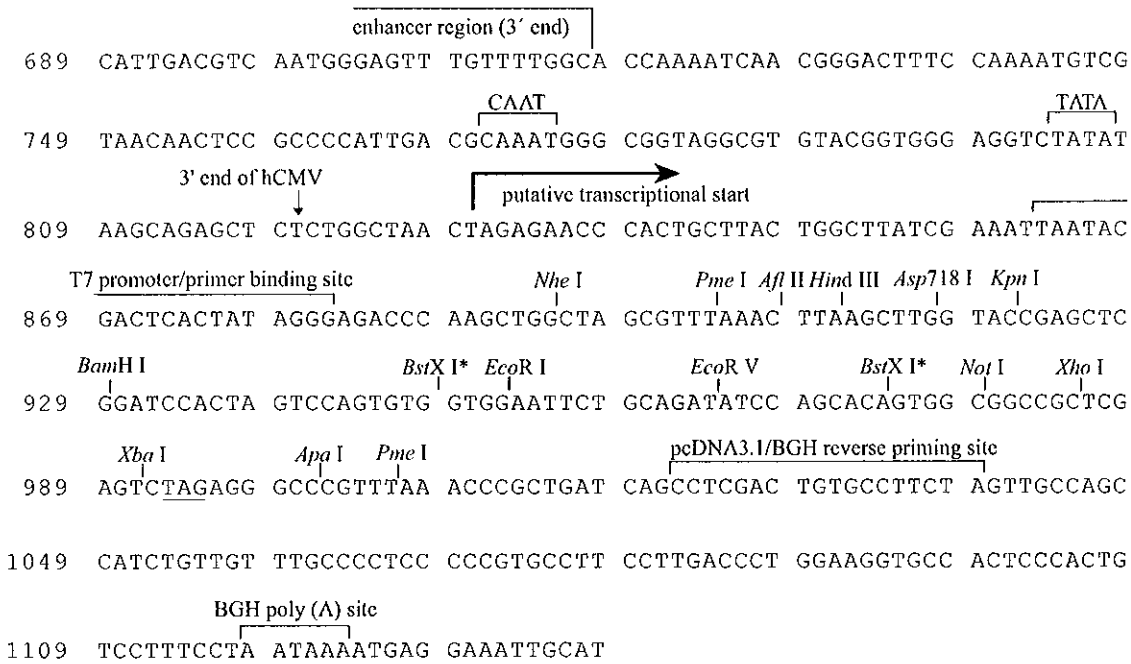
Your insert must also contain a stop codon for proper termination of your gene. Please note that the *Xba* I site contains an internal stop codon (TC<u>TAG</u>A).

continued on next page

Cloning into pcDNA3.1, continued

Multiple Cloning Site of pcDNA3.1(+)

Below is the multiple cloning site for pcDNA3.1(+). Restriction sites are labeled to indicate the cleavage site. The *Xba* I site contains an internal stop codon (TCTAGA). The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pcDNA3.1(+) is available for downloading from our web site (www.invitrogen.com) or from Technical Service (see page 13).** For a map and a description of the features of pcDNA3.1(+), please refer to the **Appendix**, pages 10-11.



*Please note that there are two *Bst*X I sites in the polylinker.

continued on next page

Cloning into pcDNA3.1, continued

Multiple Cloning Site of pcDNA3.1(-)

Below is the multiple cloning site for pcDNA3.1(-). Restriction sites are labeled to indicate the cleavage site. The *Xba* I site contains an internal stop codon (TCTAGA). The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pcDNA3.1(-) is available for downloading from our web site** (www.invitrogen.com) **or from Technical Service (see page 13).** For a map and a description of the features of pcDNA3.1(-), please see the **Appendix**, pages 10-11.

```
                                enhancer region (3′ end)
689  CATTGACGTC AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG

                                    CAAT                              TATA
749  TAACAACTCC GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT

           3' end of hCMV          putative transcriptional start
809  AAGCAGAGCT CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC

   T7 promoter/primer binding site          Nhe I      Pme I      Apa I Xba I   Xho I   Not I
869  GACTCACTAT AGGGAGACCC AAGCTGGCTA GCGTTTAAAC GGGCCCTCTA GACTCGAGCG

                    BstX I*  EcoR V        EcoR I       BstX I*          BamH I
929  GCCGCCACTG TGCTGGATAT CTGCAGAATT CCACCACACT GGACTAGTGG ATCCGAGCTC

   Asp718 I  Kpn I Hind III  Afl II   Pme I                pcDNA3.1/BGH reverse priming site
989  GGTACCAAGC TTAAGTTTAA ACCGCTGATC AGCCTCGACT GTGCCTTCTA GTTGCCAGCC

1049 ATCTGTTGTT TGCCCCTCCC CCGTGCCTTC CTTGACCCTG GAAGGTGCCA CTCCCACTGT

                BGH poly (A) site
1109 CCTTTCCTAA TAAAATGAGG AAATTGCATC
```

