The University *of* Western Ontario
BIOLOGICAL AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: October 14, 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. Stephen Ferguson**
DEPARTMENT: **Physiology and Pharmacology**
ADDRESS: **Robarts Research Institue 3250**
PHONE NUMBER: **x 24165**
EMERGENCY PHONE NUMBER(S):
EMAIL: **ferguson@robarts.ca**

Location of experimental work to be carried out :
Building : **RRI** Room(s): **3250**
Building : **RRI** Room(s): **3260**
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, Huntington's Society**
GRANT TITLE(S): **Regulation of Metabotropic Glutamate Receptor Scaffolded Signaling Complexes, Role of Metabotropic Glutamate Receptors in Huntington's Disease**
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
Harpreet Chahal	hchahal3@uwo.ca	07-Jun-2012
Tamara Cregan	tcregan@robarts.ca	22-Sept-2007
Rebecca Devries	rdevries@robarts.ca	18-Dec-2006
Henry Dunn	hdunn@uwo.ca	19-May-2009
Jessica Esseltine	jesselt@uwo.ca	27-Oct-2010

Stephen Ferguson	ferguson@robarts.ca	
Christie Godin	cgodin@robarts.ca	10-Sept-2008
Alison Hamilton	ahamil58@uwo.ca	10-May-2011
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Fitore Raka	fraka@uwo.ca	9-Oct-2011
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Ben Dickson	bdickso3@uwo.ca	26-May-2011
Fabiana Caetano	fcaetano@uwo.ca	30-Nov-2008

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.

Biological agents use are; recombinant plasmid cDNA that encodes genes of interest. These are expressed in cell lines or primary neuronal cultures. Plasmids are replicated in DHalpha5 E.coli cells, stored long term at -80 C and short term purified cDNA is stored at 4 C. All surfaces are treated with 1 % bleach and disposables are disposed of in biohazard waste for autoclave. These studies are focused on signaling complexes related to G protein-coupled receptors.

Our studies use either established cultured cell lines or primary mouse neuronal cultures. The HEK 293 and COS 7 cell lines are biohazard level 2, all other cell lines are biohazard level 1.

We have Type-5 adenovirus encoding GRK2, supplied from the Gros/Feldman Lab. Virus is replicated in HEK 293 cells and purfied from the culture medium, then stored as frozen stock at -80 C. Culture media from cells transduced with virus are treated with 10% bleach prior to disposal and flushed with water. Other contaminated lab wares are either treated with 10% bleach or disposed of in biohazard waste for autoclave. Recombinant adenovirus is used for expression of GRK2 in primary neuronal culture.
See Attachment 1.

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

Research in Ferguson Lab focuses on the signaling complexes and cascades formed from G protein-coupled receptors. G protein-coupled receptors comprise the largest family of receptors. Their roles are very divers, including hormone regulation, neurotransmission, sight and smell. Cell surface receptors are stimulated with various drugs to promote receptor activation, this elicites a second messenger signaling cascade; a series of events leading to an outcome.

Our reseach focuses on, but is not limited to Group 1 metabotropic glutamate receptors (mGluRs). mGluRs activate heterotrimeric G proteins, G alpha q/11, which then couples the receptor to 1) the activation of phospholipase C, 2) the formation of intracellular inositol phosphates and diacylglycerol, 3) the release of calcium from intracellular stores 4) the activation of other downstream effector enzymes such as protein kinase C, extracellulare regulated kinase, proline-rich kinase and CaM kinase II. Group 1 mGluRs are implicated in the regulation of higher central neurvous system functions, as well as neurological disorders. Impaired signal from the receptors is directly linked to schizophrenia, Frgile-X syndrome, Huntington's Disease, Parkinson's Disease, generalized anxiety disorders and Alzheimer disease. Consequently it is important to understand signaling of these receptors and how it can be modified.

The role of cellular prion protein in mGluR signaling and its potential role in Alzheimer disease is being explored. HEK293 cells expressing mGluR1 or 5 are contransfected with cellular prion protein and treated with or with out beta-amyloid. These cells are then assayed for inositol phosphate formation, ERK signaling and protein-protein interaction. All cells are treated with 10% bleach prior to disposal in autoclave waste. All other dishes and pipets that come into contact with these cells, are disposed of in biohazard waste for autoclave. Cellular prion protein is destroyed by autoclave.

Other signaling mechanisms from such G protein-coupled receptors as, angiotensin II type 1 receptor, 5HT 2a receptor and the corticotropin releasing factor receptor are also studied in the lab.

Experiments are carried out in cell lines transiently transfect with various plasmid cDNA. Cells are then treated with various drugs and experiments are exicuted. Conversly, experiments involving native proteins are carried out in primary mouse neuronal cultures.

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
DHalpha 5 E. coli	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	**500 mL**	**Invitrogen**	☒ 1 ☐ 2 ☐ 2+ ☐ 3
Type 5 adenovirus	☒ Yes ☐ No	☒ Yes ☐ No	☐ Yes ☒ No	**100 mL**	**Generated by Gros/Feldman lab**	☐ 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
	☐ 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: **See Attachment 2**

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		Not applicable
Rodent	☒ Yes ☐ No	**brain**	**2009-081**
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	**HEK293, IMR32, U87**	**1, 2 (HEK 293)**	**ATTC**
Rodent	☒ Yes ☐ No	**A10,RBL-2H3, PC12, ATT-20, CF10**	**1**	**ATTC, CF10 from Dr. M. Prado**
Non-human primate	☒ Yes ☐ No	**COS7**	**2**	**ATTC**
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: **See Attachment 3**

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		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Blood (fraction) or other Body Fluid		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (unpreserved)		☐ Yes ☐ Unknown		☐1 ☐2 ☐2+ ☐3
Human Organs or Tissues (preserved)		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 Transfected	Will there be a change due to transformation of the bacteria?	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?
DHalpha 5 E.coli	**See attached list**	**See attached list**	**See attachment 4**	**no**	**no**	**Bacteria amplify the plasmid**

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

***** Please attach a plasmid map.***

******No Material Safety Data Sheet is required for the following strains of E. coli:***
http://www.uwo.ca/humanresources/docandform/docs/ohs/CFIA_Ecoli_list.pdf

4.3 Will genetic modification(s) of bacteria and/or cells involving viral vectors 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
Type 5 adenovirus	**see attachment**	**See attachment**	**GRK2**	**expression of GRK2 under investigation Ribiero et al., 2009 see attachment**

**** 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: **See attachment 4**

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 7.0

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 8.0

7.2 Name of animal species to be used **mouse**

7.3 AUS protocol # **2009-081**

7.4 List the location(s) for the animal experimentation and housing. **Housed in MSB rm. 4457, brought to RRI 1291**

7.5 Will any of the agents listed in section 4.0 be used in live animals
☒ NO ☐ YES, specify:

7.6 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:

9.0 Biological Toxins and Hormones

9.1 Will toxins or hormones of biological origin be used? ☒ YES ☐ NO If **NO**, please proceed to Section 10.0

9.2 If YES, please name the toxin(s) or hormones(s) **AngII, CRF, 5HT,**
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.3 What is the LD_{50} (specify species) of the toxin or hormone **30.8mg/kg intravenous, 19g/kg oral, 12.8mg/kg intravenous, respectively**

9.4 How much of the toxin or hormone is handled at one time*? **50-100 mmolar**

9.5 How much of the toxin or hormone is stored*? **1 mg, 200 ug, 100 mg respectively**

9.6 Will any biological toxins or hormones be used in live animals? ☐ YES ☒ NO
If **YES**, Please provide details:

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

Additional Comments: **See attachment 5**

10.0 Insects

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

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

10.3 What is the origin of the insect?

10.4 What is the life stage of the insect?

10.5 What is your intention?
☐ Initiate and maintain colony, give location:
☐ "One-time" use, give location:

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

10.7 Do you use insects that require a permit from the CFIA permit? ☐ YES ☐ NO
If **YES**, Please attach the CFIA permit & describe any CFIA permit conditions:

11.0 Plants

11.1 Do you use plants? ☐ YES ☒ NO - If **NO**, please proceed to Section 12.0

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 13.0

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 Stephen Ferguson ***Date:*** August 30 2012

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: April 09, 2012
☐ NO, please certify
☐ NOT REQUIRED for Level 1 containment

14.3 Please indicate permit number (not applicable for first time applicants): **BIO-RRI-0005**

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:

Wound should be flushed with water and cleaned thoroughly, needles are disoposed of in sharps waste material. A report should be filed with the health and safety officer.

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/workplacehealth.html

An X in the check box indicates you agree with the above statement... ☒
Enter Your Name **Stephen Ferguson** ***Date:*** **August 30 2012**

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: Ronald Noseworthy
Date: Sept. 05, 2012

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

Special Conditions of Approval:

Attachment 1:

Ferguson Laboratory Guidelines for the Safe Handling of Adenovirus Vectors.

Safety equipment: Lab coats, gloves and safety glasses are worn while handling adenoviral vectors

Adenoviral containing materials are handled inside the biological safety cabinet (RRI 3260C)

All experiments and materials are handled inside the biological safety cabinet

All seriological pipettes, tips are decontaminated in virucide (clidox quadticide or 10% bleach) for 30 minutes prior to disposal in biohazard wasted container.

Upon completion of work inside the biological safety cabinet, surface is cleaned with virucide and then with 70% ethanol

All solid waste materials related to adenoviral experiments are placed in biohazard waste bags and sealed for disposal

Vaccum lines for liquid wasted are filtered with a HEPA filter before entering the vaccum system. For aspirated liquid waste, aspirate full strength bleach through the tubing to a final concentration of 10 % and soak for 30 minutes. Empty contents down the drain and flush with water. Clean liquid waste flask with 70% ethanol.

All centrifugation containing adenovirus is done in swinging bucket rotor which is sealed with aerosol tight screw caps.

Incase of adenoviral spill outside the biological safety cabinet , warn everyone in the immediate area, contain the spill and soak with bleach and cover with paper towel. Mop up spill with paper towel, reapply bleach and soak for 30 minutes. All waste materials are placed in biohazard waste and area is cleaned further with 70% ethanol.

Virus used for Transduction	**Vector**	**Source**	**Gene transferred**	**Changes that result**
Adenovirus type-5	pDC316	Vector Biolabs (Dr. Gros)	GRK2	expression of GRK2

Attachment 2:

MSDS for DHalpha 5 E. coli

MSDS for Adenovirus Type 5

Reference: Ribiero et al., 2009

VECTOR BIOLABS
THE ADENOVIRUS COMPANY

MATERIAL SAFETY DATA SHEET

EMERGENCY TELEPHONES: 1- 877-Biolabs 1-215-966-6045
http://www.vectorbiolabs.com

MATERIAL SAFETY DATA SHEET - INFECTIOUS SUBSTANCES

SECTION I - INFECTIOUS AGENT

PRODUCT IDENTIFICATION:
All pre-made adenovirus made by Vector BioLabs.

BIOLOGICAL NAME: **Adenovirus - Type 5**
CHARACTERISTICS: Adenoviridae; non-enveloped, icosahedral virions, 75-80 nm diameter, doubledstranded, linear DNA genome. The recombinant viruses are based on human adenoviral backbone which is deleted in the essential E1 gene as well as the E3 gene. The viruses produced are thus non-replicative.

SECTION II - HEALTH HAZARD

PATHOGENICITY: Varies in clinical manifestation and severity; symptoms include fever, rhinitis, pharyngitis, cough and conjunctivitis. The risk from infection by defective recombinant adenoviral vectors depends both on the dose of virus and on the nature of the transgene. Adenovirus does not integrate into the host cell genome but can produce a strong immune response.
HOST RANGE: Humans and animals
INCUBATION PERIOD: from 1-10 days
MODE OF TRANSMISSION: In the laboratory, care must be taken to avoid spread of infectious material by aerosol, direct contact or accidental injection
CHEMICAL LISTED AS CARCINOGEN OR POTENTIAL CARCINOGEN: None

SECTION III - VIABILITY

DRUG SUSCEPTIBILITY: No specific antiviral available
SUSCEPTIBILITY TO DISINFECTANTS: Susceptible to 1% sodium hypochlorite, 2% glutaraldehyde. Recommend use of 1/3 volume of bleach for 30 minutes.
PHYSICAL INACTIVATION: Sensitive to heat; 1 hour at 56°C is used to inactivate virus.
SURVIVAL OUTSIDE HOST: Adenovirus type 5 survived from 3-8 weeks on environmental surfaces at room temperature.

SECTION IV - MEDICAL

SURVEILLANCE: Monitor for symptoms; confirm by serological analysis
FIRST AID/TREATMENT:
Contact: Immediately flush eyes and skin with plenty of water for at least 15 minutes. Call a physician.
Inhalation: N/A
Ingestion: Wash out mouth with water. Call a physician
Accidental injection: wash area with soap and water. Call a physician.

SECTION V – ACCIDENTAL RELEASE PROCEDURES

Pour 1 volume of Javel water over the leak(s) and wait for 15 minutes.
Wipe up carefully.
Hold for autoclave waste disposal and decontaminate work surfaces with 70% alcohol.

SECTION VI - RECOMMENDED PRECAUTIONS

CONTAINMENT REQUIREMENTS: Biosafety level 2 practices and containment facilities for all activities involving the virus and potentially infectious body fluids or tissues. This level consists of etiological agents considered to be of ordinary potential harm.

PROTECTIVE CLOTHING: Recombinants Adenovirus: Laboratory coat; gloves.

OTHER PRECAUTIONS:

Access to the laboratory is limited.
Work surfaces are decontaminated before and after each procedure
Mechanical pipetting devices are used for all procedures; mouth pipetting is prohibited.
Eating, drinking, and smoking are not permitted in the laboratory; food is not stored in laboratory areas.
Laboratory coats are worn in and are removed before leaving the laboratory.
Hands are washed before and after handling virus.

SECTION VII - HANDLING INFORMATION

DISPOSAL: Decontaminate all wastes before disposal; steam sterilization
STORAGE: In sealed containers that are appropriately labeled

SECTION VIII - MISCELLANEOUS INFORMATION

The above information and recommendations are believed to be accurate and represent the most complete information currently available to us. All materials and components may present unknown hazards and should be used with caution. Vector BioLabs, Inc assumes no liability resulting from use of the above products.

Date of revision: May 24, 2004

invitrogen™ **Material Safety Data Sheet** **Revision Date:** 19-Mar-2010

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code	18265017
Product name	Subcloning Efficiency ™ DH5alpha™ Competent Cells

Company/Undertaking Identification

INVITROGEN CORPORATON
5791 VAN ALLEN WAY
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
5250 MAINWAY DRIVE
BURLINGTON, ONT
CANADA L7L 6A4
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

24 hour Emergency Response (Transport):
866-536-0631
301-431-8585
Outside of the U.S. ++1-301-431-8585

For research use only

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health. We recommend handling all chemicals with caution.

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health

3. HAZARDS IDENTIFICATION

Form
Liquid

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	May be harmful if swallowed.

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects	No information available

HMIS

Health	0
Flammability	0
Reactivity	0

4. FIRST AID MEASURES

Skin contact	Wash off immediately with plenty of water. If symptoms persist, call a physician.
Eye contact	Rinse thoroughly with plenty of water, also under the eyelids. If symptoms persist, call a physician.
Ingestion	Never give anything by mouth to an unconscious person. If symptoms persist, call a physician.
Inhalation	Move to fresh air. If symptoms persist, call a physician.
Notes to physician	Treat symptomatically.

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media	Dry chemical
Special protective equipment for firefighters	Wear self-contained breathing apparatus and protective suit

6. ACCIDENTAL RELEASE MEASURES

Personal precautions	Use personal protective equipment
Methods for cleaning up	Soak up with inert absorbent material.

7. HANDLING AND STORAGE

Handling	No special handling advice required
Storage	Keep in properly labelled containers

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Occupational exposure controls

Exposure limits

Engineering measures	Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory Protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing.
Hygiene measures	Handle in accordance with good industrial hygiene and safety practice
Environmental exposure controls	Prevent product from entering drains.

9. PHYSICAL AND CHEMICAL PROPERTIES

General Information

Form	Liquid

Important Health Safety and Environmental Information

Boiling point/range	**°C** No data available	**°F** No data available
Melting point/range	**°C** No data available	**°F** No data available
Flash point	**°C** No data available	**°F** No data available
Autoignition temperature	**°C** No data available	**°F** No data available
Oxidizing properties	No information available	
Water solubility	No data available	

10. STABILITY AND REACTIVITY

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur.

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available

Ingestion	May be harmful if swallowed.

Specific effects	**(Long Term Effects)**
Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects	No information available

12. ECOLOGICAL INFORMATION

Ecotoxicity effects	No information available.
Mobility	No information available.
Biodegradation	Inherently biodegradable.
Bioaccumulation	Does not bioaccumulate.

13. DISPOSAL CONSIDERATIONS

Dispose of in accordance with local regulations

14. TRANSPORT INFORMATION

IATA

Proper shipping name	Not classified as dangerous in the meaning of transport regulations
Hazard Class	No information available
Subsidiary Class	No information available
Packing group	No information available
UN-No	No information available

15. REGULATORY INFORMATION

International Inventories

U.S. Federal Regulations

SARA 313

This product is not regulated by SARA.

Clean Air Act, Section 112 Hazardous Air Pollutants (HAPs) (see 40 CFR 61)

This product does not contains HAPs.

U.S. State Regulations

California Proposition 65

This product does not contain chemicals listed under Proposition 65

WHMIS hazard class:

Non-controlled

This product has been classified according to the hazard criteria of the CPR and the MSDS contains all of the information required by the CPR

16. OTHER INFORMATION

For research use only

The above information was acquired by diligent search and/or investigation and the recommendations are based on prudent application of professional judgment. The information shall not be taken as being all inclusive and is to be used only as a guide. All materials and mixtures may present unknown hazards and should be used with caution. Since the Company cannot control the actual methods, volumes, or conditions of use, the Company shall not be held liable for any damages or losses resulting from the handling or from contact with the product as described herein. THE INFORMATION IN THIS MSDS DOES NOT CONSTITUTE A WARRANTY, EXPRESSED OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY PARTICULAR PURPOSE.

End of Safety Data Sheet

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Phosphorylation-independent Regulation of Metabotropic Glutamate Receptor 5 Desensitization and Internalization by G Protein-coupled Receptor Kinase 2 in Neurons*

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The uncoupling of metabotropic glutamate receptors (mGluRs) from heterotrimeric G proteins represents an essential feedback mechanism that protects neurons against receptor overstimulation that may ultimately result in damage. The desensitization of mGluR signaling is mediated by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs). Unlike mGluR1, the attenuation of mGluR5 signaling in HEK 293 cells is reported to be mediated by a phosphorylation-dependent mechanism. However, the mechanisms regulating mGluR5 signaling and endocytosis in neurons have not been investigated. Here we show that a 2-fold overexpression of GRK2 leads to the attenuation of endogenous mGluR5-mediated inositol phosphate (InsP) formation in striatal neurons and siRNA knockdown of GRK2 expression leads to enhanced mGluR5-mediated InsP formation. Expression of a catalytically inactive GRK2-K220R mutant also effectively attenuates mGluR5 signaling, but the expression of a GRK2-D110A mutant devoid in $G\alpha_{q/11}$ binding increases mGluR5 signaling in response to agonist stimulation. Taken together, these results indicate that the attenuation of mGluR5 responses in striatal neurons is phosphorylation-independent. In addition, we find that mGluR5 does not internalize in response to agonist treatment in striatal neuron, but is efficiently internalized in cortical neurons that have higher levels of endogenous GRK2 protein expression. When overexpressed in striatal neurons, GRK2 promotes agonist-stimulated mGluR5 internalization. Moreover, GRK2-mediated promotion of mGluR5 endocytosis does not require GRK2 catalytic activity. Thus, we provide evidence that GRK2 mediates phosphorylation-independent mGluR5 desensitization and internalization in neurons.

Glutamate is the major excitatory neurotransmitter in the mammalian brain and functions to activate two distinct classes of receptors (ionotropic and metabotropic) to regulate a variety of physiological functions (1–3). Ionotropic glutamate receptors, such as NMDA, AMPA, and kainate receptors, are ligand-gated ion channels, whereas metabotropic glutamate receptors (mGluRs)[5] are members of the G protein-coupled receptor (GPCR) superfamily (4–7). mGluRs modulate synaptic activity via the activation of heterotrimeric G proteins that are coupled to a variety of second messenger cascades. Group I mGluRs (mGluR1 and mGluR5) are coupled to the activation of $G\alpha_{q/11}$ proteins, which stimulate the activation of phospholipase $C\beta1$ resulting in diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) formation, release of Ca^{2+} from intracellular stores and subsequent activation of protein kinase C.

The attenuation of GPCR signaling is mediated in part by G protein-coupled receptor kinases (GRKs), which phosphorylate GPCRs to promote the binding of β-arrestin proteins that uncouple GPCRs from heterotrimeric G proteins (8–10). GRK2 has been demonstrated to contribute to the phosphorylation and desensitization of both mGluR1 and mGluR5 in human embryonic kidney (HEK 293) cells (11–17). GRK4 is also implicated in mediating the desensitization of mGluR1 signaling in cerebellar Purkinje cells, but does not contribute to the desensitization of mGluR5 (14, 15). In addition, GRK4 plays a major role in mGluR1 internalization (13, 14). A role for GRK2 in promoting mGluR1 internalization is less clear as different laboratories have obtained discordant results (11, 14, 15, 16). However, the only study examining the role of GRK2 in regulating mGluR1 endocytosis in a native system reported that GRK2 knockdown had no effect upon mGluR1 internalization in cerebellar Purkinje cells (14).