*Please note that there are two *Bst*X I sites in the polylinker.

continued on next page

Cloning into pcDNA3.1, continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F', DH5α™-T1^R, TOP10) and select transformants on LB plates containing 50 to 100 µg/ml ampicillin. Select 10-20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct with the T7 Promoter and BGH Reverse primers (Catalog nos. N560-02 and N575-02, respectively) to confirm that your gene is in the correct orientation for expression and contains an ATG and a stop codon. Please refer to the diagrams on pages 3-4 for the sequences and location of the priming sites. The primers are available separately from Invitrogen in 2 µg aliquots.

Preparing a Glycerol Stock

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. You should keep a DNA stock of your plasmid at -20°C.

- Streak the original colony out on an LB plate containing 50 µg/ml ampicillin. Incubate the plate at 37°C overnight.
- Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin.
- Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
- Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

Transfection

Introduction

Once you have verified that your gene is cloned in the correct orientation and contains an initiation ATG and a stop codon, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. HeLa), please 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). Invitrogen offers the Calcium Phosphate Transfection Kit (Catalog no. K2780-01) and a large selection of reagents for transfection. For more information, please refer to our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 13).

Positive Control

pcDNA3.1/CAT is provided as a positive control vector for mammalian transfection and expression (see page 12) and may be used to optimize transfection conditions for your cell line. The gene encoding chloramphenicol acetyl transferase (CAT) is expressed in mammalian cells under the control of the CMV promoter. A successful transfection will result in CAT expression that can be easily assayed (see below).

Assay for CAT Protein

You may assay for CAT expression by ELISA assay, western blot analysis, fluorometric assay, or radioactive assay (Ausubel *et al.*, 1994; Neumann *et al.*, 1987). If you wish to detect CAT protein using western blot analysis, you may use the Anti-CAT Antiserum (Catalog no. R902-25) available from Invitrogen. Other kits to assay for CAT protein using ELISA assay are available from Roche Molecular Biochemicals (Catalog no. 1 363 727) and Molecular Probes (Catalog no. F-2900).

Creation of Stable Cell Lines

Introduction

The pcDNA3.1(+) and pcDNA3.1(-) vectors contain the neomycin resistance gene for selection of stable cell lines using neomycin (Geneticin®). We recommend that you test the sensitivity of your mammalian host cell to Geneticin® as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

Geneticin® Selective Antibiotic

Geneticin® Selective Antibiotic blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, in mammalian cells results in detoxification of Geneticin® (Southern and Berg, 1982).

Geneticin® Selection Guidelines

Geneticin® Selective Antibiotic is available from Invitrogen (Catalog no. 10486-025). Use as follows:

- Prepare Geneticin® in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
- Use 100 to 800 µg/ml of Geneticin® in complete medium.
- Calculate concentration based on the amount of active drug (check the lot label).
- Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (see below). Cells differ in their susceptibility to Geneticin®.

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 up to 3 weeks of growth in selective media.

Determination of Antibiotic Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pcDNA3.1, you need to determine the minimum concentration of Geneticin® required to kill your untransfected host cell line. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Geneticin® (0, 50, 100, 200, 400, 600, 800 µg/ml Geneticin®).
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of Geneticin® that prevents growth within 2-3 weeks after addition of Geneticin®.

continued on next page

Creation of Stable Cell Lines, continued

Possible Sites for Linearization of pcDNA3.1(+)

Prior to transfection, we recommend that you linearize the pcDNA3.1(+) vector. Linearizing pcDNA3.1(+) will decrease the likelihood of the vector integrating into the genome in a way that disrupts the gene of interest or other elements required for expression in mammalian cells. The table below lists unique restriction sites that may be used to linearize your construct prior to transfection. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Invitrogen, Catalog no. 15213-028
Mfe I	161	Upstream of CMV promoter	New England Biolabs
*Bst*1107 I	3236	End of SV40 polyA	AGS*, Fermentas, Takara, Roche Mol. Biochemicals
*Eam*1105 I	4505	Ampicillin gene	AGS*, Fermentas, Takara
Pvu I	4875	Ampicillin gene	Invitrogen, Catalog no. 25420-019
Sca I	4985	Ampicillin gene	Invitrogen, Catalog no. 15436-017
Ssp I	5309	*bla* promoter	Invitrogen, Catalog no. 15458-011

*Angewandte Gentechnologie Systeme

Possible Sites for Linearization of pcDNA3.1(-)

The table below lists unique restriction sites that may be used to linearize your pcDNA3.1(-) construct prior to transfection. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Invitrogen, Catalog no. 15213-028
Mfe I	161	Upstream of CMV promoter	New England Biolabs
*Bst*1107 I	3235	End of SV40 polyA	AGS*, Fermentas, Takara, Roche Mol. Biochemicals
*Eam*1105 I	4504	Ampicillin gene	AGS*, Fermentas, Takara
Pvu I	4874	Ampicillin gene	Invitrogen, Catalog no. 25420-019
Sca I	4984	Ampicillin gene	Invitrogen, Catalog no. 15436-017
Ssp I	5308	*bla* promoter	Invitrogen, Catalog no. 15458-011

*Angewandte Gentechnologie Systeme

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Creation of Stable Cell Lines, continued

Selection of Stable Integrants

Once you have determined the appropriate Geneticin® concentration to use for selection in your host cell line, you can generate a stable cell line expressing your gene of interest.

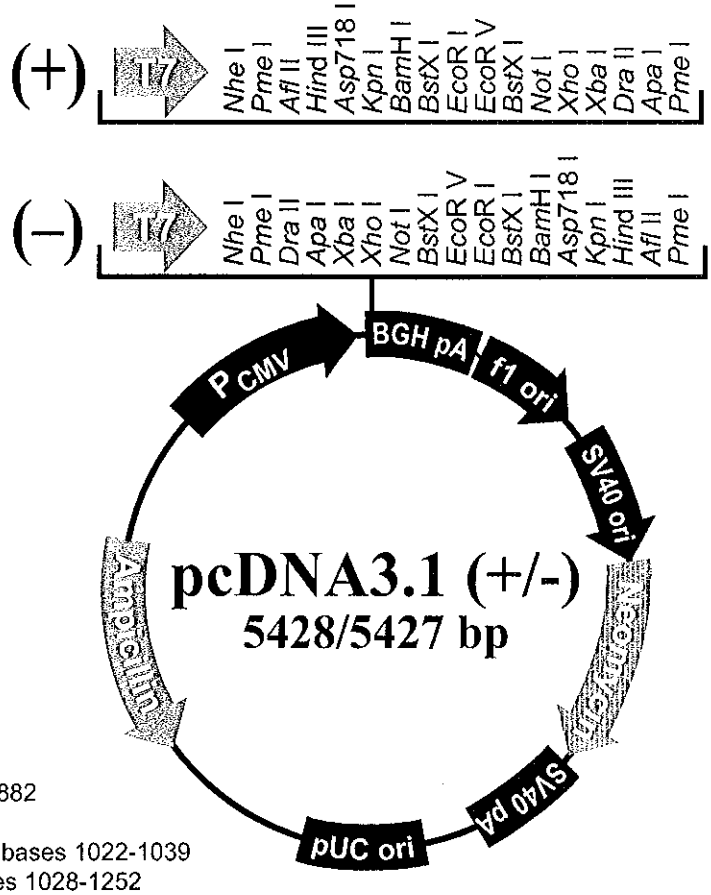
1. Transfect your mammalian host cell line with your pcDNA3.1 construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control and the pcDNA3.1/CAT plasmid as a positive control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Geneticin® at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent.
4. Feed the cells with selective medium every 3-4 days until Geneticin®-resistant foci can be identified.
5. Pick and expand colonies in 96- or 48-well plates.

Appendix

pcDNA3.1 Vectors

Map of pcDNA3.1(+) and pcDNA3.1(-)

The figure below summarizes the features of the pcDNA3.1(+) and pcDNA3.1(-) vectors. **The complete sequences for pcDNA3.1(+) and pcDNA3.1(-) are available for downloading from our World Wide Web site (**www.invitrogen.com**) or from Technical Service (see page 13).** Details of the multiple cloning sites are shown on page 3 for pcDNA3.1(+) and page 4 for pcDNA3.1(-).