GRK2 is composed of three functional domains: an N-terminal regulator of G protein signaling (RGS) homology (RH) domain, a central catalytic domain, and a C-terminal $G_{\beta}\gamma$ binding pleckstrin homology domain (18). In HEK 293 cells,

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[5] The abbreviations used are: mGluR, metabotropic glutamate receptor; DHPG, (*S*)-3,5-dihydroxylphenylglycine; DIV, days *in vitro*; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; InsP, inositol phosphate; NC, non-coding; PKC, protein kinase C; RGS, regulator of G protein signaling; RH, regulator of G protein signaling homology; siRNA, small interfering RNA; PBS, phosphate-buffered saline.

mGluR1 desensitization is not dependent on GRK2 catalytic activity. Rather the GRK2 RH domain interacts with both the second intracellular loop domain of mGluR1 and the α-subunit of $G\alpha_{q/11}$ and attenuates second messenger responses by disrupting the mGluR1/$G\alpha_{q/11}$ signaling complexes (12, 19–21). Although the molecular mechanism underlying GRK2-mediated attenuation of mGluR1 signaling is relatively well established in HEK 293 cells, the role of GRK2 in regulating the desensitization of mGluRs in neurons remains to be determined. Moreover, it is not known whether GRK2-dependent attenuation of mGluR5 signaling is mediated by the same phosphorylation-independent mechanism that has been described for mGluR1. In a previous study, GRK2-mediated mGluR5 desensitization was reported to be phosphorylation-dependent, based on the observation that the overexpression of a catalytically inactive GRK2 (K220R) did not attenuate mGluR5 signaling (15). In the present study, we examined whether a 2-fold overexpression of GRK2 in primary mouse striatal neurons to match GRK2 expression levels found in the cortex results in increased agonist-stimulated desensitization and internalization of endogenous mGluR5. We report here that GRK2 mediates phosphorylation-independent mGluR5 desensitization and internalization. Furthermore, GRK2 knockdown causes an increase in mGluR5 signaling, demonstrating that endogenous GRK2 plays a role in mGluR5 desensitization.

EXPERIMENTAL PROCEDURES

Materials—(*S*)-3,5-Dihydroxylphenylglycine (DHPG) was purchased from Tocris Cookson Inc. (Ellisville, MO). *myo*-[^{3}H]Inositol and [^{32}P]orthophosphate were acquired from PerkinElmer Life Sciences (Waltham, MA). The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad (Mississauga, ON). Bovine serum albumin was obtained from BioShop Canada Inc. (Mississauga, ON). Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody was from Bio-Rad. ECL Western blotting detection reagents were from GE Healthcare (Oakville, Ontario, Canada). EZ-Link Sulfo-NHS-SS-Biotin and immobilized NeutrAvidin beads were from Pierce Biotechnology. Hiperfect transfection reagent was purchased from Qiagen (Mississauga, ON). QuikChange^{TM} site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Rabbit anti-mGluR1 and anti-mGluR5 antibodies were from Upstate/Millipore (Billerica, MA). Mouse anti-NeuN monoclonal antibody, Alexa Fluor 568 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit secondary antibodies were purchased from Invitrogen/Molecular Probes (Burlington, ON). Rabbit anti-GRK2 and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-clathrin antibody was from BD Biosciences (Mississauga, ON). Rabbit anti-FLAG antibody and all other biochemical reagents were purchased from Sigma-Aldrich.

mGluR5 Knock-out Mice—B6;129-Grm5^{tm1Rod}/J (mGluR5$^{-/-}$) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in an animal care facility at 23 °C on a 12 h light/12 h dark cycle with food and water provided *ad libitum*. Animal care was in accordance with the University of Western Ontario Animal Care Committee.

Adenovirus Construction—The mouse GRK2 cDNA used for adenovirus construction was FLAG-tagged in the N-terminal region (22). Mutations (K220R and D110A) introduced to mouse GRK2 construct were created using the QuikChange^{TM} site-directed mutagenesis kit. cDNA encoding FLAG-tagged GRK2, GRK2-K220R, and GRK2-D110A or green fluorescent protein (GFP) were used to generate adenoviral constructs (AdMax) as per the manufacturer's instructions (Microbix Biosystems, Toronto, ON).

Neuronal Primary Culture Preparation—Neuronal cultures were prepared from the striatal region of E15 mouse embryo brains. Animal procedures were approved by The University of Western Ontario Animal Care Committee. After dissection, either striatal or cortical tissue was submitted to trypsin digestion followed by cell dissociation using a fire-polished pasteur pipette. Cells were plated on poly-L-ornithine-coated dishes in neurobasal media supplemented with N2 and B27 supplements, 2 mM glutamax, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated at 37 °C and 5% CO_2 in a humidified incubator and cultured for 12 days *in vitro* (DIV) with media replenishment every 4 days. The cultures were infected with the different adenoviruses (GFP, wild-type GRK2, GRK2-K220R, or GRK2-D110A) at DIV9 and used for experiments at DIV12.

Small Interfering RNA (siRNA) Transfection—Striatal neurons seeded in 12-well plates were transfected in cultured medium using Hiperfect transfection reagent. Neurons were transfected with either non-coding (NC) or GRK2 siRNA as per the manufacturer's instructions. siRNA (20 nM final concentration) was added to 100 μl of Neurobasal medium, followed by addition of 6 μl of Hiperfect. Transfection mix was vortexed, incubated for 5–10 min at room temperature, and added to neuronal cultures. Neurons were transfected twice at DIV5 and DIV7 and used for experiments at DIV9. Both the GRK2 siRNA and NC siRNA (siGENOME control siRNA-non-targeting siRNA 1) were purchased from Dharmacon Research (Lafayette, CO). The GRK2 mouse antisense oligonucleotide had the following sequence: 5′-AAGAAATATGAGAAGCTGGAG-3′ (23). The effect of siRNA transfection was assessed by measuring the total GRK2 protein expression by Western blotting analysis.

Inositol Phosphate Formation—Inositol lipids were radiolabeled by incubating the striatal neurons infected with different adenoviruses (GFP, GRK2, GRK2-K220R, or GRK2-D110A) overnight with 1 μCi/ml *myo*-[^{3}H]inositol in Neurobasal medium. Unincorporated *myo*-[^{3}H]inositol was removed by washing cells with Hank's balanced salt solution (HBSS). Cells were preincubated for 1 h in HBSS at 37 °C and then preincubated in 500 μl of the same buffer containing 10 mM LiCl for an additional 2 min at 37 °C. Cells were then incubated in the presence of either DHPG or carbachol for 5 min at 37 °C. Drug concentration is indicated in the figures. The reaction was stopped on ice by the addition of 500 μl of perchloric acid and then neutralized with 400 μl of 0.72 M KOH, 0.6 M $KHCO_3$. Total [^{3}H]inositol incorporated into cells was determined by counting the radioactivity present in 50 μl of cell lysate. Total inositol phosphate was purified from cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form)

200–400 mesh anion exchange resin. [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system (20, 21).

Co-immunoprecipitation—Striatal neurons infected with either GFP or GRK2 adenovirus were incubated at 37 °C in HBSS in the presence of 10 μM DHPG for 5 min. Cells were washed with ice-cold HBSS and solubilized in lysis buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF and 10 μg/ml of both leupeptin and aprotinin). mGluR5 was immunoprecipitated from 500–1000 μg of total cell lysate using anti-mGluR5 antibody and protein G-Sepharose beads by 2 h rotation at 4 °C. Afterward, beads were washed one time with lysis buffer and two times with PBS, and proteins were eluted in SDS-PAGE loading buffer by warming the samples at 55 °C for 5 min. Eluted samples were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes.

Internalization Assay—To evaluate the effect of GRK2 overexpression on mGluR5 internalization, either cortical or striatal neurons infected with different adenoviruses (GFP, GRK2, GRK2-K220R, or GRK2-D110A) were incubated at 37 °C in HBSS in the presence of 10 μM DHPG for varying times (0, 5, or 10 min). Cells were washed with HBSS and incubated on ice. Plasma membrane proteins were biotinylated with sulfo-NHS-SS-Biotin for 1 h on ice, as described previously (24). To quench the biotinylation reaction, cells were washed and incubated for 30 min with cold 100 mM glycine in HBSS, followed by three washes with cold HBSS. Cells were then lysed in radioimmune precipitation assay buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 0.05 M EDTA, 1% nonidet P40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM AEBSF and 10 μg/ml of both leupeptin and aprotinin). Biotinylated proteins were separated from non-biotinylated proteins by Neutravidin bead pull-down from equivalent amounts of total cellular protein from each sample (100 μg per sample). Biotinylated proteins were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes.

Recycling Assay—To evaluate the effect of GRK2 overexpression on mGluR5 recycling, striatal neurons infected with either GFP or GRK2 adenovirus were incubated at 37 °C in HBSS in the presence of 10 μM DHPG for 10 min to stimulate internalization of plasma membrane mGluR5. Agonist was washed out, and cells were incubated in HBSS at 37 °C for 10 or 30 min to allow internalized mGluR5 to recycle to the plasma membrane. Cells were washed with HBSS and incubated on ice. Plasma membrane proteins were biotinylated with sulfo-NHS-SS-Biotin, and subsequent steps were conducted as described in internalization assay section.

Immunoblotting—Membranes were blocked with 10% milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) for 1 h and then incubated with rabbit anti-GRK2 (1:2000), rabbit anti-mGluR5 (1:4000), rabbit anti-mGluR1 (1:1000), mouse anti-clathrin (1:1000), or rabbit anti-actin (1:10,000) antibodies in wash buffer containing 3% milk overnight. Membranes were rinsed three times with wash buffer and then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG diluted 1:10,000 in wash buffer containing 3% skim milk for 1 h. Membranes were rinsed three times with wash buffer and incubated with ECL Western blotting detection reagents.

Whole Cell Phosphorylation—Whole cell phosphorylation experiments were performed as described previously (25). To label the intracellular ATP pool with [^{32}P]orthophosphate, striatal neurons infected with either GFP or GRK2 adenoviruses were incubated for 1 h at 37 °C in HBSS containing [^{32}P]orthophosphate (100 μCi/ml). Subsequently, the neurons were incubated at 37 °C in the presence of 10 μM DHPG for varying times (0, 5, or 10 min). Cells were solubilized in radioimmune precipitation assay buffer containing protease and phosphatase inhibitors (1 mM AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM NaF, 10 mM sodium pyrophosphate, and 0.1 mM phenylmethylsulfonyl fluoride). Samples were normalized to total protein content. mGluR5 was immunoprecipitated using a rabbit anti-mGluR5 antibody and subsequently subjected to SDS-PAGE followed by autoradiography. The extent of receptor phosphorylation was quantitated by densitometry of the resulting autoradiographs.

Immunofluorescence and Confocal Imaging—Striatal neurons either uninfected or infected with FLAG-GRK2 adenovirus were washed twice in PBS and fixed with 3% formaldehyde in PBS for 20 min. After fixation, cells were washed with PBS and preincubated with a permeabilization solution (PBS, 0.05% Triton, and 3% bovine serum albumin) for 10 min. Subsequently, rabbit anti-FLAG (1:500) and mouse anti-NeuN (1:1000) antibodies were added to cells and incubated for 1 h in permeabilization solution. Cells were washed and incubated with goat anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 568 and Alexa Fluor 488, respectively for 40 min in permeabilization solution at 1:1000 dilution. Detection of immunolabeled proteins was performed using dual excitation (488 and 543 nm) and emission (505–530 nm for Alexa Fluor 488-labeled anti-FLAG antibody and GFP, and 590–610 nm for Alexa Fluor 555-labeled anti-NeuN antibody) filter sets. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope using a Zeiss 63 × 1.3 NA oil immersion lens.

Data Analysis—Non-saturated, immunoreactive mGluR5 bands from both internalization and whole cell phosphorylation assays were quantified by scanning densitometry using Scion Image software. Means ± S.E. are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data for statistical significance and for curve fitting. Statistical significance was determined by analysis of variance (ANOVA) testing followed by post-hoc multiple comparison testing.

RESULTS

Expression of mGluR5 in Striatal Neurons—Because the role of GRK2 in regulating Group I mGluR desensitization and internalization in neurons has not been investigated, we first examined which neuronal culture preparation represented the most appropriate model to study GRK2-dependent regulation of endogenous mGluR5 signaling. Primary striatal neurons obtained from E15 mouse embryos cultured 12 days *in vitro* expressed high levels of mGluR5 protein, whereas cortical neurons cultured under the

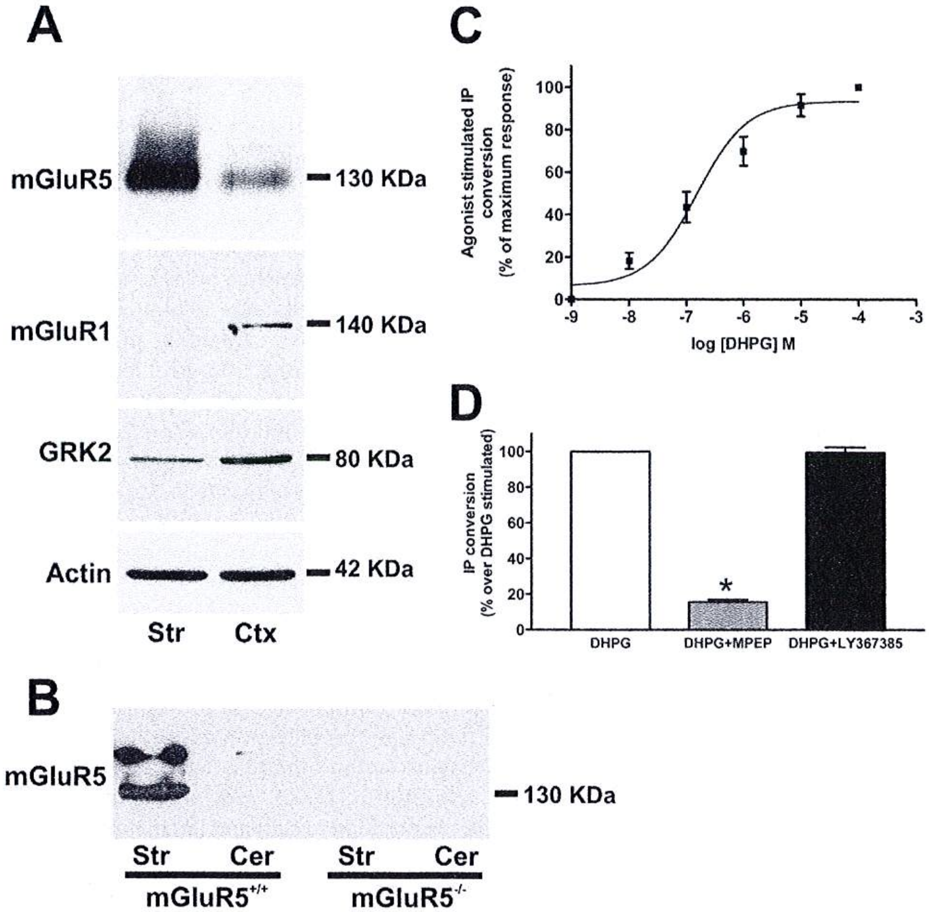

FIGURE 1. **Striatal neurons as a model for mGluR5 signaling studies.** *A*, shown are representative immunoblots for mGluR5, mGluR1, GRK2, and actin protein expression in either striatal (*Str*) or cortical (*Ctx*) neuronal cultures. 50 μg of cell lysate were used for each sample. *B*, shown is a representative immunoblot for mGluR5 protein expression in either striatal (*Str*) or cerebellum (*Cer*) from either wild-type or mGluR5$^{-/-}$ mice. 50 μg of cell lysate were used for each sample. *C*, shown is DHPG-stimulated inositol phosphate formation in striatal neurons stimulated with increasing concentrations of agonist for 5 min at 37 °C. The data points represent the means ± S.E. of five independent experiments, normalized to the maximum DHPG-stimulated response. *D*, shown is DHPG-stimulated inositol phosphate formation in response to 10 μM agonist for 5 min at 37 °C, either in the presence or absence of mGluR1- (LY36785) or mGluR5-antagonists (MPEP). Data represent the means ± S.E. of four independent experiments. The *asterisk* indicates a significant difference compared with control ($p < 0.05$).

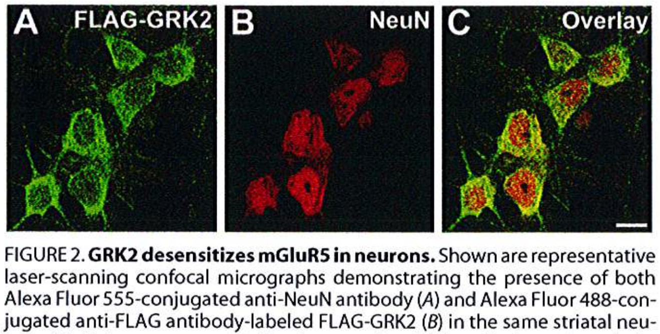

FIGURE 2. **GRK2 desensitizes mGluR5 in neurons.** Shown are representative laser-scanning confocal micrographs demonstrating the presence of both Alexa Fluor 555-conjugated anti-NeuN antibody (*A*) and Alexa Fluor 488-conjugated anti-FLAG antibody-labeled FLAG-GRK2 (*B*) in the same striatal neurons (*C*). Data are representative images of multiple cells from three independent experiments. *Bar*, 10 μM.

same conditions expressed lower levels of mGluR5, but also expressed mGluR1 (Fig. 1*A*). In contrast, mGluR1 protein expression was not detected in striatal cultures (Fig. 1*A*). GRK2 protein expression levels were also ~2-fold higher in the cortical neuronal cultures as compared with the striatal neuronal cultures (Fig. 1*A*).

To determine whether anti-mGluR5 antibody was specific to mGluR5 protein, we compared whole cell lysates obtained from the cerebellum and cortex of either wild-type or mGluR5$^{-/-}$ mice. Anti-mGluR5 antibody detected a 130-kDa band only in wild-type striatal lysate (Fig. 1*B*). No band was detected in the cerebellum, which does not express mGluR5, or in the striatum of mGluR5$^{-/-}$ lysates (Fig. 1*B*). DHPG, a specific group I mGluR agonist, promoted a dose-dependent increase in InsP formation in striatal neuronal cultures, demonstrating the expected signaling function of endogenous group I mGluRs (Fig. 1*C*). Based on the results from the dose response curve, in all subsequent experiments either 1 or 10 μM DHPG were used. The specificity of the group I mGluR-mediated InsP formation in striatal neurons was further established by the pretreatment of cells with specific mGluR5 (10 μM MPEP) and mGluR1 (100 μM LY367385) antagonists. The pretreatment of cells with MPEP reduced DHPG-stimulated InsP formation to 15.5 ± 1.3% of control and LY367385 pretreatment had no effect on DHPG-stimulated InsP formation (Fig. 1*D*). Taken together, these data indicated that mGluR5 was the primary group I mGluR expressed and activated by DHPG in embryonic-derived striatal cultures. Consequently, in all subsequent experimentation striatal neurons were used to examine GRK2-mediated regulation of mGluR5 desensitization.