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

continued on next page

pcDNA3.1 Vectors, continued

Features of pcDNA3.1(+) and pcDNA3.1(-)

pcDNA3.1(+) (5428 bp) and pcDNA3.1(-) (5427 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits 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
Multiple cloning site in forward or reverse orientation	Allows insertion of your gene and facilitates cloning
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen
Neomycin resistance gene	Selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 early polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

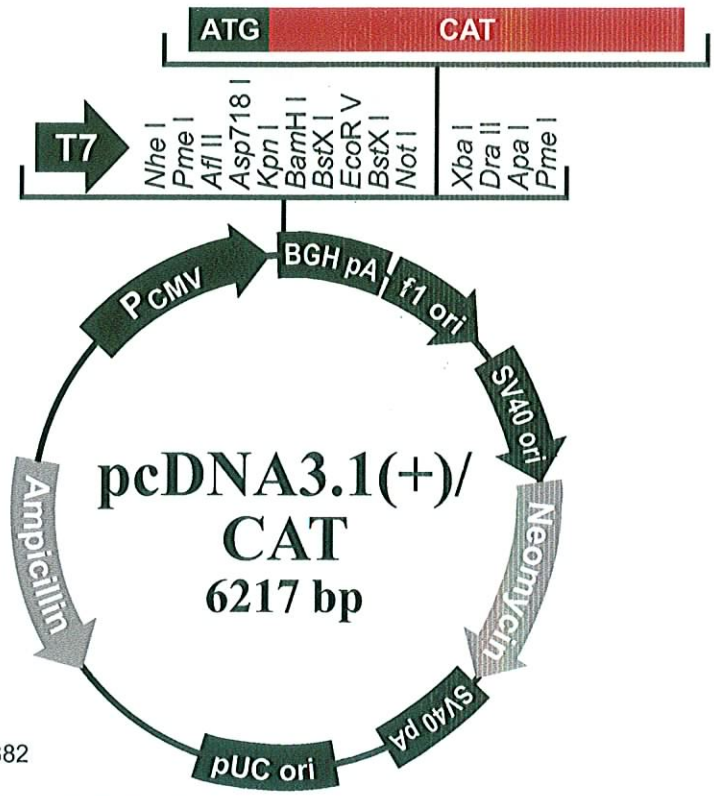
pcDNA3.1/CAT

Description

pcDNA3.1/CAT is a 6217 bp control vector containing the gene for CAT. It was constructed by digesting pcDNA3.1(+) with *Xho* I and *Xba* I and treating with Klenow. An 800 bp *Hin*d III fragment containing the CAT gene was treated with Klenow and then ligated into pcDNA3.1(+).

Map of Control Vector

The figure below summarizes the features of the pcDNA3.1/CAT vector. **The complete nucleotide sequence for pcDNA3.1/CAT is available for downloading from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (see page 13).**



Comments for pcDNA3.1(+)/CAT
6217 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
CAT ORF: bases 1027-1686
pcDNA3.1/BGH reverse priming site: bases 1811-1828
BGH polyadenylation sequence: bases 1817-2041
f1 origin: bases 2087-2515
SV40 early promoter and origin: bases 2520-2863
Neomycin resistance gene (ORF): bases 2925-3719
SV40 early polyadenylation sequence: bases 3893-4023
pUC origin: bases 4406-5076 (complementary strand)
Ampicillin resistance gene (ORF): bases 5221-6081 (complementary strand)

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our web page (www.invitrogen.com).

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Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
E-mail: eurotech@invitrogen.com

MSDS Requests

To request an MSDS, please visit our web site (www.invitrogen.com) and follow the instructions below.

1. On the home page, go to the left-hand column under 'Technical Resources' and select 'MSDS Requests'.
2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

continued on next page

Technical Service, continued

Emergency Information

In the event of an emergency, customers of Invitrogen can call the 3E Company, 24 hours a day, 7 days a week for disposal or spill information. The 3E Company can also connect the customer with poison control or with the University of California at San Diego Medical Center doctors.

3E Company
Voice: 1-760-602-8700

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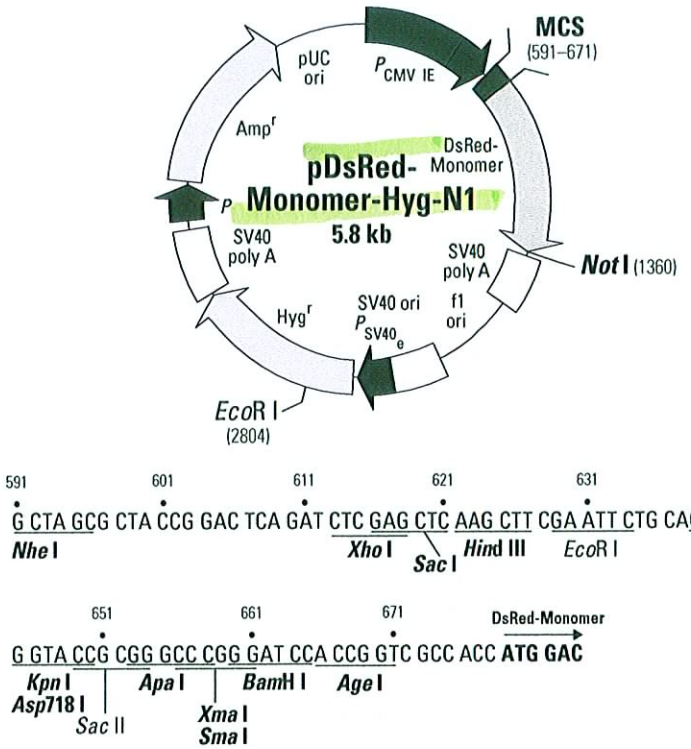
Vector Information

pDsRed-Monomer-Hyg-N1 Vector Information

PT3843-5

Cat. No. 632494



Restriction Map and Multiple Cloning Site (MCS) of pDsRed-Monomer-Hyg-N1 Vector. Unique restriction sites are in bold. The *Not* I site follows the DsRed-Monomer stop codon. NOTE: The *Xba* I and *Bcl* I sites are methylated in the DNA provided by Clontech Laboratories, Inc. If you wish to digest the vector with these enzymes, you will need to transform the vector into a dam– host and make fresh DNA.

Description

pDsRed-Monomer-Hyg-N1 is a mammalian expression vector that encodes DsRed-Monomer (DsRed.M1), a monomeric mutant derived from the tetrameric *Discosoma sp.* red fluorescent protein DsRed (1). DsRed-Monomer contains forty-five amino acid substitutions (listed on page 2). When DsRed-Monomer is expressed in mammalian cell cultures, red fluorescent cells can be detected by either fluorescence microscopy or flow cytometry 12–16 hr after transfection (DsRed-Monomer excitation and emission maxima = 557 nm and 592 nm, respectively). The DsRed-Monomer coding sequence is human codon-optimized for high expression in mammalian cells (2).

DsRed-Monomer is well suited for use as a fusion tag. The multiple cloning site (MCS) in pDsRed-Monomer-Hyg-N1 is positioned between the immediate early promoter of CMV ($P_{CMV\ IE}$) and the DsRed-Monomer coding sequence. Genes cloned into the MCS are expressed as fusions to the N-terminus of DsRed-Monomer if they are in the same reading frame as DsRed-Monomer and there are no intervening stop codons. A Kozak consensus sequence is located immediately upstream of the DsRed-Monomer gene to enhance translational efficiency in eukaryotic systems (3). SV40 polyadenylation signals downstream of the DsRed-Monomer gene direct proper processing of the 3' end of the DsRed-Monomer mRNA. The vector backbone contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen, a pUC origin of replication for propagation in *E. coli*, and an f1 origin for single-stranded DNA production. A hygromycin resistance cassette (Hyg^r)—consisting of the SV40 early promoter, the hygromycin resistance gene, and SV40 polyadenylation signals—allows stably transfected eukaryotic cells to be selected using hygromycin. A bacterial promoter-resistance gene cassette confers ampicillin resistance in *E. coli*.

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Use

pDsRed-Monomer-Hyg-N1 can be used to construct fusions to the N-terminus of DsRed-Monomer. If a fusion construct retains the fluorescent properties of the native DsRed-Monomer protein, its expression can be monitored by flow cytometry and its localization *in vivo* can be determined by fluorescence microscopy. The target gene must be cloned into pDsRed-Monomer-Hyg-N1 so that it is in frame with the DsRed-Monomer coding sequence, with no intervening in-frame stop codons. The inserted gene must include an initiating ATG codon. pDsRed-Monomer-Hyg-N1 can be transfected into mammalian cells using any standard transfection method. If required, stable transfectants can be selected using hygromycin. pDsRed-Monomer-Hyg-N1 can also be used as a cotransfection marker; the unmodified vector will express DsRed-Monomer.

This vector can also be cotransfected with pAcGFP1-N1 (Cat. No. 632469) or pAcGFP1-C1 (Cat. No. 632470) to establish stable cell lines expressing two different fluorescent proteins. Different selection markers (hygromycin for pDsRed-Monomer-Hyg-N1, neomycin for pAcGFP1-N1 and pAcGFP1-C1) allow for the generation of cell lines that simultaneously express red and green fluorescent proteins.