GRK2-mediated Attenuation of mGluR5 Signaling—GRK2 protein expression in cortical neurons (which express mGluR5) was 2-fold higher than that of striatal neurons (Fig. 1*A*). Therefore, we tested whether a physiologically similar 2-fold increase in GRK2 expression would result in attenuated mGluR5 signaling in striatal neurons. GRK2 was overexpressed in neurons using a FLAG epitope-tagged GRK2 adenovirus. To determine whether the FLAG-GRK2 adenovirus efficiently infected neurons, we immunolabeled striatal neuronal cultures with both an antibody that recognizes the FLAG epitope tag and an antibody directed against the neuron-specific marker NeuN (neuronal nuclei) (26). We found that the FLAG-GRK2 adenoviral construct was able to effectively infect striatal neurons as the NeuN-positive neurons also exhibited FLAG-positive labeling (Fig. 2). Infection of striatal neurons with a GRK2 adenovirus increased GRK2 expression to 193 ± 7% of control neurons

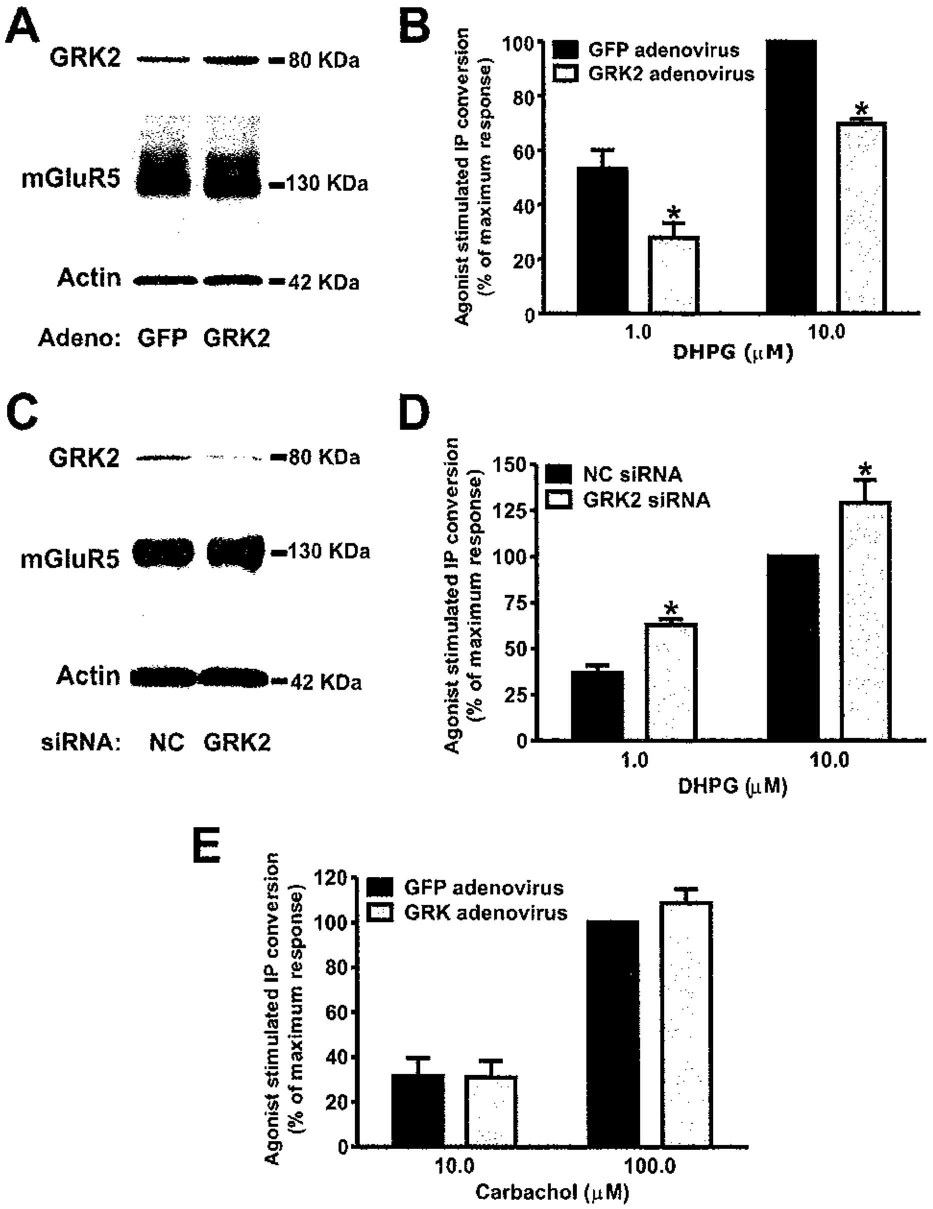

FIGURE 3. *A*, shown are representative immunoblots for mGluR5, GRK2, and actin protein expression in neurons infected with either GFP or GRK2 adenovirus. 50 μg of cell lysate were used for each sample. *B*, shown is mGluR5-stimulated inositol phosphate formation in response to either 1 or 10 μM DHPG for 5 min at 37 °C in striatal neurons infected with either GFP or GRK2 adenovirus. Data represent the means ± S.E. of four independent experiments, normalized to DHPG-stimulated GFP-infected neurons maximum response. *Asterisks* indicate significant differences compared with GFP adenovirus infected neurons at the corresponding agonist concentration ($p < 0.05$). *C*, shown are representative immunoblots for mGluR5, GRK2, and actin protein expression in neurons transfected with either NC or GRK2 siRNA. 50 μg of cell lysate were used for each sample. *D*, shown is mGluR5-stimulated inositol phosphate formation in response to either 1 or 10 μM DHPG for 5 min at 37 °C in striatal neurons transfected with either NC or GRK2 siRNA. Data represent the means ± S.E. of five independent experiments, normalized to DHPG-stimulated NC siRNA-transfected neurons maximum response. *Asterisks* indicate significant differences compared with NC siRNA transfected neurons at the corresponding agonist concentration ($p < 0.05$). *E*, shown is mGluR5-stimulated inositol phosphate formation in response to either 10 or 100 μM carbachol for 5 min at 37 °C in striatal neurons infected with either GFP or GRK2 adenovirus. Data represent the means ± S.E. of four independent experiments, normalized to carbachol-stimulated GFP-infected neurons maximum response.

infected with GFP adenovirus (Fig. 3*A*). Neurons infected with either GRK2- or GFP adenovirus were submitted to InsP formation assay and stimulated with DHPG for 5 min. GRK2 overexpression resulted in a significant attenuation of InsP formation in striatal neurons following stimulation with either 1 or 10 μM DHPG (Fig. 3*B*). Because the expression of mGluR5 was not altered by the moderate overexpression of GRK2 (Fig. 3*A*), the observed decrease in DHPG-stimulated InsP formation was the result of GRK2-mediated attenuation of mGluR5 responsiveness. To further establish the role of endogenously expressed GRK2 on mGluR5 desensitization, GRK2 siRNA was used to knockdown GRK2 protein expression in striatal neurons. Transfection of GRK2 siRNA into striatal neurons decreased GRK2 protein expression to 37 ± 9% of that in control neurons transfected with non-coding (NC) siRNA (Fig. 3*C*). siRNA transfection did not alter mGluR5 expression (Fig. 3*C*), but siRNA-mediated GRK2 knockdown led to an increase in DHPG-stimulated InsP formation (Fig. 3*D*). Thus, providing evidence that endogenously expressed GRK2 regulates mGluR5 activity in striatal neurons. A 2-fold increase in GRK2 expression was not sufficient to promote muscarinic receptor desensitization, as InsP formation stimulated by carbachol was not different between neurons infected with GFP or GRK2 adenovirus (Fig. 3*E*). However, it is possible that higher levels of GRK2 expression might lead to muscarinic receptor desensitization. Taken together, these data indicate that at the level of GRK2 expression tested mGluR5 but not muscarinic receptor signaling was desensitized by GRK2.

Assessment of mGluR5 Phosphorylation—The most common mechanism underlying GPCR desensitization is GRK-mediated phosphorylation followed by binding of β-arrestin proteins, which function to uncouple GPCRs from heterotrimer G proteins (8–10). Therefore, we examined whether GRK2 plays a role in mGluR5 phosphorylation. DHPG stimulation of neurons led to a small increase in mGluR5 phosphorylation when compared with control untreated neurons at both 5 and 10 min of agonist stimulation (Fig. 4, *A* and *B*). This increase in DHPG-stimulated mGluR5 phosphorylation was enhanced in GRK2-overexpressing neurons as compared with GFP-infected neurons. However, the apparent increase in

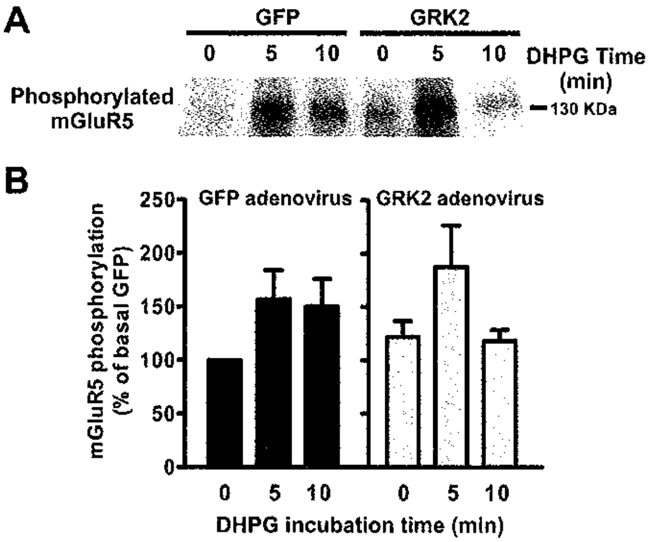

FIGURE 4. **DHPG-induced mGluR5 phosphorylation in striatal neurons.** *A*, shown is a representative autoradiograph demonstrating mGluR5 phosphorylation in striatal neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 0, 5, or 10 min. *B*, graph shows the densitometric analysis of mGluR5 phosphorylation autoradiograms. Data represent the means ± S.E. of seven independent experiments, normalized to mGluR5 phosphorylation in GFP-infected neurons not treated with agonist.

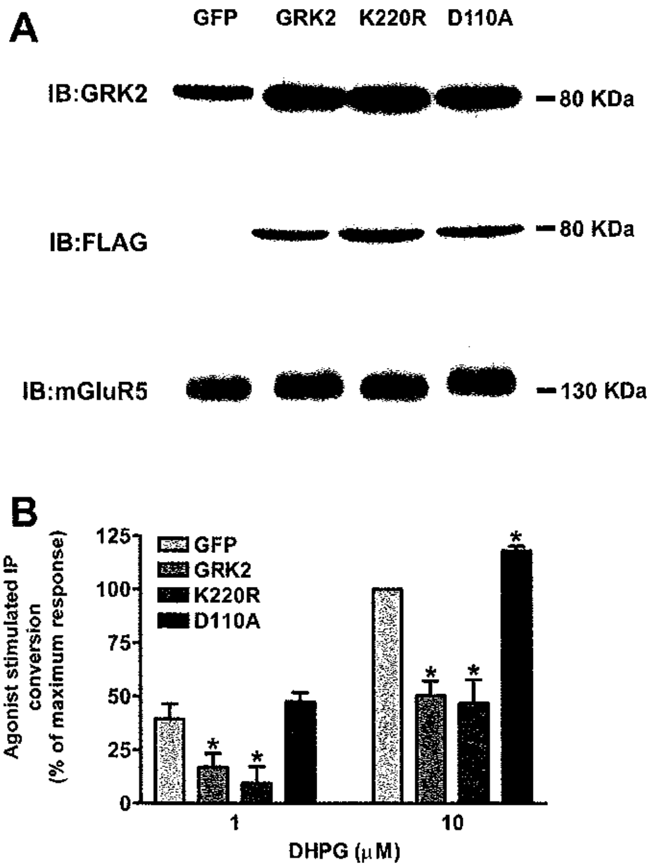

FIGURE 5. **GRK2-mediated mGluR5 desensitization in striatal neurons.** *A*, shown are representative immunoblots for mGluR5 and GRK2 protein expression in neurons infected with GFP, GRK2, GRK2-K220R, or GRK2-D110A adenovirus. 50 μg of cell lysate were used for each sample, and the samples were immunoblotted for the expression of GRK2, FLAG, and mGluR5 protein using anti-GRK2, anti-FLAG, and anti-mGluR5 antibodies. *B*, shown is mGluR5-stimulated inositol phosphate formation in response to either 1 or 10 μM DHPG for 5 min at 37 °C in striatal neurons infected with GFP, GRK2, GRK2-K220R, or GRK2-D110A adenovirus. Data represent the means ± S.E. of five independent experiments, normalized to DHPG-stimulated GFP-infected neurons maximum response. *Asterisks* indicate significant differences compared with GFP adenovirus-infected neurons at the corresponding agonist concentration ($p < 0.05$).

mGluR5 phosphorylation was again not statistically significant different between control and GRK2-infected cultures at any time point tested (Fig. 4*B*).

Phosphorylation-independent GRK2-mediated Attenuation of mGluR5 Signaling—Because we did not observe a significant increase in GRK2-mediated mGluR5 phosphorylation, we sought to investigate whether phosphorylation was required for GRK2-dependent attenuation of mGluR5 signaling in striatal neurons. To address this, we developed adenoviral constructs to express two GRK2 mutants in striatal neurons: a catalytically inactive GRK2 mutant (K220R) (27) and a GRK2 mutant impaired in $G\alpha_{q/11}$ binding (D110A) (28, 29). Adenoviral titers were chosen that resulted in a 2-fold above basal overexpression of wild-type GRK2, GRK2-K220R, and GRK2-D110A protein (Fig. 5*A*). Expression of mGluR5 in the striatal neuronal cultures was not altered by infection with any of the adenoviral constructs (Fig. 5*A*). InsP formation assays were performed to determine whether either GRK2 mutant retained the capacity to attenuate mGluR5 signaling. Overexpression of either wild-type GRK2 or GRK2-K220R resulted in a significant reduction of DHPG-stimulated InsP formation at both 1 μM and 10 μM concentrations tested (Fig. 5*B*). In contrast, mGluR5-stimulated InsP formation in response to 1 μM DHPG treatment was not attenuated in striatal neurons overexpressing GRK2-D110A (Fig. 5*B*). However, DHPG-mediated InsP formation in neurons infected with GRK2-D110A adenovirus was significantly higher than that of GFP adenovirus infected neurons following treatment of cells with 10 μM DHPG (Fig. 5*B*). Thus, unlike what was previously reported for GRK2-dependent regulation of mGluR5 signaling in HEK 293 cells (15), GRK2-dependent attenuation of mGluR5 signaling was mediated by a phosphorylation-independent mechanism in striatal neurons.

GRK2 Regulation of mGluR5 Trafficking—Internalization of a number of GPCRs can be altered by the expression of GRK2 (30, 31). Therefore, we examined mGluR5 internalization in striatal neurons overexpressing GRK2 and cortical neurons. To measure mGluR5 internalization, a modified cell surface biotinylation assay was utilized (24). Neuronal cultures were first stimulated with DHPG at varying times (0, 5, and 10 min) and then biotinylated on ice. Cell surface loss of biotinylated mGluR5 protein indicated receptor internalization. We found that DHPG treatment (10 μM) of striatal neurons expressing endogenous levels of GRK2 did not result in mGluR5 internalization at either the 5- or 10-min time points of agonist stimulation tested (Fig. 6, *A* and *B*). However, mGluR5 internalization was observed both in striatal neurons that were infected with adenovirus to overexpress GRK2 and in cortical neurons expressing endogenous levels of GRK2 protein (Fig. 6, *A* and *B*). Thus, the overexpression of GRK2 in striatal neurons at levels equivalent to endogenous GRK2 expression levels found in cortical neurons (Fig. 6*C*) established a striatal neuron mGluR5 internalization phenotype that resembled mGluR5 internalization in cortical neurons. These data suggest that different levels

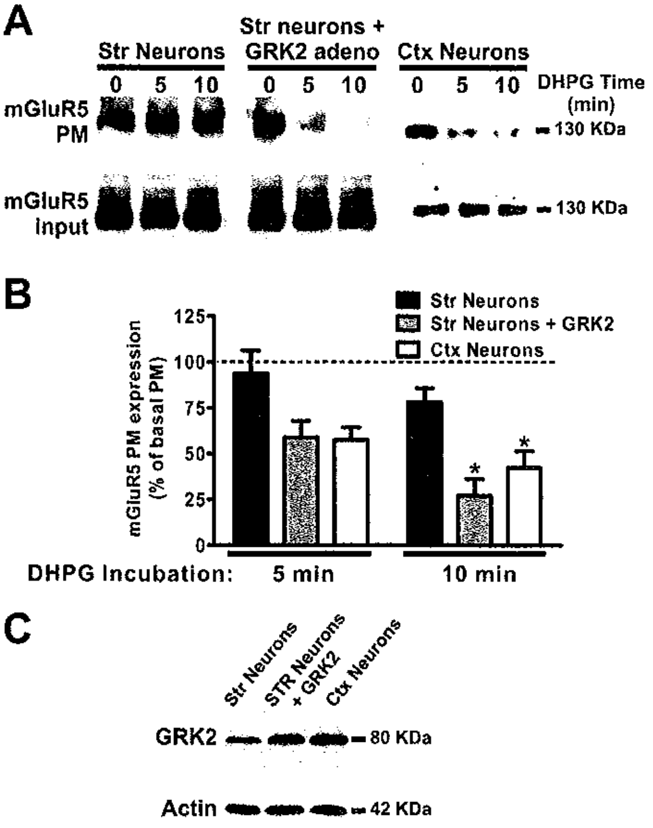

FIGURE 6. **GRK2-mediated mGluR5 internalization in striatal neurons.** *A*, shown is a representative immunoblot for cell surface biotin-labeled mGluR5 (*upper panel*) in cortical (*Ctx*) and striatal (*Str*) neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 0, 5, or 10 min. Total cell lysates (50 μg) are used to determine mGluR5 total cell expression (*input*) for each sample (*lower panel*). *B*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot. Data represent the mean ± S.E. of four independent experiments, normalized to cell surface mGluR5 in neurons not treated with agonist. *Asterisks* indicate significant difference as compared with mGluR5 cell surface expression in striatal neurons expressing endogenous levels of GRK2 ($p < 0.05$). *C*, shown are representative immunoblots for GRK2 and actin protein expression in cortical and striatal neurons infected with either GFP or GRK2 adenovirus. 50 μg of cell lysate were used for each sample.

of endogenous expression of GRK2 protein in distinct tissues may differentially affect mGluR5 internalization accordingly.

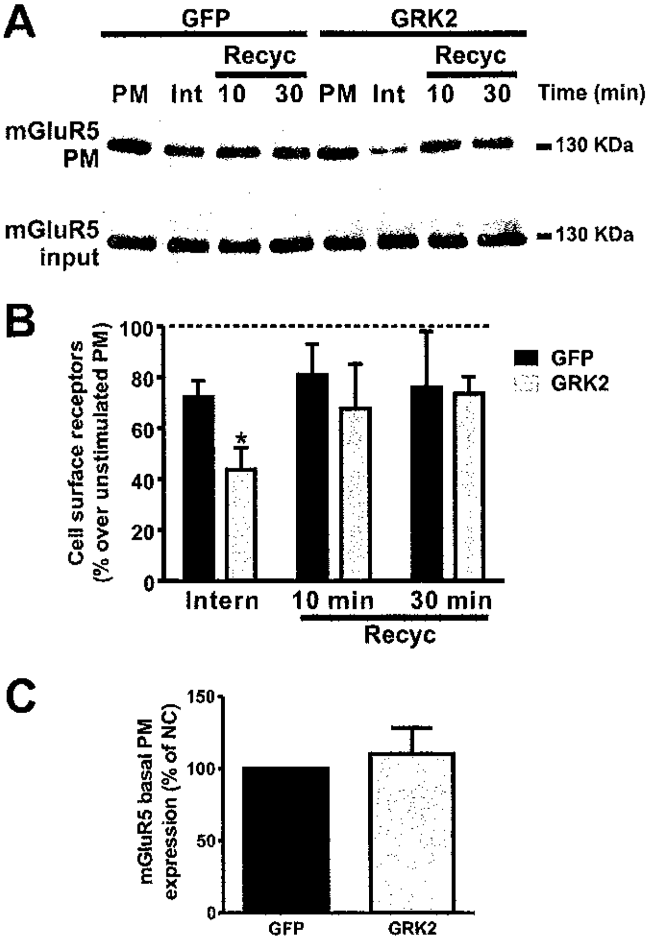

FIGURE 7. **GRK2 does not affect recycling or basal plasma membrane expression of mGluR5.** *A*, shown is a representative immunoblot for cell surface biotin-labeled mGluR5 (*upper panel*) in striatal neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 10 min and allowed to recover for 10 or 30 min. Total cell lysates (50 μg) were used to determine mGluR5 total cell expression (*input*) for each sample (*lower panel*). *B*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot. Data represent the mean ± S.E. of four independent experiments, normalized to cell surface mGluR5 in neurons not treated with agonist. *Asterisk* indicates significant difference as compared with mGluR5 cell surface expression in striatal neurons expressing endogenous levels of GRK2 ($p < 0.05$). *C*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot from unstimulated neurons. Data represent the mean ± S.E. of five independent experiments, normalized to total mGluR5 expression for each sample and expressed as percentage of cell surface mGluR5 in neurons infected with GFP adenovirus.

To determine whether GRK2 could affect mGluR5 recycling, we performed a modified cell surface biotinylation assay. Neuronal cultures were first incubated with DHPG for 10 min to stimulate mGluR5 internalization. Agonist was washed out and cells were incubated at 37 °C to allow internalized mGluR5 to recycle to the plasma membrane, and cells were then biotinylated on ice. mGluR5 internalization was increased in GRK2-infected neurons, as compared with GFP-infected neurons (Fig. 7, *A* and *B*). However, when agonist was washed out and neurons were allowed to recover for 10 and 30 min, the same levels of mGluR5 plasma membrane expression were observed in neurons infected with either GFP or GRK2 adenovirus, indicating that mGluR5 recycling is not affected by GRK2 overexpression (Fig. 7, *A* and *B*). Furthermore, basal levels of mGluR5 plasma membrane expression were the same in GFP- and GRK2-infected neurons, indicating that GRK2 overexpression does not alter the potential constitutive internalization and recycling of mGluR5 (Fig. 7*C*).

Previous studies demonstrated that GRK2-mediated receptor phosphorylation contributed to GPCR endocytosis (30, 31, 32, 33). Furthermore, GRK2-mediated mGluR1 internalization appears to be phosphorylation-dependent, because GRK2-K220R overexpression was reported to decrease mGluR1 internalization in HEK 293 cells (28, 29). Therefore, we examined whether GRK2-promoted endocytosis of mGluR5 in striatal neurons requires GRK2 catalytic activity. Following treatment with DHPG for either 5 or 10 min, mGluR5 internalization in striatal neurons was increased following the overexpression of either wild-type GRK2, GRK2-K220R, or GRK2-D110A (Fig. 8, *A* and *B*). These data indicated that, unlike what was previously

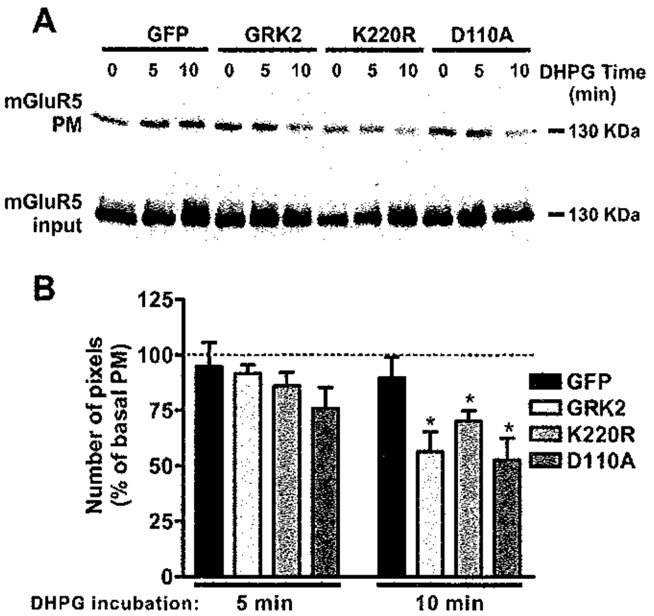

FIGURE 8. **GRK2 mutants promote mGluR5 internalization.** *A*, shown is a representative immunoblot for cell surface biotin-labeled mGluR5 (*upper panel*) in striatal neurons infected with GFP, GRK2, GRK2-K220R, or GRK2-D110A adenovirus and stimulated with 10 μM DHPG for 0, 5, or 10 min. Total cell lysates (50 μg) were used to determine mGluR5 total cell expression (*input*) for each sample (*lower panel*). *B*, graph shows the densitometric analysis of biotin-labeled cell surface mGluR5 immunoblot. Data represent the mean ± S.E. of five independent experiments, normalized to cell surface mGluR5 in neurons not treated with agonist. *Asterisks* indicate significant differences as compared with untreated matched controls ($p < 0.05$).