The DsRed1-N Sequencing Primer (Cat. No. 632387) can be used to sequence genes cloned adjacent to the 5' end of the DsRed-Monomer coding region.

For Western blotting, the Living Colors® DsRed Polyclonal Antibody (Cat. No. 632496) can be used to recognize the DsRed-Monomer protein. However, to generate optimal results it may be necessary to use a higher concentration of antibody than recommended on the DsRed Polyclonal Antibody Certificate of Analysis.

Location of features

- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  - Enhancer region: 59–465; TATA box: 554–560
  - Transcription start point: 583
  - C→G mutation to remove *Sac* I site: 569
- Multiple Cloning Site: 591–671
- Human codon-optimized DsRed-Monomer gene
  - Kozak consensus translation initiation site: 672–682
  - Start codon (ATG): 679–681; Stop codon: 1354–1356
  - Amino acid substitutions (DsRed→DsRed-Monomer)
  - GCC→GAC (Ala-2 to Asp) mutation: 682–684
  - TCC→AAC (Ser-3 to Asn) mutation: 685–687
  - TCC→ACC (Ser-4 to Thr) mutation: 688–690
  - AAG→GAG (Lys-5 to Glu) mutation: 691–693
  - AAC→GAC (Asn-6 to Asp) mutation: 694–696
  - CGC→CAG (Arg-13 to Gln) mutation: 715–717
  - ACC→TCC (Thr-21 to Ser) mutation: 739–741
  - GAG→TAC (Glu-26 to Tyr) mutation: 754–756
  - CGC→AAG (Arg-36 to Lys) mutation: 784–786
  - CAC→ACC (His-41 to Thr) mutation: 799–801
  - AAC→CAG (Asn-42 to Gln) mutation: 802–804
  - GTG→GCC (Val-44 to Ala) mutation: 808–810
  - AAG→CAG (Lys-47 to Gln) mutation: 817–819
  - GTG→GCC (Val-71to Ala) mutation: 889–891
  - AAG→ATG (Lys-83 to Met) mutation: 925–927
  - AAG→ACC (Lys-92 to Thr) mutation: 952–954
  - GTG→TCC (Val-96 to Ser) mutation: 964–966
  - ACC→GAG (Thr-106 to Glu) mutation: 994–996
  - ACC→CAG (Thr-108 to Gln) mutation: 1000–1002
  - TCC→ACC (Ser-117 to Thr) mutation: 1027–1029
  - ATC→AAG (Ile-125 to Lys) mutation: 1051–1053
  - TCC→GCC (Ser-131 to Ala) mutation: 1069–1071
  - ATG→GCC (Met-141 to Ala) mutation: 1099–1101
  - GCC→CCC (Ala-145 to Pro) mutation: 1111–1113
  - CGC→AAG (Arg-149 to Lys) mutation: 1123–1125
  - CGC→CAG (Arg-153 to Gln) mutation: 1135–1137
  - CAC→TCC (His-162 to Ser) mutation: 1162–1164
  - AAG→CAC (Lys-163 to His) mutation: 1165–1167
  - CTG→ACC (Leu-174 to Thr) mutation: 1198–1200

GTG→TGC (Val-175 to Cys) mutation: 1201–1203
GAG→GAC (Glu-176 to Asp) mutation: 1204–1206
TCC→ACC (Ser-179 to Thr) mutation: 1213–1215
ATC→GTG (Ile-180 to Val) mutation: 1216–1218
ATG→AAG (Met-182 to Lys) mutation: 1222–1224
TAC→AAC (Tyr-192 to Asn) mutation: 1252–1254
TAC→CAC (Tyr-193 to His) mutation: 1255–1257
TCC→AAC (Ser-203 to Asn) mutation: 1285–1287
ATC→GTG (Ile-210 to Val) mutation: 1306–1308
CGC→CAC (Arg-216 to His) mutation: 1324–1326
ACC→GCC (Thr-217 to Ala) mutation: 1327–1329
GGC→GCC (Gly-219 to Ala) mutation: 1333–1335
CAC→TCC (His-222 to Ser) mutation: 1342–1344
CTG→GGC (Leu-223 to Gly) mutation: 1345–1347
TTC→TCC (Phe-224 to Ser) mutation: 1348–1350
CTG→CAG (Leu-225 to Gln) mutation: 1351–1353

- SV40 early mRNA polyadenylation signal
  Polyadenylation signals: 1510–1515 & 1539–1544; mRNA 3' ends: 1548 & 1560
- f1 single-strand DNA origin: 1607–2062 (Packages the noncoding strand of DsRed-Monomer)
- SV40 origin of replication: 2403–2538
- SV40 early promoter
  Enhancer (72-bp tandem repeats): 2236–2307 & 2308–2379
  21-bp repeats: 2383–2403, 2404–2424 & 2426–2446
  Early promoter element: 2459–2465
  Major transcription start points: 2455, 2493, 2499 & 2504
- Hygromycin resistance gene:
  Start codon (ATG): 2560–2562; stop codon: 3583–3585
- SV40 early mRNA polyadenylation signal: 3732–3737 & 3761–3766; mRNA 3' ends: 3770 & 3782
- Bacterial promoter for expression of Amp^r gene:
  –35 region: 3932–3937; –10 region: 3955–3960
- Ampicillin resistance gene:
  Start codon (ATG): 4002–4004; stop codon: 4860–4862
- pUC plasmid replication origin: 5025–5668

Sequencing primer location

- DsRed1-N Sequencing Primer (Cat. No. 632387; 5'-GTACTGGAACTGGGGGGACAG-3'): 879–859

Propagation in *E. coli*

- Suitable host strains: DH5α, HB101 and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid, such as the JM109 or XL1-Blue strains.
- Selectable marker: plasmid confers resistance to ampicillin (100 µg/ml) in *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: high

Excitation and emission maxima of DsRed-Monomer

- Excitation maximum = 557 nm
- Emission maximum = 592 nm

References

1. Matz, M. V., *et al.* (1999) *Nature Biotech.* **17**:969–973.
2. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
3. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.

Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by Clontech Laboratories, Inc. This vector has not been completely sequenced.

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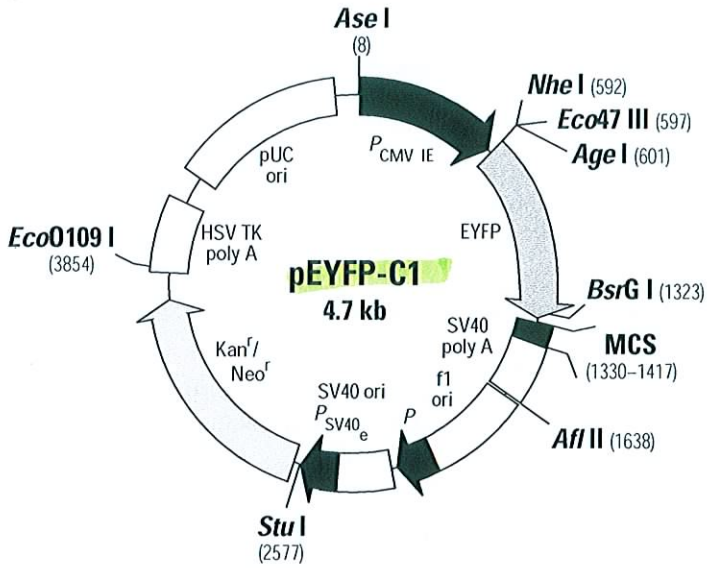
The DsRed Monomer and the Fruit Fluorescent Proteins are covered by one or more of the following U.S. Patents: 7,157,566; 7,393,923; 7,005,511 and 7,250,298.

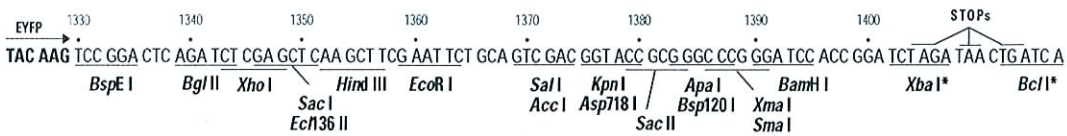
Vector Information

pEYFP-C1 Vector Information

PT3176-5

Catalog #6005-1





Restriction map and multiple cloning site (MCS) of pEYFP-C1. All restriction sites are shown are unique. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vectors with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

Description:

pEYFP-C1 encodes an enhanced yellow-green variant of the *Aequorea victoria* green fluorescent protein (GFP). The EYFP gene contains the four amino acid substitutions previously published as GFP-10C (1): Ser-65 to Gly; Val-68 to Leu; Ser-72 to Ala; and and Thr-203 to Tyr. The fluorescence excitation maximum of EYFP is 513 nm; the emission spectrum has a peak at 527 nm (in the yellow-green region). When excited at 513-nm, the E_m of EYFP is 36,500 $cm^{-1}M^{-1}$ and the fluorescent quantum yield is 0.63 (1), resulting in a bright fluorescent signal. The fluorescence observed is roughly equivalent to that from EGFP.