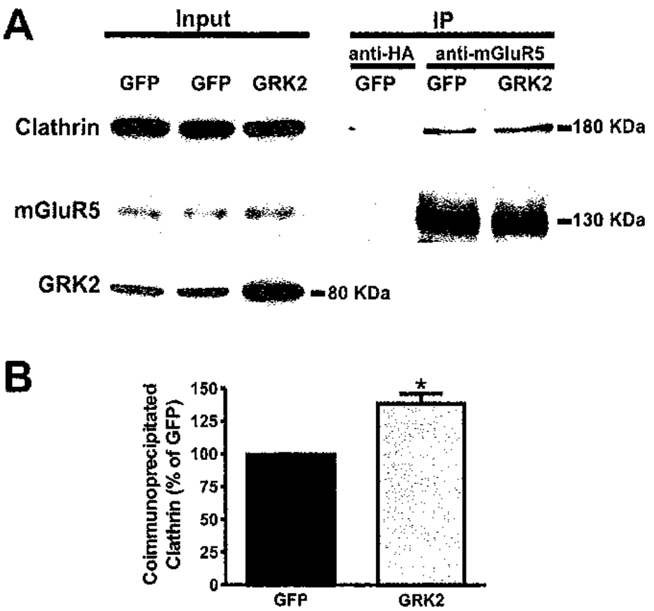

FIGURE 9. **GRK2 overexpression leads to an increase in clathrin recruitment to mGluR5.** *A*, shown is a representative immunoblot for the co-immunoprecipitation of clathrin with mGluR5 in striatal neurons infected with either GFP or GRK2 adenovirus and stimulated with 10 μM DHPG for 5 min (*right panel*). mGluR5 immunoprecipitation (*IP*) was performed by using either anti-mGluR5 or anti-HA (negative control) antibodies from an equivalent amount of protein (500–1000 μg of protein). Total cell lysates (30 μg) were used to determine clathrin, mGluR5, and GRK2 total cell expression (*input*) for each sample (*left panel*). *B*, graph shows the densitometric analysis of immunoblots for clathrin that was co-immunoprecipitated with mGluR5. Data represent the mean ± S.E. of three independent experiments, normalized to clathrin co-immunoprecipitated in GFP-infected neurons. The *asterisk* indicates a significant difference as compared with GFP-infected neurons ($p < 0.05$).

reported for mGluR1 internalization in HEK 293 cells (11, 14, 15, 16), GRK2-mediated mGluR5 endocytosis does not require GRK2 catalytic activity in striatal neurons.

GRK2 can function as a clathrin adaptor, facilitating the internalization of a number of receptors in a mechanism that is β-arrestin-independent (34, 35, 36, 37). To investigate the GRK2-mediated facilitation of mGluR5 endocytosis, we tested whether GRK2 could modulate clathrin-mGluR5 interaction. GRK2 overexpression led to an increase in the recruitment of clathrin to mGluR5 (Fig. 9). The recruitment of clathrin by GRK2 might represent the mechanism underlying the phosphorylation-independent internalization of mGluR5.

DISCUSSION

GPCR desensitization represents an important regulatory mechanism by which acute and/or chronic receptor overstimulation is avoided. For many GPCRs, receptor desensitization correlates with GRK-mediated receptor phosphorylation followed by the binding of β-arrestin proteins (8–10). However, this simple correlation does not necessarily hold for all GPCRs. In the present study, we provide evidence that GRK2 contributes to the phosphorylation-independent desensitization of endogenously expressed mGluR5 in primary mouse striatal neurons. Overexpression of GRK2 by ~2-fold to match GRK2 expression levels found in other brain regions that express mGluR5 (*e.g.* cortical neurons) leads to pronounced attenuation of mGluR5-stimulated InsP formation in response to DHPG stimulation. Furthermore, mGluR5-stimulated InsP formation is increased following the siRNA knockdown of GRK2 protein demonstrating a role for endogenous GRK2 in the regulation of mGluR5 activity. In addition, agonist treatment induces little mGluR5 internalization in striatal neurons when compared with cortical neurons. In contrast, agonist-stimulated mGluR5 internalization is significantly increased in striatal neurons expressing levels of GRK2 that are comparable to cortical neurons. Furthermore, expression of a catalytically inactive GRK2 promotes mGluR5 desensitization and internalization, indicating that GRK2-dependent mGluR5 desensitization and internalization are mediated by a phosphorylation-independent mechanism.

We find that GRK2-dependent mGluR5 desensitization is independent of GRK2 catalytic activity. Rather, intact interactions between the GRK2 RH domain and $G\alpha_{q/11}$ are essential for the attenuation of mGluR5 signaling. Thus, the mechanism underlying GRK2-mediated mGluR5 desensitization in striatal neurons is similar to what we previously reported for mGluR1 expressed in HEK 293 cells (12, 20, 21, 28). The present findings are distinct from a previous report that GRK2-dependent mGluR5 desensitization in HEK 293 cells requires GRK2 catalytic activity (15). In the previous study, overexpression of GRK2-K220R led to enhanced mGluR5-mediated activation of a G protein-coupled inward rectifier potassium channel and also resulted in altered mGluR5 expression. In contrast, we find that GRK2-K220R expression not only effectively attenuates mGluR5 signaling but it does not affect the level of endogenous mGluR5 protein expression in striatal neurons. Unexpectedly, a

2-fold overexpression of GRK2-D110A resulted in a statistically significant increase in InsP formation in striatal neurons. Thus, it is likely that the GRK2-D110A mutant functions to compete with endogenous GRK2 to bind mGluR5, but does not functionally uncouple the receptor from $G\alpha_{q/11}$. Thus, it appears that the interaction of the GRK2 RH domain with $G\alpha_{q/11}$ represents the primary mechanism by which GRK2 functions to attenuate mGluR5 signaling. The observed difference in GRK2-mediated regulation of mGluR5 activity in HEK 293 cells and primary striatal neurons highlights the importance of studying the regulation of GPCR in their natural environment.

We found that in cultured striatal neurons, agonist treatment did not promote internalization of endogenously expressed mGluR5. However, mGluR5 endocytosis was significantly higher in cortical neurons, which express twice as much GRK2 protein than striatal neurons (38). Overexpression of GRK2 in striatal neurons to match GRK2 expression levels found in cortical neurons increased mGluR5 internalization to the same level as that of cortical neurons. These data demonstrate that GRK2 protein expression level in different tissues might result in altered rates of mGluR5 endocytosis. The observed differences in GRK2 protein expression and mGluR5 endocytosis may be important physiologically in determining the sensitivity of neurons to excitotoxic cell death. For example, heterozygous GRK2 knock-out mice exhibit increased neuronal cell loss following unilateral carotid artery occlusion and hypoxia (39). Moreover, hippocampal slices and cerebellar granular neurons derived from heterozygous GRK2 knock-out mice are more sensitive to glutamate-induced death. GRK2 protein is expressed at levels in the hippocampus and cerebellum that are comparable to the cortex and are twice the expression levels found in the striatum (38). Consequently, the relatively reduced GRK2 protein expression found in the striatum may make striatal neurons more susceptible to neuronal loss following either injury or in diseases associated with excitotoxic cell death such as Huntington disease. Interestingly, mutant huntingtin has also previously been demonstrated to regulate Group I mGluR signaling via its association with optineurin (38).

Internalization of muscarinic (M2-M5 receptors) (40), β2 adrenergic (41), and dopamine D2 receptors (30) is also enhanced by expression of specific GRKs. Furthermore, co-expression of arrestins and GRKs seems to synergistically improve internalization of some GPCRs (23, 31, 42). These data are consistent with findings that arrestins seem to preferentially bind to phosphorylated receptors and facilitate clathrin-mediated endocytosis (8–10). Although mGluR1 and mGluR5 share the same mechanism of phosphorylation-independent GRK2 desensitization, the mechanism by which GRK2 regulates the internalization of these two receptors appears to be different. We demonstrate here that GRK2 plays a crucial role in promoting mGluR5 endocytosis. We have reported that the co-expression of GRK2 with β-arrestin 1 leads to increased agonist-stimulated mGluR1 internalization, suggesting that GRK2-mediated mGluR1 phosphorylation may be important for its internalization (16). In contrast to what is reported for mGluR1, we demonstrate here that a 2-fold increase in either GRK2 or GRK2-K220R protein expression is sufficient to support mGluR5 internalization in striatal neurons. These data suggest that receptor phosphorylation is not required for mGluR5 endocytosis. This is different from what is observed for mGluR1, where GRK2-K220R overexpression is reported to decrease mGluR1 internalization in HEK 293 cells and GRK2 knockdown in cerebellar Purkinje neurons did not alter the extent of mGluR1 internalization (11, 14, 17). Furthermore, we show here that GRK2 overexpression leads to a modest increase in clathrin recruitment to mGluR5. Although the mechanism by which GRK2 targets mGluR5 for endocytosis in striatal neurons remains to be determined, these data suggest that clathrin is involved, even though phosphorylation of the receptor does not play a role. Previously, it has been shown that GRK2 can associate directly with clathrin via a clathrin binding box that is found in many endocytic adaptor proteins including β-arrestins (34). The mutation of the GRK2 clathrin binding motif prevents GRK2-mediated phosphorylation and internalization of the β_2-adrenergic receptor (35). GRK2 has also been reported to facilitate the β-arrestin-independent internalization of the β_1-adrenergic, adrenocorticotropin, and leukotriene B4 receptors (34, 36, 37). Thus, it is possible that GRK2 replaces β-arrestin as an endocytic adaptor protein for mGluR5 endocytosis.

In summary, we have shown here that, similar to what is observed for mGluR1, GRK2-mediated attenuation of mGluR5 signaling in striatal neurons is independent of receptor phosphorylation. It is rather mediated by GRK2 RH domain interactions with $G\alpha_{q/11}$. Moreover we show that GRK2-mediated endocytosis of endogenous mGluR5 in striatal neurons is also independent of GRK2 catalytic activity and $G\alpha_{q/11}$ binding.

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Attachment 3:

Cell culture lines

From ATTCC; HEK293, A10, IMR32, U-87, RBL-2H3, PC-12, ATT-20, Cos 7

From M. Prado; CF10, reference: Griel et. Al. 2008

Cell Line Designation: COS-7
ATCC® Catalog No. CRL-1651™

Table of Contents:

Cell Line Description

Organism: *Cercopithecus aethiops* (monkey, African green)
Tissue: kidney; SV40 transformed
Morphology: fibroblast
Growth Properties: adherent
Virus Susceptibility: SV40 (lytic growth); SV40 tsA209 at 40ºC; SV40 mutants with deletions in the early region
Depositor: Y. Gluzman
Comments: This line was derived from the CV-1 cell line (ATCC® CCL-70™) by transformation with an origin defective mutant of SV40 which codes for wild-type T antigen.

Parental cell line -- ATCC® CCL-70™

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,* 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 x g for 5 to 7 minutes. Discard supernatant.

4. Resuspend the cell pellet with the recommended complete medium 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).*

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 cm^2 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 - 0.53 mM 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 suspension to new culture vessels.

   Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is recommended.

6. Incubate cultures at 37°C.

   Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 12 in ***Culture of Animal Cells: A Manual of Basic Technique*** by R. Ian Freshney, 4th edition, published by Wiley - Liss, N.Y., 2000.

Medium Renewal

Every 2 to 3 days

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%

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).

ATCC tested fetal bovine serum is available as ATCC® Catalog No. 30-2020 (500mL) or 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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ATCC Warranty

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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.

07/07

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

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

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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.

06/11

Cell Line Designation: IMR-32
ATCC Catalog No. CCL-127™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: neuroblastoma; brain; derived from metastatic site: abdominal mass
Age: 13 months
Gender: male
Ethnicity: Caucasian
DNA profile (STR analysis):
  Amelogenin: X,Y
  CSF1PO: 11,12
  D13S317: 9
  D16S539: 8
  D5S818: 11,12
  D7S820: 9,10
  TH01: 7,9.3
  TPOX: 11
  vWA: 15
Morphology: neuroblast and fibroblast
Growth properties: adherent
Doubling time: approximately 20 hours
VirusSuscept: vesicular stomatitis (Indiana); herpes simplex; vaccinia; coxsackievirus B3; poliovirus 3 (poorly)
VirusResist: echovirus 11
Depositors: W.W. Nichols

Comments: The IMR-32 cell line was established by W.W. Nichols, J. Lee and S. Dwight in April, 1967 from an abdominal mass occurring in a 13-month-old Caucasian male.

The tumor was diagnosed as a neuroblastoma with rare areas of organoid differentiation. Two cell types are present. Predominant is a small neuroblast-like cell. The other is a large hyaline fibroblast.

The cell line was submitted to the American Type Culture Collection in the 36th passage. It has been demonstrated that the cells can be propagated successfully beyond the 80th serial subculture.

Karyology: Stable male karyotype with stemline number of 49. Two large marker chromosomes with submedian centromeres. A deletion in one number 1 chromosome: One number 16 chromosome missing; two extra chromosomes in C group.

Sublines with 50 and 48 chromosomes differ from those with 49 chromosomes by having an extra or missing C group chromosome respectively.

Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.

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). *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

CCL-127™ cells may pile up and grow in patches. (Please see the photos of CCL-127™ on the ATCC website at www.atcc.org.)
CCL-127™ cells may not become 100% confluent.

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. 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. Maintain cultures at a cell concentration between $4x10^4$ and 4×10^5 cells/cm^2.
   Subcultivation Ratio: 1:3 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

Every 2 to 3 days

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.

Cryoprotectant Medium

Complete culture 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 may be available in the catalog description at www.atcc.org)

Tumilowicz JJ et al. **Definition of a continuous human cell line derived from neuroblastoma.** Cancer Res. 30: 2110, 1970 PubMed: 70293585

Rostomily RC et al. **Expression of neurogenic basic helix-loop-helix genes in primitive neuroectodermal tumors**. Cancer Res. 57: 3526-3531, 1997 PubMed: 97413638

Maestrini E et al. **A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor**. Proc. Natl. Acad. Sci. USA 93: 674-678, 1996 PubMed: 96149362

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.

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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: PC-12
ATCC Catalog No. CRL-1721™

Table of Contents:

Cell Line Description

Organism: *Rattus norvegicus* (rat)
Tissue: pheochromocytoma; adrenal gland
Gender: male
Morphology: small irregularly shaped cells
Tumorigenic: yes, in New England Deaconess Hospital strain rats
Karyotype: 40 chromosomes; 38 autosomes plus XY
Products: catecholamines; dopamine; norepinephrine
Receptors expressed: nerve growth factor (NGF)
Growth Properties: floating clusters; few scattered lightly attached cells.
Depositor: B. Patterson
Comments: The PC-12 cell line was derived from a transplantable rat pheochromocytoma. The cells respond reversibly to NGF by induction of the neuronal phenotype when plated on Collagen IV coated culture flasks. The cells do not synthesize epinephrine.
Related cell line CRL-1721.1™, PC-12 Adh (adherent variant has been adapted to Corning CellBIND flasks).

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 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 50 ml tube containing 9 ml complete growth medium. Centrifuge cells at 180 – 225 xg for 8-15 minutes at room temperature. Remove and discard supernatant. Resuspend cells in 5 ml complete growth medium. Break up cell clusters by gently aspirating cells through a 22g needle 4 or 5 times. (see the specific batch information for the culture recommended dilution ratio. *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).*

4. 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

2. Incubate the flask in an upright position for several hours at 37°C. After the temperature has equilibrated, aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove

shipping medium and save for reuse. Resuspend the cell pellet in 10 ml of this medium.

3. From this cell suspension remove a sample for a cell count and viability. Adjust the cell density of the suspension to 5 x 10^5 viable cells/ml in the shipping medium.

4. Incubate the culture, horizontally, at 37°C in a 5% CO_2 in air atmosphere. Maintain the cell density of the culture as suggested under the subculture procedure.

Subculturing Procedure

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

1. Transfer cell suspension to centrifuge tube. Centrifuge cells at 180 – 225 xg for 8-15 minutes at room temperature.
2. Remove and discard supernatant leaving cell pellet.
3. Resuspend the cell pellet in an appropriate volume of fresh medium (about one tenth of the original volume.
4. Gently aspirate each 5 ml aliquot of cells 4 or 5 times with a new 20 ml syringe outfitted with a 22g (1½ in.) needle to break up cell clusters.
5. Add appropriate aliquots of the cell suspension to new 75 cm^2 flask with 10-15 ml fresh growth medium. Seed flask 5 x 10^5 to 1 x 10^6 viable cells/ml or use subcultivation ratio of 1:2 to 1:4 .
6. Place culture vessels in incubator at 37ºC Subculture when cell density reaches between 2-4 x 10^6 viable cells/ml.

Medium Renewal

Add medium every two or three days.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, add the following components to the base medium:

- heat-inactivated horse serum to a final concentration of 10%
- fetal bovine serum to a final concentration of 5%.

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 (500 ml) and ATCC Catalog No. 30-2021 (100ml).

Cryoprotectant Medium

Complete growth medium described above supplemented with 10% (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)

Levi A et al. **Molecular cloning of a gene sequence regulated by nerve growth factor.** Science 229: 393-395, 1985 PubMed: 85244666

Greene LA and Tischler AS. **Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor.** Proc. Natl. Acad. Sci. USA 73: 2424-2428, 1976 PubMed: 76244539

Biocca S et al. **A macromolecular structure favouring microtubule assembly in NGF- differentiated pheochromocytoma cells (PC12).** EMBO J. 2: 643-648, 1983 PubMed: 84057740

Weber E et al. **Distinct functional properties of Rab3A and Rab3B in PC12 neuroendocrine cells.** J. Biol. Chem. 271: 6963-6971, 1996 PubMed: 96215126

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.

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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: RBL-2H3
ATCC® Catalog No. CRL-2256™

Table of Contents:

Cell Line Description

Organism: *Rattus norvegicus* (rat)
Strain: Wistar
Tissue: leukemia; peripheral blood; basophil; chemically induced
Morphology: fibroblast-like
Growth properties: adherent
Receptors expressed: FcERI (Fc of IgE)
Products: histamine
Depositors: R.P. Siraganian
Comments: RBL-2H3 is a basophilic leukemia cell line isolated and cloned in 1978 in the Laboratory of Immunology at the National Institute of Dental Research from Wistar rat basophilic cells that were maintained as tumors (Nat. New Biol. 244: 73-76, 1973). These cells have high affinity IgE receptors (Fc-epsilon-RI). They can be activated to secrete histamine and other mediators by aggregation of these receptors or with calcium ionophore. RBL-2H3 cells have been the model for studies of structure of Fc-epsilon-RI and have been used extensively to study Fc-epsilon-RI and the biochemical pathways for secretion in mast cells, including the role of changes in intracellular calcium, the activation of phospholipases, protein kinases and small G proteins.
Although nearly all lots of fetal bovine serum support the growth of these cells, the cells grown in some lots degranulate better after Fc-epsilon-RI aggregation. Another rat basophil line is available ATCC® CRL-1378 (RBL-1) that does not degranulate.
Histamine release capacity may be seriously reduced after too much subculturing. PubMed: 6166481.

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 x g for 5 to 10 minutes.

4. Resuspend the 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.

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 x g 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 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 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

Every 2 to 3 days.

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:

- heat-inactivated fetal bovine serum to a final concentration of 15%.

This medium is formulated for use with a 5% CO_2 in air atmosphere.

Cryoprotectant Medium

Complete culture 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 may be available in the catalog description at www.atcc.org)

Kulczycki A J et al. **The interaction of IgE with rat basophilic leukemia cells. I. Evidence for specific binding of IgE**. J. Exp. Med. 139: 600-616, 1974 PubMed: 74096455

Barsumian EL et al. **IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones.** Eur. J. Immunol. 11: 317-323, 1981. PubMed: 6166481.

Eccleston E et al. **Basophilic leukaemia in the albino rat and a demonstration of the basopoietin.** Nat. New Biol. 244: 73-76, 1973. PubMed: 4516144.

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.

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12/08

Cell Line Designation: U-87 MG
ATCC Catalog No. HTB-14™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: brain; glioblastoma; astrocytoma
Age: 44 years
Tumor Stage: classified as grade IV as of 2007
Gender: male
Ethnicity: caucasian
Morphology: epithelial
Growth properties: adherent
Tumorigenic: yes, Tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with 10(7) cells
AntigenExp: Blood Type A, Rh+
DNA profile (STR analysis):
D5S818: 11,12
D13S317: 8,11
D7S820: 8,9
D16S539: 12
vWA: 15,17
THO1: 9.3
TPOX: 8
CSF1PO: 10,11
Amelogenin: X
Depositors: J. Ponten
Comments: This is one of a number of cell lines derived from malignant gliomas (see also ATCC HTB-15™ and ATCC HTB-16™) by J. Ponten and associates from 1966 to 1969. Mycoplasma contamination was eliminated in September 1975.

Note: ATCC has confirmed that the ATCC® HTB-14™ cell line is male in origin based on STR, Y-chromosome paint and Q-band assays. However, based on the current literature, the cell line is still of glioblastoma origin and the discrepancy of gender is not unusual. It is possible that the cell line was misidentified in the depositor's original publication.

Karyology: This is a hypodiploid human cell line with the modal chromosome number of 44 occurring in 48% of cells. The rate of higher ploidy was 5.9%. Twelve markers were common to all cells, including der(1)t(1;3) (p22;q21), der(16)t(1;16) (p22;p12), del(9) (p13) and nine others. The marker der(1) had two copies in most cells. There was only one copy of normal X. N1, N6 and N9 were not found.

Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.

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 cm^2 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 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 into new culture vessels.
   Subcultivation Ratio: 1:2 to 1:5

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

2 to 3 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.

Cryoprotectant Medium

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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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Acute cellular uptake of abnormal prion protein is cell type and scrapie strain independent

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Abstract

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease, bovine spongiform encephalopathy and sheep scrapie. Although one of the earliest events during TSE infection is the cellular uptake of protease resistant prion protein (PrP-res), this process is poorly understood due to the difficulty of clearly distinguishing input PrP-res from either PrP-res or protease-sensitive PrP (PrP-sen) made by the cell. Using PrP-res tagged with a unique antibody epitope, we examined PrP-res uptake in neuronal and fibroblast cells exposed to three different mouse scrapie strains. PrP-res uptake was rapid and independent of scrapie strain, cell type, or cellular PrP expression, but occurred in only a subset of cells and was influenced by PrP-res preparation and aggregate size. Our results suggest that PrP-res aggregate size, the PrP-res microenvironment, and/or host cell-specific factors can all influence whether or not a cell takes up PrP-res following exposure to TSE infectivity.

Keywords

protein aggregation; PrP; PrP-res; PrP-sen; prion; scrapie; transmissible spongiform encephalopathy; TSE

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that include kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler-Scheinker syndrome in humans as well as bovine spongiform encephalopathy, sheep scrapie, and chronic wasting disease (CWD) in deer and elk (Priola & Vorberg, 2006). Partially protease resistant prion

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protein (PrP-res) associates closely with infectivity and is considered to be a marker of TSE infection. PrP-res is derived from the normal protease sensitive form of PrP, PrP-sen. The post-translational, conformationally driven conversion of PrP-sen to PrP-res is a critical, though poorly understood, event during TSE pathogenesis.

Certain cell types when exposed to scrapie infected brain homogenate will produce PrP-res over multiple serial passes, and when injected into experimental animals, cause disease. These cells are considered to be persistently infected. A number of studies have shown that both neuronal and non-neuronal cell lines can become persistently infected with certain strains of mouse scrapie, sheep scrapie or CWD (Baron, Magalhaes et al., 2006), (Butler, Scott et al., 1988), (Race, Fadness et al., 1987), (Raymond, Olsen et al., 2006), (Rubenstein, Carp et al., 1984), (Schatzl, Laszlo et al., 1997), (Vorberg, Raines et al., 2004a), (Vorberg, Raines et al., 2004b). The vast majority of what is understood about the process of cell infection and PrP-res formation has been the result of studies on such persistently infected cells. These studies have shown that PrP-res formation in infected cells occurs at the cell surface and/or along the endocytic pathway (Beranger, Mange et al., 2002), (Borchelt, Taraboulos et al., 1992), (Caughey & Raymond, 1991) and that cell associated PrP-res has a long half life (Borchelt, Scott et al., 1990), (Caughey & Raymond, 1991) and can accumulate in endolysosomal compartments (Caughey, Raymond et al., 1991), (McKinley, Taraboulos et al., 1991), (Pimpinelli, Lehmann et al., 2005), (Taraboulos, Scott et al., 1994). Although cells persistently infected with scrapie agent have provided important insights into the cellular biology of PrP-res formation during infection, very little is known about how PrP-res is initially taken up by cells.

Several recent studies have looked at the earlier stages of TSE infection with some data suggesting that PrP-res is taken up rapidly (i.e. within 24 hrs) (Hijazi, Kariv- Inbal et al., 2005), (Horonchik, Tzaban et al., 2005), (Mohan, Hopkins et al., 2005), (Paquet, Daude et al., 2007) and other data indicating that uptake may occur more slowly (i.e. over days) (Bergstrom, Jensen et al., 2006),(Magalhaes, Baron et al., 2005). Most of these earlier studies were limited by the difficulty of specifically distinguishing PrP-res in the inoculum from PrP-res newly made in the cells which may help to explain the conflicting results. Even in instances where partially purified PrP-res was uniquely labeled, it was not always possible to absolutely distinguish the labeled PrP-res from other labeled, but non-relevant, protein contaminants (Magalhaes, Baron et al., 2005). Additionally, few studies have examined more then one strain of TSE agent meaning that any potential differences in strain-specific PrP-res uptake early during infection remain largely unresolved.

Using uniquely epitope tagged PrP-sen molecules we have previously shown that, regardless of scrapie strain, both mouse neuroblastoma (MNB) cells as well as mouse fibroblast cells form de novo PrP-res after only 4 hrs of scrapie exposure (Vorberg, Raines et al., 2004a). This finding strongly suggests that the association of PrP-res with a cell occurs rapidly. We have now used PrP-res uniquely tagged with an epitope to the mouse monoclonal antibody 3F4 to examine the interactions between PrP-res from different strains of mouse scrapie and different cell types during the acute stage of scrapie infection (0 – 72 hrs). Our results show that PrP-res uptake is indeed very rapid and independent of cell type, scrapie strain, and host cell PrP-sen expression. However, PrP-res uptake was influenced by PrP-res aggregate size and only a certain subpopulation of cells detectably internalized PrP-res. Furthermore, PrP-res in brain homogenate was taken up more efficiently by cells then either partially purified PrP-res or PrP-res in brain microsomes. Our results suggest that, while PrP-res uptake during acute infection appears to be similar for different scrapie strains and cell types, internalization of PrP-res can be influenced by PrP-res aggregate size, PrP-res microenvironment, and/or an as yet unidentified host factor or factors.

RESULTS

PrP-res^{3F4} uptake by mouse neural cells

To determine whether or not our mouse neural cells were able to take up PrP-res^{3F4}, MoL42-CFD5 cells were exposed to 22L(3F4), ME7(3F4), Obi(3F4) or uninfected Mock(3F4) brain homogenates. MoL42-CFD5 cells are mouse neuronal PrP$^{0/0}$ cells that have been modified to express mouse PrP-sen that is recognized by the mouse monoclonal antibody L42 allowing us to distinguish it, if necessary, from mouse PrP in the brain homogenate. MoL42 PrP-sen is not recognized by the 3F4 mouse monoclonal antibody (Fig. 1).

After 24 hrs incubation time, cells were rinsed to remove any unbound PrP-res^{3F4}, lysed and assayed for PrP-res^{3F4} by Western blot as shown in Fig. 2. Unbound, excess PrP-res^{3F4} was effectively washed from the cells by four rinses of fresh media (Fig. 2A–C) and no PrP-res^{3F4} could be detected in cells exposed to Mock(3F4) brain homogenate (Fig. 2A–C, lane 1). Thus, residual, unbound PrP^{3F4} could be efficiently removed from the cell monolayer. PrP-res^{3F4} associated with detached cells, cell debris, and/or unbound brain homogenate was observed in the pellet from a low speed spin of the cellular supernatant (Fig. 2 A–C, SN pellet) but significant amounts remained cell-associated (Fig. 2A–C, Cells). By 24 hours, the amount of PrP-res^{3F4} that was cell associated was 10–15% of the total PrP-res added to the cells (Fig. 2D), a percentage that did not significantly change at later timepoints (Fig. 4 and data not shown). Thus, unbound PrP-res^{3F4} (i.e. PrP-res^{3F4} in both the SN pellet and the clarified supernatant) remained in excess throughout the course of the experiment. These data suggest that the majority of PrP-res^{3F4} in the brain homogenate did not interact with the cells.

PrP-res is internalized by cells

To determine if cell-associated PrP-res^{3F4} was bound to the cells non-specifically or internalized via an active cellular process such as endocytosis, MoL42-CFD5 cells were exposed to scrapie-infected brain homogenates and incubated either at 18°C, which blocks endocytosis, or at 37°C. Without PK digestion, some PrP^{3F4} was bound and/or internalized in the cells at both 37 and 18°C (Fig. 3A), although in the case of 22L(3F4) a longer gel exposure was required to detect it (Fig. 3B). This material was also observed when uninfected brain homogenate was overlayed onto the cells (data not shown) suggesting that at least some fraction of the PrP-sen^{3F4} in the brain homogenate was aggregated and interacting with the cells. As shown in Fig. 3A, cells exposed to scrapie-infected brain homogenates at 37°C were positive for PrP-res^{3F4} while cells which were exposed to scrapie-infected brain homogenates at 18°C appeared to be negative for PrP-res^{3F4}. However, upon longer exposure of the gel, it was clear that a small amount of PrP-res^{3F4} was also cell associated at 18°C (Fig. 3B). Given the fact that very long gel exposures were required to reliably detect PrP-res^{3F4} associated with cells at 18°C, our data suggest that the majority of the cell-associated PrP^{3F4} is likely being actively internalized by the cells and not simply attaching to the cell surface.

Rapid PrP-res^{3F4} uptake into cells is independent of scrapie strain, cell type and host cell PrP-sen expression

In order to determine the kinetics of PrP-res^{3F4} uptake into cells during exposure to TSE infectivity, MoL42-CFD5 cells were exposed to PrP-res^{3F4} in 22L, ME7, or Obihiro scrapie infected brain homogenates for 0–72 hrs. For each strain, cell-associated PrP-res^{3F4} was detected by 2 hours post-exposure and the amount of PrP-res^{3F4} internalized by the cell increased over time (Fig. 4A). Quantification of total PrP-res^{3F4} uptake into the cells for each given time point showed that there were no significant differences in PrP-res^{3F4} uptake amongst the different scrapie strains. For each strain, approximately half of the total PrP-res^{3F4} eventually taken up by the cells was cell-associated by 10–15 hrs post-inoculation and the majority was taken up by 24hrs. Although ME7 appeared to be taken up more slowly by

MoL42-CFD5 cells (Fig. 4B, ME7 solid line), this difference was not statistically significant when compared to the other strains ($p>.05$ by 2-Way ANOVA).

Previous studies have suggested that cells exposed to scrapie associate with PrP-res independently of host cell PrP expression (Hijazi, Kariv-Inbal et al., 2005),(Paquet, Daude et al., 2007). However, given that both the rabbit epithelial cells expressing doxycyline inducible sheep PrP-sen (Rov cells) (Paquet, Daude et al., 2007) and the Chinese hamster ovary (CHO) cells (Hijazi, Kariv-Inbal et al., 2005) used in these studies may still express low levels of PrP-sen (Hijazi, Kariv-Inbal et al., 2005), a role for PrP-sen in PrP-res uptake could not be completely ruled out. To determine whether or not host cell PrP-sen expression was needed for the internalization of PrP-res, we utilized a cell line (CF10) derived from PrP$^{0/0}$ mice. CF10 cells are neural cells and are the parent cell line of MoL42-CFD5 cells. However, CF10 cells do not express PrP-sen (Fig. 1). PrP-res^{3F4} was taken up by the CF10 cells similarly to MoL42-CFD5 cells (Fig. 4B, dashed lines). Thus, the uptake of PrP-res derived from different strains of mouse scrapie is independent of PrP-sen expression by the host cell.

We next determined if cell type could influence PrP-res uptake into cells. To test this, non-neuronal MoL42-ψ2A2 cells (a fibroblast cell line) were analyzed using the same kinetics assay employed on both the MoL42-CFD5 and CF10 cell lines. Results from this assay showed that, for all strains tested, the MoL42-ψ2A2 cells took up PrP-res^{3F4} from scrapie brain homogenate similarly to either MoL42-CFD5 or CF10 cells (Fig. 4C). Thus, the kinetics of PrP-res uptake were similar for both neuronal and non-neuronal cell types, at least with respect to fibroblast cells.

Increased PrP-res uptake correlates with increased cell number

The observed increase in cell-associated PrP-res^{3F4} over 72 hours could be due either to an increase in the number of cells harboring PrP-res^{3F4} or to an increase in the amount of PrP-res^{3F4} per cell. To determine if increased PrP-res^{3F4} uptake correlated with cell number, the number of cells at each time point was divided by the amount of PrP-res^{3F4} taken up by the cells as quantified by Western blot. This ratio of cell number to cell-associated PrP-res^{3F4} was then plotted against the time cell cultures were exposed to scrapie brain homogenate. For all strains and cell lines tested, the ratio of cells to total PrP-res^{3F4} was high 2 hours after exposure of the cells to the infected homogenate. However, over time that ratio decreased and, as indicated by the linear portion of the curve, after 8 hours was essentially 1:1 (Fig. 5A–C). These data suggest that the system had reached a saturation point and that the population of cells within the culture that could take up PrP-res was limited (Fig. 5A–C).

In order to determine if the number of PrP-res^{3F4} positive cells increased over time, the uptake of partially purified PrP-res^{3F4} into MoL42-CFD5 cells was assessed by immunofluorescent microscopy for 22L(3F4), ME7(3F4) and Obi(3F4) PrP-res^{3F4}. Similar to the western blot data in Fig. 4, cell-associated PrP-res^{3F4} was detected as early as 2 hours. PrP-res^{3F4} was observed as small, punctate aggregates generally localized to both cytoplasmic and perinuclear regions (Fig. 6). Consistent with the data in Figure 5, as cell numbers increased so too did the number of cells positive for PrP-res^{3F4}. However, for all time points examined, only 30%–40% of the cells were positive for PrP-res^{3F4}. Our results suggest that there is a select subpopulation of cells able to take up PrP-res which, over time, divide and increase in number leading to the observed increase in the amount of cell-associated PrP-res.

PrP-res aggregate size influences PrP-res binding and uptake

Uptake of PrP-res^{3F4} in cells in our assay was quite different from that of previous studies where large, fluorescent PrP-res aggregates were not fully internalized by SN56 neuronal cells until several days post PrP-res exposure (Baron, Magalhaes et al., 2006), (Magalhaes, Baron et al., 2005). To test whether differences in cell type or PrP-res preparation could account for these discrepancies, we exposed differentiated SN56 neuronal cells for 24 hrs to either 22L PrP-res^{3F4} or 22L PrP-res^{3F4} conjugated to an Alexa-Fluor-596 fluorescent tag (Fig. 7). SN56 cells exposed to Alexa-Fluor labeled 22L PrP-res^{3F4} showed very large PrP-res^{3F4} aggregates (Fig. 7A) that were indistinguishable from those described previously (Baron, Magalhaes et al., 2006),(Magalhaes, Baron et al., 2005). By contrast, SN56 cells exposed to non-Alexa-Fluor labeled 22L PrP-res^{3F4} (Fig. 7B) showed the same small, punctate aggregates of PrP-res^{3F4} observed in MoL42-CFD5 cells (Fig. 6). When both Alexa-Fluor labeled PrP-res from 22L scrapie-infected wild type mice and non-Alexa- Fluor labeled 22L PrP-res^{3F4} were added to the same monolayer of SN56 cells, the difference in PrP-res aggregate size was even more apparent (Fig. 7C). The difference in aggregate size between 22L PrP-res and Alexa-Fluor labeled 22L PrP-res strongly suggests that the larger PrP-res aggregates were primarily a consequence of the Alexa- Fluor labeling process.

When both Alexa-Fluor labeled 22L PrP-res from scrapie-infected wild type mice and non-Alexa-Fluor labeled 22L PrP-res^{3F4} were added to the same monolayer of SN56 cells, there was almost no co-localization between the PrP-res molecules in the two preparations (Fig. 7C). To help resolve this difference in co-localization, we conducted a temperature shift analysis of SN56 cells exposed to both Alexa-Fluor labeled 22L PrP-res from wild type mice and non-Alexa-Fluor labeled PrP-res^{3F4} (Fig. 7D). Cells incubated at 37°C (Fig. 7D) showed localization patterns similar to Figure 7C. However, cell-associated PrP-res^{3F4} was not detected at 18°C suggesting that the amount of PrP-res^{3F4} bound or internalized by the cells at was very low (Fig. 7D). By contrast, significant amounts of Alexa-Fluor labeled PrP-res were cell-associated at 18°C (Fig. 7D) suggesting that it was bound to the cell surface via either PrP-res^{3F4} or the Alexa-Flour dye moiety. The fact that Alexa-Fluor labeled PrP-res was largely out of the plane of focus of the cell when examined by immunofluorescence (note the faint halo effect around the red Alexa-Fluor labeled PrP-res aggregates in Fig. 7) also support the conclusion that these aggregates were primarily localized at the cell surface. Our data suggest that during the first 24 hrs of infection, large PrP-res aggregates are localized primarily at the cell surface while smaller aggregates are rapidly internalized.

Infectious brain homogenate PrP-res is taken up most efficiently by cells

A number of PrP-res preparation methods have been employed to infect cells *in vitro* (Baron, Magalhaes et al., 2006), (Baron, Wehrly et al., 2002), (Bendheim, Barry et al., 1984), (Vorberg & Priola, 2002). To test if different PrP-res preparation methods would alter PrP-res uptake into cells, equal amounts of Obihiro PrP-res^{3F4} derived either from partially purified PrP-res or infectious crude brain homogenate was added to MoL42- CFD5 cells and the uptake of PrP-res into the cells was assayed by Western blot. By 8 hours post infection, infectious crude brain homogenate appeared to be taken up significantly more efficiently then partially purified PrP-res (Fig. 8A, open triangles). To determine whether or not this discrepancy in PrP-res uptake was due to a difference in total protein, mock infected brain homogenate was added to the partially purified PrP-res in order to match the total protein content found in the infectious brain homogenate. Protein adjusted partially purified PrP-res was taken up with the same efficiency as partially purified PrP-res alone (Fig. 8A). Microsome PrP-res was also taken up by cells with the same efficiency as either partially purified PrP-res or total protein adjusted partially purified PrP-res (total protein adjusted with mock infected microsome preparation) (Fig. 8B). Taken together, our results suggest that there is an increased efficiency in the uptake of PrP-res when it is associated with an infectious brain homogenate.

DISCUSSION

The use of PrP-res tagged with a unique antibody epitope has allowed us to examine for the first time the cellular uptake of PrP-res present in an infectious inoculum in the absence of any confounding background from host cell derived PrP-res or PrP-sen. Our data show that PrP-res uptake is cell type and scrapie strain independent and are consistent with previous work where the acute conversion of cellular PrP-sen to PrP-res was also found to be cell type and scrapie strain independent (Vorberg, Raines et al., 2004a). Furthermore, the current study demonstrates that during the first three days post-scrapie exposure cells take up PrP-res from different strains at a similar rate (Fig. 4, Fig. 6) and that this process absolutely does not require host cell expression of PrP-sen (Fig. 4). Thus, although PrP-sen is necessary for persistent PrP-res formation and scrapie infection, its absence in cells does not inhibit acute uptake of PrP-res.

Regardless of strain, PrP-res uptake into cells was detectable by 2 hrs (Fig. 4, Fig. 6) and, after 8 hrs, was apparently restricted by total cell number (Fig. 5). This change in the kinetics curve may be related to the fact that most cells are still rapidly dividing and in log phase during the first 8 hours of exposure to PrP-res. Over time, the cells become more confluent and PrP-res uptake may be reduced as cellular division slows. This interpretation is consistent with the recent observation that cell division can also influence PrP-res levels within mouse neuroblastoma cells persistently infected with scrapie (Ghaemmaghami, Phuan et al., 2007).

For all mouse strains tested, our results show that 10–15% of PrP-res^{3F4} in the brain homogenate was taken up by the cells (Fig. 2D). This is in stark contrast to a recent study showing that the amount of PrP-res taken up by the cell during acute TSE infection was strain dependent and could exceed 80% (Paquet, Daude et al., 2007). One possible explanation for this discrepancy is that the population of cells susceptible to TSE infection is higher in the epithelial cells used in the previous study than in the cells used here. Another possibility is that strain-specific differences in the size of the PrP-res aggregate may influence how much PrP-res the cells take up. The recent observation that PrP-res particle size can influence scrapie infectivity (Silveira, Raymond et al., 2005), the demonstration that large Alexa-Fluor PrP-res aggregates are broken down over several days into smaller aggregates which are then internalized by the cell (Magalhaes, Baron et al., 2005), as well as our data demonstrating that large AlexFluor-labeled PrP-res aggregates are not rapidly internalized (Fig. 7A), are all consistent with this possibility.

Interestingly, at any given timepoint only 30–40% of the cells were able to take up detectable levels of PrP-res (Fig. 6) despite the fact that it was always supplied in excess. The most likely explanation for this result is that there is a limited population of cells which can detectably take up PrP-res. Over time, these cells divide and increase in number leading to the observed increase in the amount of cell-associated PrP-res, a process that may also be important once persistent infection has been established (Ghaemmaghami, Phuan et al., 2007), (Weissmann, 2004). Alternatively, it may be that only certain cells have the necessary cell surface ligands to endocytose PrP-res. A third possibility may relate to the observation that the pathway of cellular internalization of particles such as viruses and bacteria is influenced by both particle size and the composition of plasma membrane microdomains (Cheng, Singh et al., 2006) Perhaps only a certain subpopulation of cells within the culture have the plasma membrane microdomains needed to efficiently interact with and internalize PrP-res. Whatever the explanation, our data are consistent with the hypothesis that PrP-res aggregate size can influence not only how PrP-res initially interacts with the cell but also its uptake.