A mixture of EYFP- and EGFP-expressing cells can be sorted by flow cytometry using a single excitation wavelength (i.e., 488 nm). EYFP emission is detected using a 525-nm dichroic shortpass mirror and a 530/30-nm bandpass filter; EGFP emission is detected using a 510/20-nm bandpass filter.

In addition to the chromophore mutations, EYFP contains >190 silent mutations that create an open reading frame comprised almost entirely of preferred human codons (2). Furthermore, upstream sequences flanking EYFP have been converted to a Kozak consensus translation initiation site (3). These changes increase the translational efficiency of the EYFP mRNA and consequently the expression of EYFP in mammalian and plant cells.

The MCS in pEYFP-C1 is between the EYFP coding sequence and the stop codon. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EYFP if they are in the same reading frame as EYFP and there are no intervening in-frame stop codons. EYFP with a C-terminal fusion moiety retains the fluorescent properties of the native protein and thus can be used to localize fusion proteins *in vivo*.

The vector contains an SV40 origin for replication and a neomycin resistance (Neor) gene for selection (using G418) in eukaryotic cells. A bacterial promoter (*P*) upstream of Neor expresses kanamycin resistance in *E. coli*. The vector backbone also provides a pUC19 origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production. The recombinant EYFP vector can be

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transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (4). pEYFP-C1 can also be used simply to express EYFP in a cell line of interest (e.g., as a transfection marker).

Location of features:

- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  Enhancer region: 59–465; TATA box: 554–560; transcription start point: 583
  C→G mutation to remove *Sac* I site: 569
- Enhanced yellow fluorescent protein (EYFP) gene:
  Kozak consensus translation initiation site: 606–616
  Start codon (ATG): 613–615; stop codon: 1408–1410
  Insertion of Val at position 2: 616–618
  GFP-10C mutations (Ser-65 to Gly: 808–810; Val-68 to Leu: 817–819; Ser-72 to Ala: 829–831; Thr-203 to Tyr: 1222–1224)
  His-231 to Leu mutation (A→T): 1307
  Last amino acid in wild-type GFP coding region: 1327–1329
- MCS: 1330–1417
- SV40 early mRNA polyadenylation signal:
  Polyadenylation signals: 1550–1555 & 1579–1584; mRNA 3' ends: 1588 & 1600
- f1 single-strand DNA origin: 1647–2102 (Packages the noncoding strand of EYFP.)
- Bacterial promoter for expression of Kan^r gene:
  –35 region: 2164–2169; –10 region: 2187–2192
  Transcription start point: 2199
- SV40 origin of replication: 2443–2578
- SV40 early promoter:
  Enhancer (72-bp tandem repeats): 2276–2347 & 2348–2419
  21-bp repeats: 2423–2443, 2444–2464 & 2466–2486
  Early promoter element: 2499–2505
  Major transcription start points: 2495, 2533, 2539 & 2544
- Kanamycin/neomycin resistance gene:
  Neomycin phosphotransferase coding sequences:
  Start codon (ATG): 2627–2629; stop codon: 3419–3421
  G→A mutation to remove *Pst* I site: 2809
  C→A (Arg to Ser) mutation to remove *Bss*H II site: 3155
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  Polyadenylation signals: 3657–3662 & 3670–3675
- pUC plasmid replication origin: 4006–4649

Primer Locations:

- EGFP-N Sequencing Primer (#6479-1): 679–658
- EGFP-C Sequencing Primer (#6478-1): 1266–1287

Propagation in *E. coli*:

- Suitable host strains: DH5α, HB101, and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as JM109 or XL1-Blue.
- Selectable marker: plasmid confers resistance to kanamycin (30 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: ≈500
- Plasmid incompatibility group: pMB1/ColE1

References:

1. Ormö, M., *et al.* (1996) *Science* **273**:1392–1395.
2. Haas, J., *et al.* (1996) *Curr. Biol.* 6:315–324.
3. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
4. Gorman, C. (1985) In *DNA Cloning: A Practical Approach, Vol. II,* Ed. Glover, D. M. (IRL Press, Oxford, UK), pp. 143–190.

Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by BD Biosciences Clontech. This vector has not been completely sequenced.

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