Mouse PrP-res from all strains tested formed small, punctate aggregates that appeared to localize primarily in the perinuclear and cytoplasmic regions of the cell (Fig. 6). This localization is consistent with earlier studies of PrP-res in persistently infected cells (Caughey, Raymond et al., 1991), (McKinley, Taraboulos et al., 1991), (Pimpinelli, Lehmann et al., 2005), (Taraboulos, Scott et al., 1994) and these punctate aggregates likely act as PrP-res seeds that help propagate new PrP-res formation (Vorberg, Raines et al., 2004a). Despite the evidence that cell-type can influence establishment of a persistent infection (Vorberg, Raines et al., 2004a), for all cell types and strains tested we were unable to detect any differences in the cellular localization of PrP-res. For example, both ME7 and 22L PrP-res appeared to be in similar cytoplasmic and perinculear locations (Fig. 6), despite the fact that the 22L scrapie strain infects fibroblast cells while ME7 does not (Vorberg, Raines et al., 2004a). This suggests that the initial cellular location of the inoculum PrP-res may not be a determining factor in whether or not a cell becomes persistently infected. However, given the fact that our current results do not allow us to clearly distinguish different cellular compartments, further studies looking at the co-localization of input PrP-res^{3F4} with different cellular markers will be needed to determine if this is the case.

Previous studies using the hamster scrapie strain 263K and CHO cells found that hamster PrP-res remained cell associated at 18°C and likely bound to cells via cell surface heparan sulfate (Hijazi, Kariv-Inbal et al., 2005). It was therefore somewhat surprising that we found that only a small amount of mouse PrP-res^{3F4} was cell-associated at 18°C (Fig. 3 and Fig. 7D). Our data comparing AlexaFluor labeled PrP-res to unlabeled PrP-res demonstrate that PrP-res aggregate size can influence whether or not PrP-res can detectably bind to the cell surface. Thus, one explanation for the difference between the mouse and hamster results could be that hamster PrP-res aggregates are larger than mouse PrP-res aggregates. Alternatively, it is possible that at 18°C the receptor for internalization of mouse PrP-res is largely absent from the cell surface but still present for hamster PrP-res. However, the low level of mouse PrP-res cell surface binding and uptake at 18°C (Fig. 3, Fig. 7D) suggests that mouse PrP-res is taken up into cells through an active cellular process such as endocytosis that may not necessarily be mediated by a specific cell surface ligand.

PrP-res within an infectious brain homogenate was taken up more efficiently then either partially purified PrP-res or PrP-res in microsome preparations (Fig. 8). This suggests that there may be a co-factor or some type of PrP-res associated microenvironment that is specific to infectious brain homogenate which is removed or disrupted during either of the alternative PrP-res preparations. Consistent with the idea that PrP-res may have to be in a specific microenvironment to efficiently infect cells (Baron, Magalhaes et al., 2006), the simple addition of brain homogenate protein to partially purified PrP-res preparations did not enhance the uptake of partially purified PrP-res (Fig. 8A). It is also unlikely that microsomes provide the appropriate microenvironment given that microsome PrP-res was not taken up as efficiently as PrP-res in brain homogenate (Fig. 8B). Our results do not negate the possibility that microsomes may be more infectious (Baron, Magalhaes et al., 2006), but suggest that the apparent increased efficiency with which microsomes infect cells is not due to more efficient uptake of PrP-res during the early stages of infection as previously proposed (Baron, Magalhaes et al., 2006).

In combination with earlier studies (Vorberg, Raines et al., 2004a), our data suggest that there may be at least two blocks to the persistent infection of a cell with scrapie. The first occurs during the acute stage of infection (0–72hrs) when factors such as PrP-res aggregate size, the microenvironment of the PrP-res inoculum, and/or the presence of a specific population of cells help to determine whether or not PrP-res is bound to the cell and internalized. This block would not be strictly dependent upon scrapie strain or cell type but rather would depend upon heterogeneity in both the PrP-res and cell populations. Once a cell culture becomes persistently infected, cellular heterogeneity may also influence both the level and stability of infectivity over long term passage (Bosque & Prusiner, 2000), (Enari, Flechsig et al., 2001), (Weissmann, 2004).

We have previously shown that the mouse Ψ2 fibroblast cells used in the present study can be infected with the 22L, but not the ME7, strain of mouse scrapie (Vorberg, Raines et al., 2004a). The fact that ME7 acts similarly to 22L during acute infection (Fig. 4–Fig. 5 and (Vorberg, Raines et al., 2004a)) suggests that the second block to infection is scrapie-strain dependent and occurs 1) after uptake and localization of the inoculum PrP-res and, 2) after an acute burst of new PrP-res formation in the cell (Vorberg, Raines et al., 2004a).

MATERIAL AND METHODS

$PrP^{0/0}$ Neural Cell Line

Pregnant mice in which the PRNP gene has been knocked out ($PrP^{0/0}$) (Manson, Clarke et al., 1994) were anesthetized with isofluorane at gestation day 15. Embryos were isolated, decapitated and the heads were transferred to cold dissecting solution (phosphate buffered saline (PBS), Ca^{2+}/Mg^{2+} free, 5% glucose). Brains were removed and freed of meninges and blood vessels, cut into 5 mm^2 pieces, and disassociated by aspiration in Dulbecco's phosphate buffered saline (DPBS) with 10% FBS (Gibco Inc.), 0.5 mM glutamine, and B27. The cell suspension was centrifuged at 160 × g, 4°C, 5 min and cells were resuspended in DMEM with 5% FBS, and F12. Cells were adjusted such that 40,000 cells/well were plated on poly-L-lysine coated 24 well plates. After 4 hrs incubation at 37°C, 5% CO_2, medium was replaced with 1 ml/well of DMEM with growth supplements FBS (3%), B27, and N2. Transfection of cells for immortalization was done 48 hrs after plating, using the plasmid vector pSV3-neo (ATCC, #37150) (Southern & Berg, 1982) and lipofectamine (Invitrogen) according to the manufacturer's instructions. Two days after transfection, G418 was added to the medium to select for transfected, immortalized cells. Immortalized clones were pooled and single cell clones were established. A single cell clone (CF10) was selected and maintained in Opti-MEM supplemented with 10% FBS and 1% penicillin streptomycin (PS). CF10 cells are strongly positive for nestin (data not shown) which is predominantly found in stem cells of the CNS. Therefore, it is likely that they are neural stem cells.

MoL42-CFD5 and Mo3F4-CF10 cell lines were derived by limiting dilution cloning from CF10 cells that had been transduced with a retrovirus encoding the mutant PrP molecule of interest (Mann, Mulligan et al., 1983). The Mo3F4-CF10 cells express mouse PrP with the epitope to the mouse monoclonal antibody 3F4 (Mo3F4), while the MoL42-CFD5 cells express mouse PrP with the epitope to the mouse monoclonal antibody L42 (MoL42) (Vorberg, Buschmann et al., 1999). The L42 epitope can be used to distinguish mouse PrP in the cells from mouse PrP in brain homogenate.

Fibroblast cells (ψ2) are susceptible to infection with the mouse scrapie strain 22L but not with the ME7 strain (Vorberg, Raines et al., 2004a). MoL42-ψ2A2 is a cell line derived by limiting dilution cloning from ψ2 fibroblast cells (Mann, Mulligan et al., 1983;Miller & Buttimore, 1986) that had been transduced by the same retrovirus used to make the MoL42-CFD5 cells. MoL42-ψ2A2 cells express both endogenous mouse PrP as well as mouse PrP with the L42 epitope. MoL42-ψ2A2 cells were maintained in RPMI with 10% FBS supplemented with 1% penicillin/streptomycin (final concentration of 100 units/ml penicillin G and 100 ug/ml streptomycin) (Invitrogen). SN56 cells are neuronal derived cells from mouse septum neurons (Hammond, Lee et al., 1990) and were used with the kind permission of Dr. Bruce Wainer (Department of Pathology, Emory University of Medicine, Atlanta, GA).

PrP-res preparation

Scrapie strains 22L and ME7 were the kind gift of Dr. James Hope (Vitechnologies, Boston, Ma) while the Obihiro scrapie strain (Shinagawa, Takahashi et al., 1985) was the kind gift of Dr. Motohiro Horiuchi University of Obihiro, Hokkaido, Japan). Tg(WT-E1) mice were from the laboratory of Dr. David Harris (Washington University, St. Louis, Mo). These mice express high levels of PrP-sen with an epitope tag (Bolton, Seligman et al., 1991) for the mouse monoclonal antibody 3F4 (PrP-sen^{3F4}).

The mouse-adapted scrapie strains 22L, ME7, and Obihiro (Obi) in DPBS were passaged once in Tg(WT-E1) mice (Chiesa, Piccardo et al., 1998) to generate the brain homogenates 22L (3F4), ME7(3F4), and Obi(3F4). Tg(Wt-E1) mice inoculated with uninfected C57Bl/10 mouse brains were designated as mock controls (Mock(3F4)). Brains from terminally ill Tg(WT-E1) mice or mock infected, age matched controls were harvested and stored at −70°C. To study the uptake of PrP-res from crude brain homogenate, brains were dounce homogenized in DPBS (10% w/v), sonicated, and then stored at −70°C as described previously (Vorberg, Raines et al., 2004a). Crude brain microsome fractions were prepared as described in detail by Baron and colleagues (Baron, Wehrly et al., 2002), while PrP-res was partially purified as previously described (Raymond & Chabry, 2004) but without proteinase K (PK) digestion. Partially purified PrP-res was quantified by western blot using a standard protein concentration curve derived from recombinant hamster PrP-sen. For experiments where partially purified PrP-res preparations were compared to less purified microsome or brain homogenate preparations, total protein content was normalized using microsomes or brain homogenate from mock-infected Tg(WT-E1) mice. Alexa-Fluor-596 labeled PrP-res preparations were made as previously described by Magalhães and collegues (Magalhaes, Baron et al., 2005).

Cellular uptake of PrP-res

To analyze PrP-res uptake over time, experiments were performed in 24 well microtiter plates as previously described (Vorberg, Raines et al., 2004a). Briefly, different cell numbers were initially plated in order to ensure similar cell numbers at time of harvest. The number of cells plated were: MoL42-CFD5 cells (0–24 hrs, 3 × 10^5 cells/ml; 48hrs 2 × 10^5 cells/ml; and 72 hrs 1.5 × 10^5 cells/ml), CF10 and MoL42-ψ2A2 cells (0–24 hrs 4 × 10^5 cells/ml; 48hrs 2 × 10^5 cells/ml; and 72 hrs 1.5 × 10^5 cells/ml). After incubation at 37°C for 24 hrs, the medium was removed and replaced with 200 μl of brain homogenate, microsomes, or partially purified PrP-res diluted in Opti-MEM. The cells were exposed to equivalent amounts of PrP-res for each strain (10 ng for each preparation). Mock preparations were diluted 1:10 in Opti-MEM. Samples were then incubated at 37°C for 2–4 hrs followed by the addition of 400 μl/well of fresh media. At each time point, cells were washed 4 times with 300 μl of fresh medium and either removed from the plate using trypsin-EDTA (Invitrogen) and counted with a hemacytometer or lysed directly in 3X lysis buffer (3 mM Tris-HCl, pH 7.4, 420 mM NaCl, 15 mM EDTA, 1.5% sodium deoxycholate, and 1.5% Triton x–100). Lysates were treated with benzonase (0.167 U/ul) (Novagen) for 30 min at 37°C, then PK (60 μg/ml) (Roche) for 1 hr at 37°C followed by the addition of 1.2 μg of 4-(2-aminoethyl)benzenesulfonylfluoride (PEFABLOC) (Roche). PK digested lysates were precipitated in four volumes of cold methanol for 2 hrs at −20°C followed by centrifugation at 20,800 × *g* for 30 min. Pellets were sonicated into sample buffer (2.5% SDS, 3 mM EDTA, 2% β-mercaptoethanol, 5% glycerol, 0.02% bromphenol blue, and 63 mM Tris-HCL, pH 6.8), boiled for 3 min, optionally PNGaseF treated for 12 hours according to the manufacturer's instructions (New England Biolabs), and loaded on 16% Tris-Glycine precast gels (Invitrogen). PrP was detected by western blot analysis using the mouse monoclonal antibody 3F4 (1:3,000) followed by secondary ECL-anti-mouse IgG (1:5,000) (Amersham) or anti-mouse IR-dye 800CW (1:10,000) (Li-Cor).

Quantitative ECL data was generated using the UN-SCAN-IT software (Silk Scientific Corp.) according to the manufacturer's instructions. In order to quantify the data within the linear range of the film, the first three lanes of each gel were used as internal standards to establish a constant film exposure time which was then used for every experiment. PrP-res levels were quantified from gels exposed to film for a set time (4 min) using a fixed parameter box (i.e. the box was the same volume for every experiment) and the UN-SCAN-IT software. Pixel count totals within the fixed parameter box were summed and presented as pixels. When the secondary antibody anti-mouse IR-dye 800CW was used to develop the western blot, quantitative data were obtained using the Li-Cor Odyssey IR scanner and the software provided with the system (Li-Cor).

To quantify the percentage of the input brain homogenate PrP-res^{3F4} that was taken up by the cells, MoL42-CFD5 cells were plated into 24 well plates as detailed above and scrapie-infected brain homogenate containing 8ng of PrP-res^{3F4} was added to the cells for 24 or 48 hrs. Following removal of the supernatant and four rinses of the cells with PBBS, the cells were lysed and cell associated PrP-res^{3F4} was isolated as described above. For each strain, a standard curve consisting of serial two-fold dilutions of the input PrP-res^{3F4} brain homogenate was used to quantify the samples. These serial two-fold dilutions (8ng-1ng) were loaded on every gel and used to establish a standard curve to quantify only the samples run on the same gel. Two experiments were run for a total of 12 samples for each strain and timepoint. Quantitative data were obtained using the Li-Cor Odyssey IR scanner and the software provided with the system (Li-Cor).

Analysis of PrP-res uptake by fluorescent microscopy

Cells were plated in Lab-Tek II chamber slides (8-well) (Nalge Nunc Inc.) at the densities listed above. After 24 hrs at 37°C, the media was removed and immediately replaced with 200 μl of Opti-MEM containing 3 ng of partially purified PrP-res from 22L(3F4), ME7(3F4), or Obi (3F4) or volume matched partially purified PrP-res Mock(3F4) preparations. Following incubation from 2 to 48 hrs, the media was aspirated and the cells were washed 4 times with fresh medium. 200ul of 10% formaldehyde was added to each well for 30 min, followed by 200 μl of 0.4% triton-X-100 for 10 min. The wells were then rinsed with 500 μl of PBS (2X), and select chambers were treated with 200 μl of PK (10ug/ml) for 9 min at 37°C. The PK was then removed and excess PK blocked with 200 μl of PEFABLOC (10mM). Wells were washed again with PBS and treated with guanidine thiocyanate (4M, 200 μl/chamber) for 30 min at 25°C. After rinsing in PBS, 200 μl of the mouse 3F4 monoclonal antibody (1:200 dilution) was added for 30 min followed by PBS washes (3X) and the addition of 200 μl of goat anti-mouse FITC conjugated antibody (1:400 dilution) for 30 min. Following the antibody incubations, the slides were rinsed in PBS and coverslip mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Slides were viewed on an Olympus BX51 fluorescent microscope with both 20X and 40X air immersion objectives. All images were taken with an Olympus DP71 camera and were processed and analyzed with Microsuite Software (Olympus).

For comparison of PrP-res to Alexa-Fluor-596 conjugated PrP-res, SN56 cells were plated in Lab-Tek II chamber slides (8-well) (Nalge Nunc Inc.) at a 1:20 dilution from a confluent 25 cm^2 flask and differentiated with cAMP (1mm) in serum free Opti-MEM for 24 hrs. PrP-res or Alexa-Fluor-596 conjugated PrP-res (8 ng) was added to the cells and the cells were incubated for 24 hrs at 37°C. Cells were then fixed, labeled and observed by florescent microscopy as described above.

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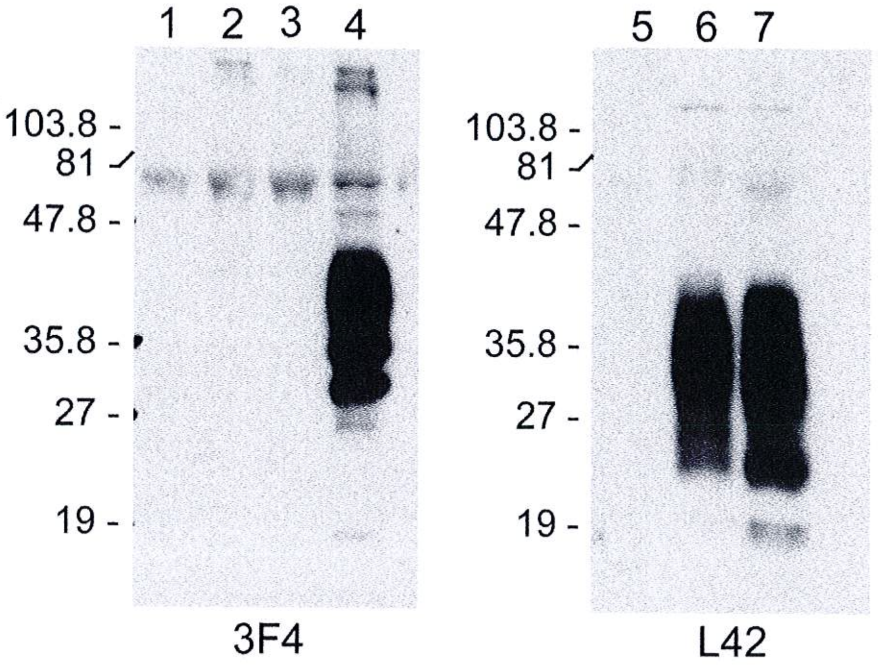


Figure 1. Cell line expression of PrP

Western blot detection of PrP-sen with either mouse monoclonal antibody 3F4 (left panel) or mouse monoclonal antibody L42 (right panel) for cell lines CF10 (lanes 1, 5), MoL42-CFD5 (lanes 2, 6), and MoL42-Ψ2A2 (lanes 3, 7). As a positive control for the specificity of the 3F4 antibody, Lane 4 shows CF10 cells expressing Mo3F4.

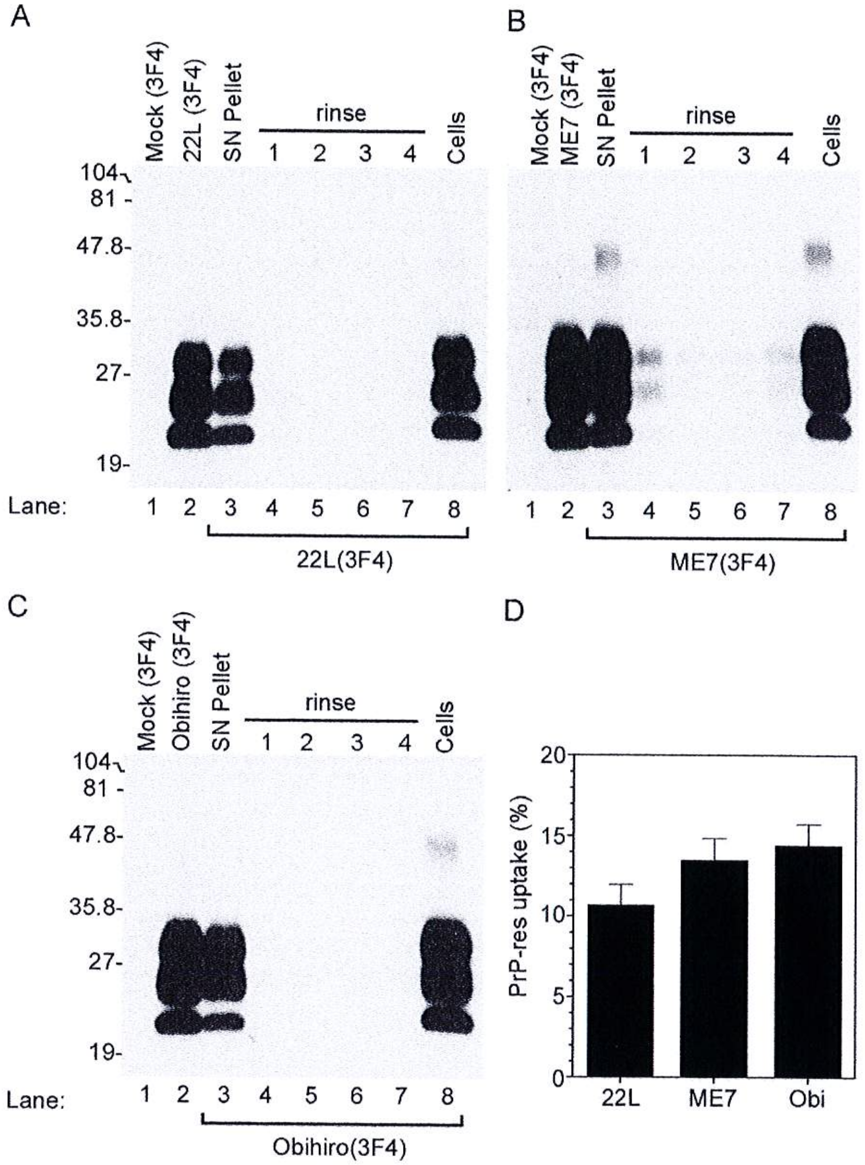


Figure 2. Mouse neural cells take up PrP-res^{3F4}

Western blot analysis of PrP-res^{3F4} uptake into MoL42-CFD5 cells. Brain homogenate from 22L, ME7 or Obihiro scrapie-infected Tg(WT-E1) mice were PK treated and used as positive controls (Panels A–C, lane 2) while PK treated brain homogenate from mock-infected Tg(WT-E1) mice was used as negative controls (Panel A–C, lane 1). For both the positive and negative controls, 1/20th of the total homogenate used was loaded onto the gel. Brain homogenates (200μl of a 1% brain homogenate) from 22L(3F4) (panel A), ME7(3F4) (panel B), or Obi(3F4) (panel C) were added to MoL42-CFD5 cells for 24 hours and then removed from the cell monolayer. Cell debris and dead cells were spun out of the supernatant and the supernatant pellet was PK treated, methanol precipitated and the entire sample loaded onto the gel (SN Pellet). Cells were then rinsed with PBS four times (rinse 1–4), lysed, and both the cell lysate and rinses were PK treated, methanol precipitated and the entire sample loaded onto the gel. PrP-res^{3F4} was detected in the lysed and PK treated cells (Cells). All blots were analyzed using the mouse monoclonal antibody 3F4 and developed using ECL (Amersham). The percentage of total PrP-res^{3F4} taken up by the cells after 24 hours is shown in Panel D. PrP-res^{3F4} was quantified as detailed in the materials and methods. There was no significant difference between the three strains in the amount of PrP-res taken up by the MoL42-CFD5 cells ($p>0.05$ using 1-way Anova with Bonferroni's multiple comparison test). Data are shown as mean ± S.D. for N=12.

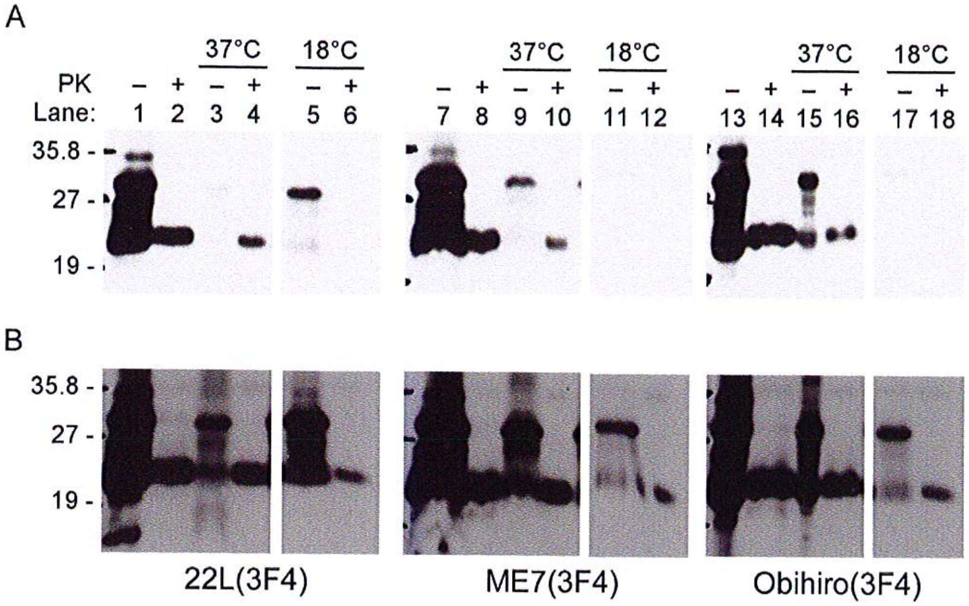


Figure 3. PrP-res^{3F4} uptake into neural cells is significantly decreased at 18°C

MoL42-CFD5 cells were exposed to infected brain homogenate from 22L(3F4), ME7(3F4) or Obi(3F4) scrapie for 24 hours at either 37°C or 18°C. Samples were PNGaseF treated to remove complex glycans. Non-PK digested infected brain homogenates were run to illustrate total PrP levels (lanes 1,7,13) while PK-digested brain homogenates were run as a positive control for PrP-res (lanes 2,8,14). Some PrP-sen^{3F4} was taken up by cells at 37°C (−PK lanes 3, 9, 15) and PrP-res^{3F4} was detected in cells which had been exposed to scrapie brain homogenate and incubated at 37°C (+PK lanes 4, 10, 16). Exposure time for Panel A was 8 minutes. After overnight exposures of the gels, some PrP-sen^{3F4} (−PK lanes 5, 11, 17) and a low level of PrP-res^{3F4} (+PK lanes 6, 12, 18) were detected in cells which had been exposed to scrapie brain homogenate and incubated at 18°C (Panel B). All blots were analyzed using the mouse monoclonal antibody 3F4 and developed using ECL (Amersham).

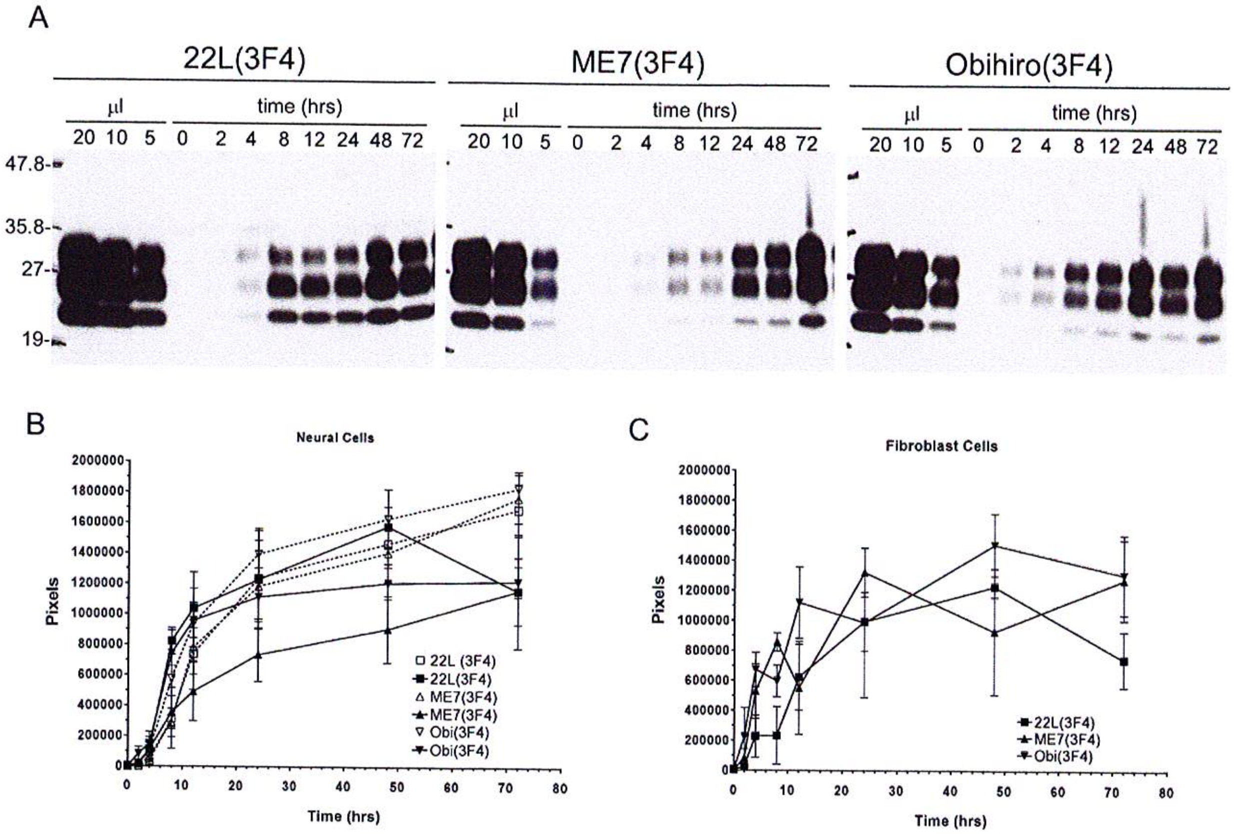


Figure 4. Kinetics of PrP-res^{3F4} uptake into cells

A) Representative Western blot analysis of PrP-res^{3F4} uptake into MoL42-CFD5 cells from 0 – 72 hrs. Scrapie-infected brain homogenates from 22L(3F4), ME7(3F4) or Obi(3F4) were analyzed. The first three lanes of each gel show PK-treated scrapie-infected brain homogenate for the appropriate strain loaded in 20 ul, 10 ul, and 5 ul volumes. These lanes were used as internal standards to establish a constant film exposure time from experiment to experiment (4min) in order to quantify the data within the linear range of the film. The amount of PrP-res internalized by the cells increased over time. B) Graphical representation of total cell-associated 22L(3F4), ME7(3F4), or Obi(3F4) taken up by either MoL42-CFD5 cells (solid lines N=5 or 6) or CF10 cells (dotted lines N=3) from 0–72 hrs. The error bars represent SEM. No statistically significant differences were found between the scrapie strains (*p>0.05, Two-way ANOVA*) or between the different cell types (*p>0.05, Mann Whitney test*) for each scrapie strain. C) Graphical representation of total cell-associated 22L, ME7 or Obihiro PrP-res^{3F4} taken up by MoL42-ψ2A2 fibroblast cells (N=3) from 0 – 72 hrs where the error bars represent SEM. No statistically significant differences were found between the scrapie strains (*p>0.05, Two-way ANOVA*) or between the fibroblast and neuronal cell types (*p>0.05, Mann Whitney test*). All blots were analyzed using the mouse monoclonal antibody 3F4. For all data, the amount of PrP-res is expressed in pixels and was quantified by analysis of ECL developed western blots using the UN-SCAN-IT software as detailed in the Methods.

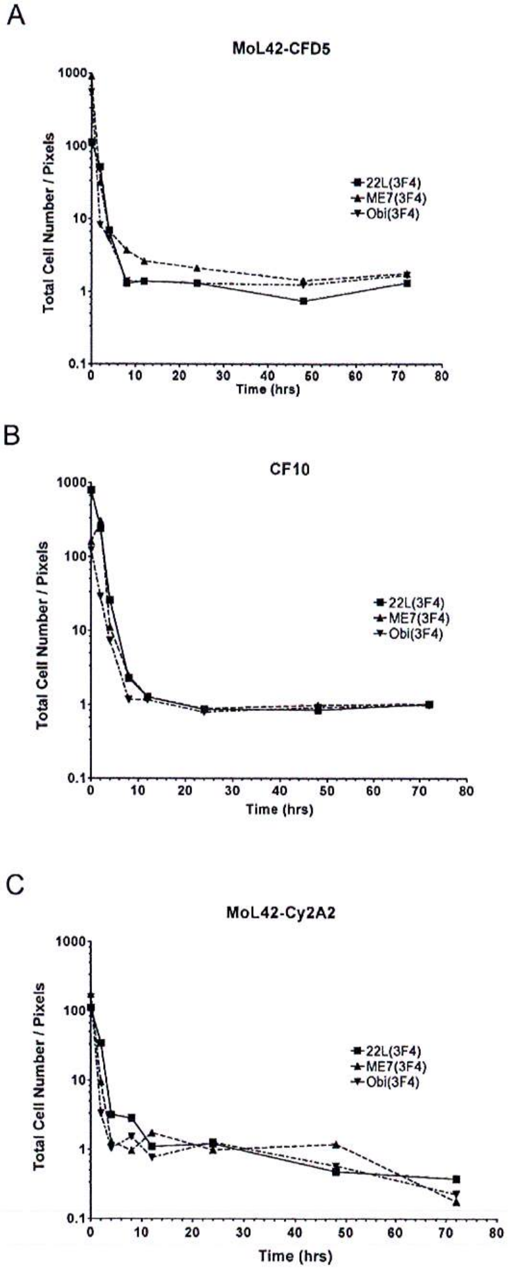


Figure 5. Increased levels of cellular PrP-res^{3F4} correlate with increased cell number

Ratio of total cell number to total cell-associated PrP-res^{3F4} for scrapie strains 22L(3F4), ME7 (3F4) or Obi(3F4). PrP-res^{3F4} was quantified using ECL developed western blots analyzed with the UN-SCAN-IT software. A) Ratio of the number of MoL42-CFD5 cells to total PrP-res^{3F4} (N=5). B) Ratio of the number of CF10 cells to total PrP-res^{3F4} (N=6). C) Ratio of the number of MoL42-ψ2A2 cells to total PrP-res^{3F4} (N=6). The variability of the data at later time points was likely due to increased cell death over time (data not shown) rather than any cell-type differences.

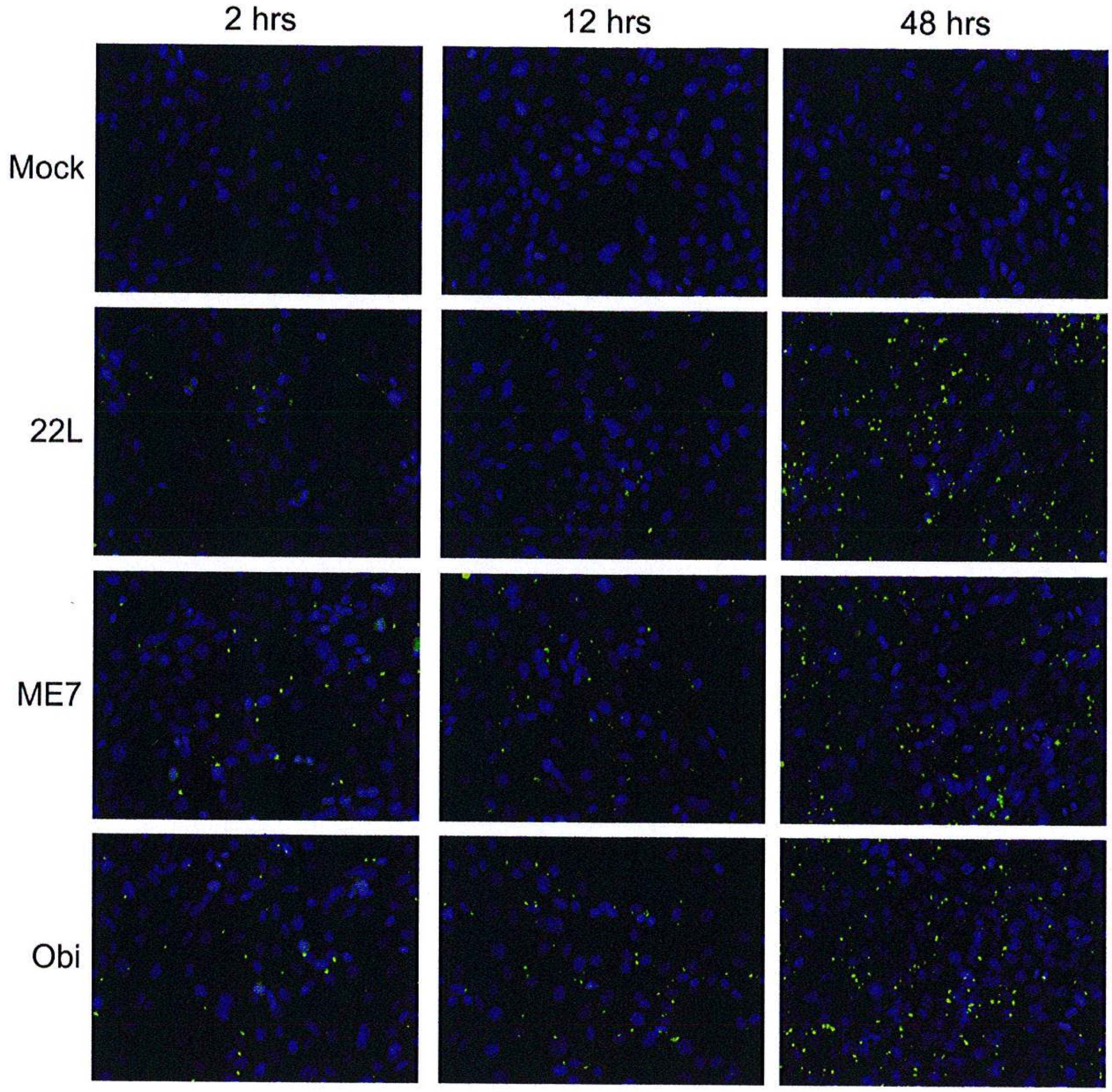


Figure 6. PrP-res^{3F4} uptake into neural cells using immunofluorescence microscopy

PrP-res^{3F4} partially purified from 22L(3F4), ME7(3F4), Obi(3F4) or mock brain homogenates was added to MoL42-CFD5 cells for 2 – 48 hrs. Cells were rinsed, fixed and immunolabeled with the mouse monoclonal antibody 3F4. Anti-mouse FITC labeled antibody (green) was used to detect PrP-res^{3F4} while DAPI stain (blue) denotes cell nuclei. All images were taken with a 40X objective.

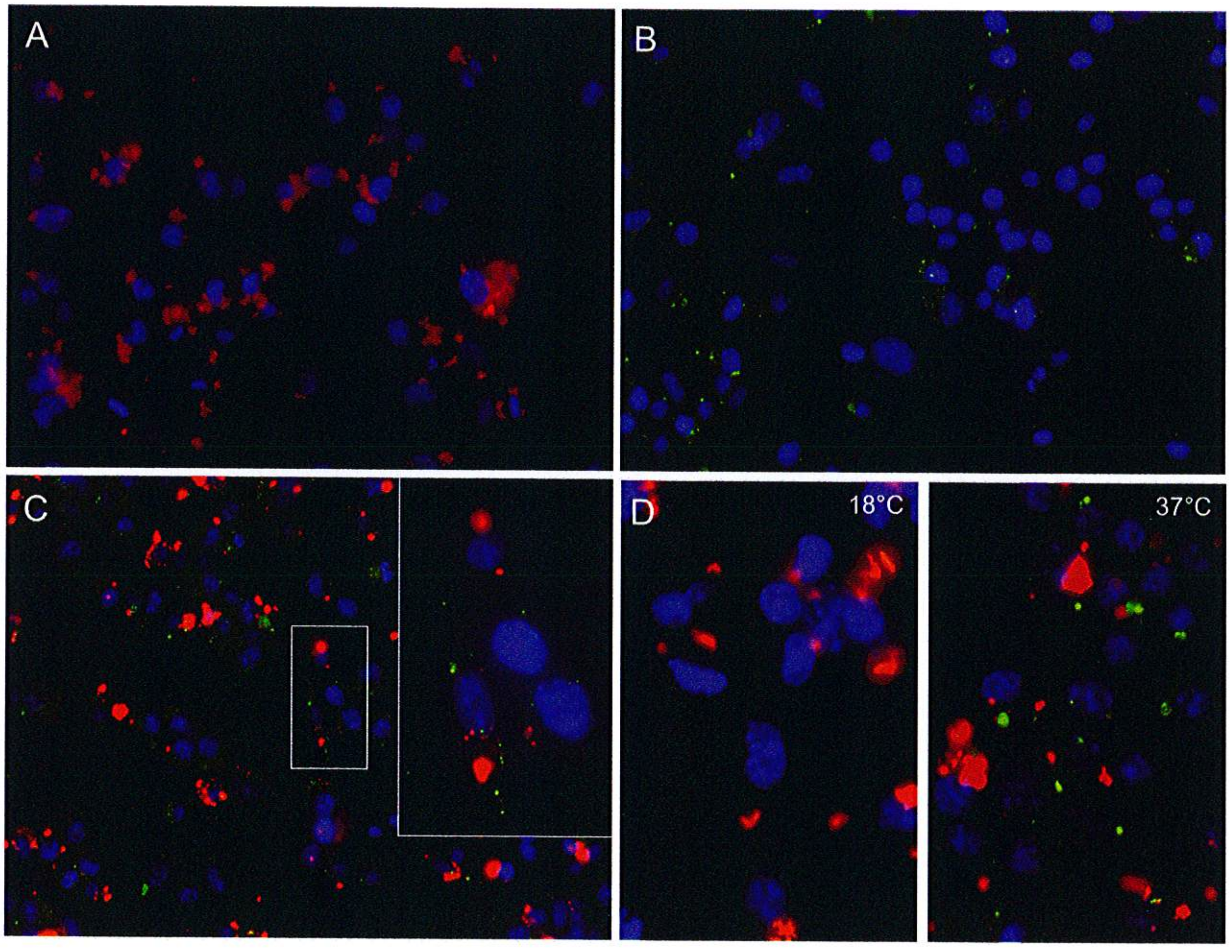


Figure 7. Large PrP-res aggregates bind to the cell surface and are not rapidly internalized

Differentiated SN56 cells were exposed to Alexa-Fluor-596 labeled (red) or unlabeled (green) 22L PrP-res^{3F4} for 24 hours. Cells were then rinsed, fixed and when necessary, immunolabeled with the mouse monoclonal antibody 3F4 (Panels B–D). Anti-mouse FITC labeled antibody (green) was used to detect 22L PrP-res^{3F4}, while DAPI stain (blue) denotes cell nuclei. Alexa-Fluor-596 labeled proteins are red. All images were taken with a 40X objective. Cells were exposed to A) Alexa-Fluor labeled partially purified 22L PrP-res^{3F4} (red), B) partially purified 22L PrP-res^{3F4} (green), C) Alexa- Fluor labeled partially purified PrP-res from a 22L scrapie-infected wild type mouse (red) and partially purified 22L PrP-res^{3F4} (green) (inset taken with a 60X objective). In panel D, cells were exposed for 24 hours at either 18°C or 37°C to Alexa-Fluor labeled partially purified PrP-res from a 22L scrapie-infected wild type mouse (red) and 22L PrP-res^{3F4} (green). The larger Alexa-Fluor labeled PrP-res aggregates do not colocalize with the smaller, more punctate PrP-res^{3F4} aggregates.

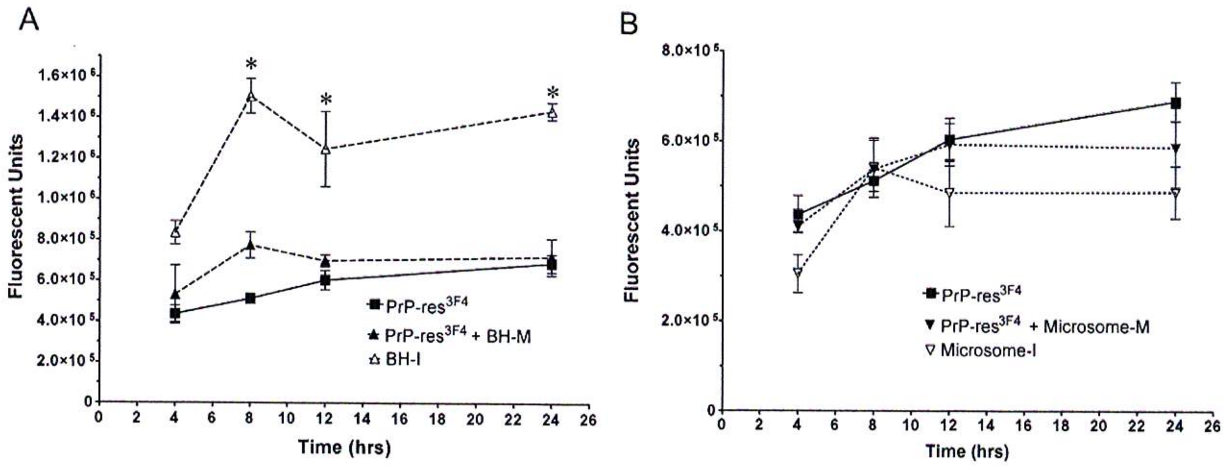


Figure 8. Infectious brain homogenate PrP-res^{3F4} is taken up more efficiently then either microsome or partially purified PrP-res^{3F4}

Graphical representation of PrP-res^{3F4} uptake from 0 – 24 hrs into MoL42-CFD5 cells (N = 6) where error bars represent SEM. A) Kinetics of PrP-res^{3F4} uptake using either infectious brain homogenate PrP-res^{3F4} (BH-I), partially purified PrP-res^{3F4} (PrP-res^{3F4}) or partially purified PrP-res^{3F4} with mock infected brain homogenate (PrP-res^{3F4} + BH-M) added to match the total protein content of BH-I (* $p<0.05$, $t = 8$–*24 hrs, Bonferroni test*). B) Kinetics of PrP-res^{3F4} uptake using either infectious microsome PrP-res^{3F4} (Microsome-I), purified PrP-res^{3F4} (PrP-res^{3F4}) or partially purified PrP-res^{3F4} with mock infected microsomes (PrP-res^{3F4} + Microsome-M) added to match total protein content of infectious microsomes ($p>0.05$ *for all time points, Bonferroni test*). All data was obtained with IR-dye 800CW developed western blots where the PrP-res^{3F4} level was quantified as fluorescent units using the Li-Cor Odyssey imaging system and associated software.

Attachment 4:

List of vector plasmids used in the laboratory and relevant MSDS

List of plasmids in use and vector maps

Plasmid	**Source**
pcDNA1-amp	Invitrogen
pcDNA3	Invitrogen
pcDNA3-HA1	Invitrogen
pcDNA3-myc	Invitrogen
pEGFP-N1	Clonetech
pEGFP-N2	Clonetech
pEGFP-N3	Clonetech
pEGFP-C1	Clonetech
pEGFP-C2	Clonetech
pEGFP-C3	Clonetech
pEGFP w/o ATG	Clonetech
pEGFP-1 delta CMV	Clonetech
pEGFP-C2 link2	Clonetech
pEGFP-C2 link1	Clonetech
DsRed1-C1	Clonetech
DsRed1-C2	Clonetech
DsRed1-N1	Clonetech
DsRed2-N1	Clonetech
DsRed2-C1	Clonetech
DsRed2-C3	Clonetech
pECFP-N1	Clonetech
pEYFP-C1	Clonetech
pEYFP-N1	Clonetech
pEBG	?
pEBG (3-4)	?
pEBG 2-5	?
pEBG 4	?
pGEX4T1	Amersham
pGEX4T link 1	Amersham
pGEX4T link2	Amersham
pMal c2x	New England Biolabs
pCDNA2.1 His B	Invitrogen
pGAD 10	?
pGAD 10 link A	?
pGAD 10 LinkB	?
pAS2-1	ATCC
pAS2-2-3	ATCC
pRluc-N1	ATCC
pRluc-N2	Perkin Elmer
pRluc-N3	Perkin Elmer
pmRFP	Perkin Elmer
pmRFP-N1	?
PA-GFP-N1	?
PA-GFP-C1	?
pBFP-N1	Clonetech

#42 - 44

BRET²™ TECHNICAL DATA SHEET

BIOLUMINESCENCE RESONANCE ENERGY TRANSFER

RENILLA LUCIFERASE FUSION PROTEIN EXPRESSION VECTOR

Product: Codon Humanized pRluc-N Vectors

Catalog number:	6310220
Description:	**The codon humanized pRluc-N vectors** contain a multiple cloning site (MCS) located upstream of the codon humanized *Renilla* Luciferase gene (Rluc(h)) which acts as the Donor moiety in a BRET² assay. The MCS allows for the subcloning of a gene of interest in order to create a fusion protein having the structure [gene of interest:Rluc(h)]. The Rluc codons have been humanized to ensure higher expression levels of the fusion protein in mammalian cells. The fusion protein gene is placed under the control of the cytomegalovirus (CMV) promoter thus assuring a very high constitutive expression in a variety of cells.
Amount:	10 µg lyophilized plasmid DNA (store lyophilized plasmid at –20°C)

Reconstitution Protocol

Reconstitution:	• Centrifuge briefly to recover contents • Reconstitute to 0.4 µg/µl with 25 µl of 10 mM Tris-HCl pH 8.0, 1 mM EDTA
Storage conditions:	• Store reconstituted plasmid at -20°C • After thawing, centrifuge briefly to recover contents
Shelf life:	• 1 year from date of receipt under recommended storage conditions

Quality Control Procedures

- The identity of the codon humanized pRluc-N plasmids and the presence of the MCS restriction sites are confirmed by sequence analysis.
- The presence of RNA and chromosomal DNA as well as the proportion of superhelical DNA are determined by agarose gel electrophoresis using 1 µg of plasmid DNA.
- The absence of nuclease contamination is determined by agarose gel electrophoresis following incubation of 1 µg of plasmid DNA in standard restriction buffer for 16 hours at 37°C.
- The quantity and purity of DNA are determined by UV spectroscopy.
- The functionality of the plasmids is assessed by measuring luciferase activity upon transfection of CHO or BHK cells with LipofectAMINE™. The intensity of the luciferase signal is compared to the signal of reference plasmids using a Fusion™ Universal Microplate Analyzer.

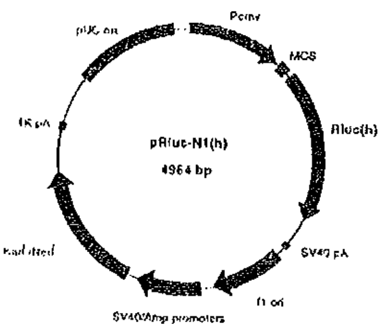
Renilla Luciferase Substrate

BRET² requires a modified form of the Rluc coelenterazine substrate, called DeepBlueC™. DeepBlueC has been selected for its ability to confer superior spectral properties to the reaction, resulting in excellent discrimination of the Rluc and GFP² signals.

Codon Humanized pRluc-N1 Vector Map & Notes

Plasmid size:	4964 bp
Cloning sites:	BglII, XhoI, MluI, PstI, EcoRV, HindIII, KpnI, SacII, ApaI, SmaI, BamHI
Antibiotic resistance:	Prokaryotic: Kanamycin (25 µg/ml for *E.coli*) Eukaryotic: G418/Neomycin (concentration is cell type dependent)

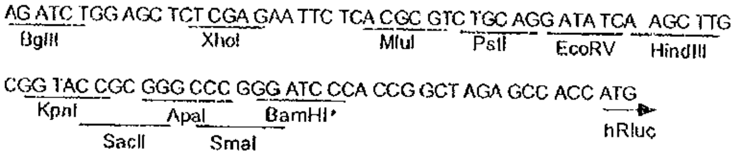
Plasmid map pRluc-N1(h)



P_{CMV}	1 - 583
SV40 early poly (A) signal	1783 - 1833
Rluc (h) gene	698 - 1633
TK poly (A) signal	3890 - 3953
Multiple cloning site (MCS)	609 - 680
P_{SV40}/P_{amp}	2397 - 2811
Kan/Neo^{R}	2860 - 3654
f1 origin	1883 - 2335
pUC sequences (ori)	4239 - 4882

Codon Humanized pRluc-N1 Vector Multiple Cloning Site

Codon humanized pRluc-N1 Vector



* Frame changes characterizing humanized pRluc-N1, N2 and N3 vectors occur after this site.

Quality Control Data

- The identity of the codon humanized pRluc-N1 plasmid and the presence of the MCS restriction sites have been confirmed by sequence analysis.
- Incubation in standard restriction enzyme buffer at 37°C for 16 hours showed no evidence of nuclease activity as detected by agarose gel electrophoresis.
- No RNA and chromosomal DNA were detected in a 1 µg sample of plasmid DNA following agarose gel electrophoresis.
- Percent DNA in Superhelical form: > 75%
- Purity (A_{260}/A_{280}) at pH 8.0: 1.76
- Transfection of CHO cells showed that the codon humanized pRluc-N1 vector is functional and expressed Rluc levels within 25% of the corresponding reference plasmids.

6310220
Rev. A 11/00

invitrogen

Material Safety Data Sheet

Revision Date: 17-Apr-2006

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code	350484
Product name	pcDNA3.1/(+)

Contact manufacturer
INVITROGEN CORPORATON
1600 FARADAY AVENUE
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
2270 INDUSTRIAL STREET
BURLINGTON, ONT
CANADA L7P 1A1
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health.

Form
Solid

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available

Sensitization	No information available
Target Organ Effects	No information available

4. FIRST AID MEASURES

Skin contact	Wash off immediately with plenty of water
Eye contact	Rinse thoroughly with plenty of water, also under the eyelids.
Ingestion	Never give anything by mouth to an unconscious person
Inhalation	Move to fresh air
Notes to physician	Treat symptomatically

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media	Dry chemical
Special protective equipment for firefighters	Wear self-contained breathing apparatus and protective suit

6. ACCIDENTAL RELEASE MEASURES

Personal precautions	Use personal protective equipment
Methods for cleaning up	Soak up with inert absorbent material

7. HANDLING AND STORAGE

Handling	No special handling advice required
Storage	Keep in properly labelled containers

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Occupational exposure controls

Exposure limits

Engineering measures	Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing
Hygiene measures	Handle in accordance with good industrial hygiene and safety practice
Environmental exposure controls	Prevent product from entering drains

9. PHYSICAL AND CHEMICAL PROPERTIES

General Information

Form	Solid

Important Health Safety and Environmental Information

Boiling point/range	°C No data available	°F No data available
Melting point/range	°C No data available	°F No data available
Flash point	°C No data available	°F No data available
Autoignition temperature	°C No data available	°F No data available
Oxidizing properties	No information available	

Water solubility	No data available

10. STABILITY AND REACTIVITY

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available
Target Organ Effects	No information available

12. ECOLOGICAL INFORMATION

Ecotoxicity effects	No information available.
Mobility	No information available.
Biodegradation	Inherently biodegradable.
Bioaccumulation	Does not bioaccumulate.

13. DISPOSAL CONSIDERATIONS

Dispose of in accordance with local regulations

14. TRANSPORT INFORMATION

IATA

Proper shipping name	Not classified as dangerous in the meaning of transport regulations
Hazard Class	No information available
Subsidiary Class	No information available
Packing group	No information available
UN-No	No information available

15. REGULATORY INFORMATION

International Inventories

U.S. Federal Regulations

SARA 313
Not regulated

Clean Air Act, Section 112 Hazardous Air Pollutants (HAPs) (see 40 CFR 61)
This product contains the following HAPs:

U.S. State Regulations

California Proposition 65
This product contains the following Proposition 65 chemicals:

WHMIS hazard class:
Non-controlled

This product has been classified according to the hazard criteria of the CPR and the MSDS contains all of the information required by the CPR

16. OTHER INFORMATION

This material is sold for research and development purposes only. It is not for any human or animal therapeutic or clinical diagnostic use. It is not intended for food, drug, household, agricultural, or cosmetic use. An individual technically qualified to handle potentially hazardous chemicals must supervise the use of this material.

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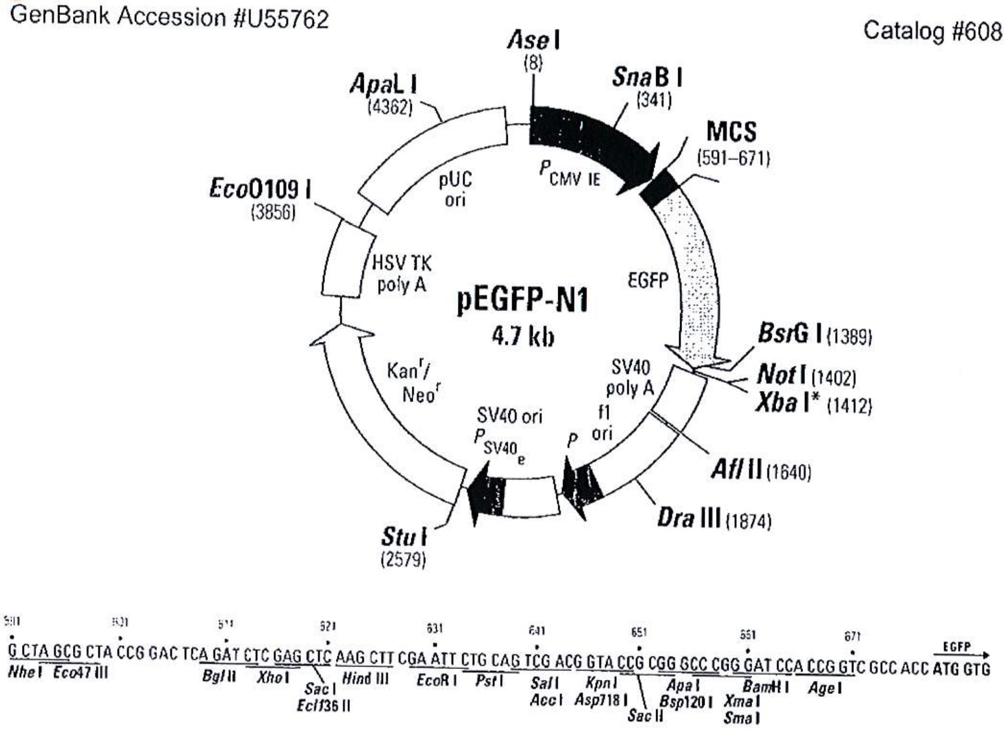
End of Safety Data Sheet

pEGFP-N1 Vector Information

GenBank Accession #U55762

PT3027-5

Catalog #6085-1



Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N1 Vector. All restriction sites shown are unique. The *Not* I site follows the EGFP stop codon. The *Xba* I site (*) is methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with this enzyme, you will need to transform the vector into a *dam*⁻ and make fresh DNA.

Description

pEGFP-N1 encodes a red-shifted variant of wild-type GFP (1–3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP-N1 encodes the GFPmut1 variant (4) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (5). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-N1 is between the immediate early promoter of CMV ($P_{CMV\ IE}$) and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. The pEGFP-N1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

(PR29972; published 03 October 2002)

Clontech

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A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

Use

Fusions to the N terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo*. The target gene should be cloned into pEGFP-N1 so that it is in frame with the EGFP coding sequences, with no intervening in-frame stop codons. The inserted gene should include the initiating ATG codon. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (7). pEGFP-N1 can also be used simply to express EGFP 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
- MCS: 591–671
- Enhanced green fluorescent protein (EGFP) gene
  - Kozak consensus translation initiation site: 672–682
  - Start codon (ATG): 679–681; Stop codon: 1396–1398
  - Insertion of Val at position 2: 682–684
  - GFPmut1 chromophore mutations (Phe-64 to Leu; Ser-65 to Thr): 871–876
  - His-231 to Leu mutation (A→T): 1373
- SV40 early mRNA polyadenylation signal
  - Polyadenylation signals: 1552–1557 & 1581–1586; mRNA 3' ends: 1590 & 1602
- f1 single-strand DNA origin: 1649–2104 (Packages the noncoding strand of EGFP.)
- Bacterial promoterfor expression of Kan^r gene:
  - –35 region: 2166–2171; –10 region: 2189–2194
  - Transcription start point: 2201
- SV40 origin of replication: 2445–2580
- SV40 early promoter
  - Enhancer (72-bp tandem repeats): 2278–2349 & 2350–2421
  - 21-bp repeats: 2425–2445, 2446–2466 & 2468–2488
  - Early promoter element: 2501–2507
  - Major transcription start points: 2497, 2535, 2541 & 2546
- Kanamycin/neomycin resistance gene
  - Neomycin phosphotransferase coding sequences: start codon (ATG): 2629–2631; stop codon: 3421–3423
  - G→A mutation to remove *Pst* I site: 2811
  - C→A (Arg to Ser) mutation to remove *Bss*H II site: 3157
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  - Polyadenylation signals: 3659–3664 & 3672–3677
- pUC plasmid replication origin: 4008–4651

Primer Locations

- EGFP-N Sequencing Primer (#6479-1): 745–724
- EGFP-C Sequencing Primer (#6478-1): 1332–1353

Propagation in *E. coli*

- Suitable host strains: DH5a, HB101 and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as JM101 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. Prasher, D. C., *et al.* (1992) *Gene* 111:229–233.
2. Chalfie, M., *et al.* (1994) *Science* 263:802–805.
3. Inouye, S. & Tsuji, F. I. (1994) *FEBS Letters* 341:277–280
4. Cormack, B., *et al.* (1996) *Gene* 173:33–38.
5. Haas, J., *et al.* (1996) *Curr. Biol.* 6:315–324.
6. Kozak, M. (1987) *Nucleic Acids Res.* 15:8125–8148.
7. Gorman, C. (1985). In *DNA cloning: A practical approach, vol. II.* Ed. D.M. Glover. (IRL Press, Oxford, U.K.) 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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Please contact BD Biosciences Clontech directly for any other assistance, including purchasing and technical support. All companies and institutions purchasing Living Colors™ products will be included in a quarterly report to Aurora Biosciences, as required by the BD Biosciences Clontech/Aurora Biosciences license agreement.

Vector Backbone: pEBG

Vector Type:	Mammalian
Viral/Non-viral:	Non-viral
Promoter:	EF-1alpha
Backbone Size (bp):	6100
Tag:	GST (N terminal)
Bacteria Resistance:	Amp

Comments: Derived from pEF-BOS, BstXI-NotI stuffer fragment of pEF-BOS replaced with polylinker containing BamHI site, PCR to generate GST fragment from pGEX-2T with 5' BglII site and eukaryotic ribosome binding site and 3' BamHI site, inserted into BamHI site to generate pEBG.

More information: Mayer et al. 1995 Current Biology 5(3):296-305.

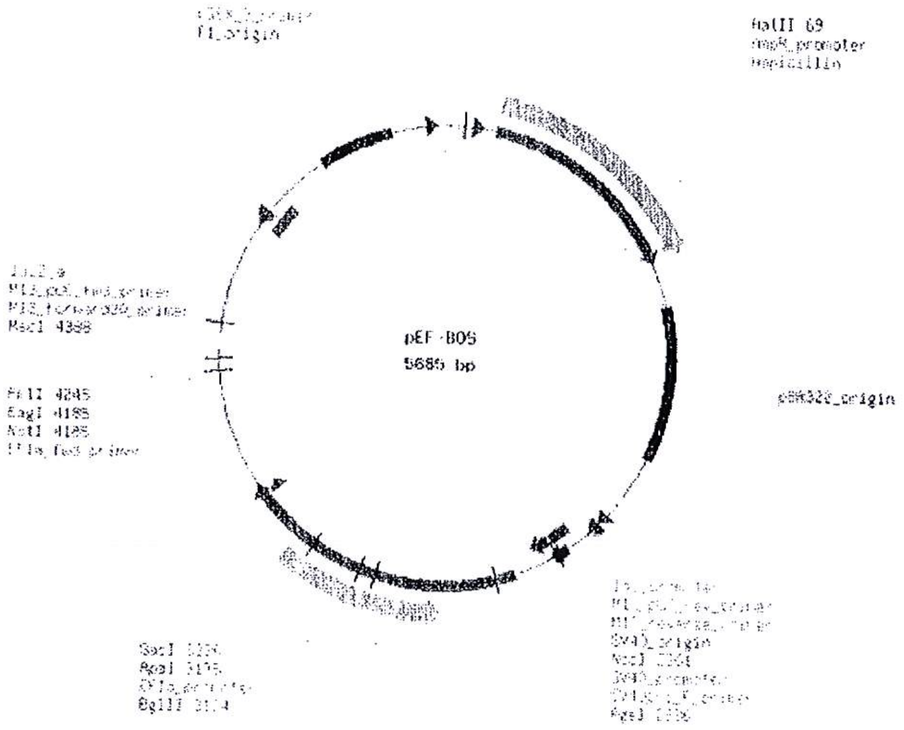
Vector Backbone: pEF-BOS

Vector Type:	Mammalian
Viral/Non-viral:	Non-viral
Backbone Size (bp):	5800
Bacteria Resistance:	Amp
Sequence and Map:	Sequence (Click to see features and cutters)

Comments: SV40 ori (311 bp), human EF-1alpha promoter (1.2 kb), stuffer fragment from CDM8 vector (450 bp, cDNA to be expressed can be inserted by removing the stuffer using BstXI or XbaI), poly-A from human G-CSF (700 bp Eco81I-EcoRI fragment), into HindIII-EcoRI site of pUC119.

More information: Mizushima and Nagata, Nucleic Acids Research, 18(17)5322.

Click on map to enlarge

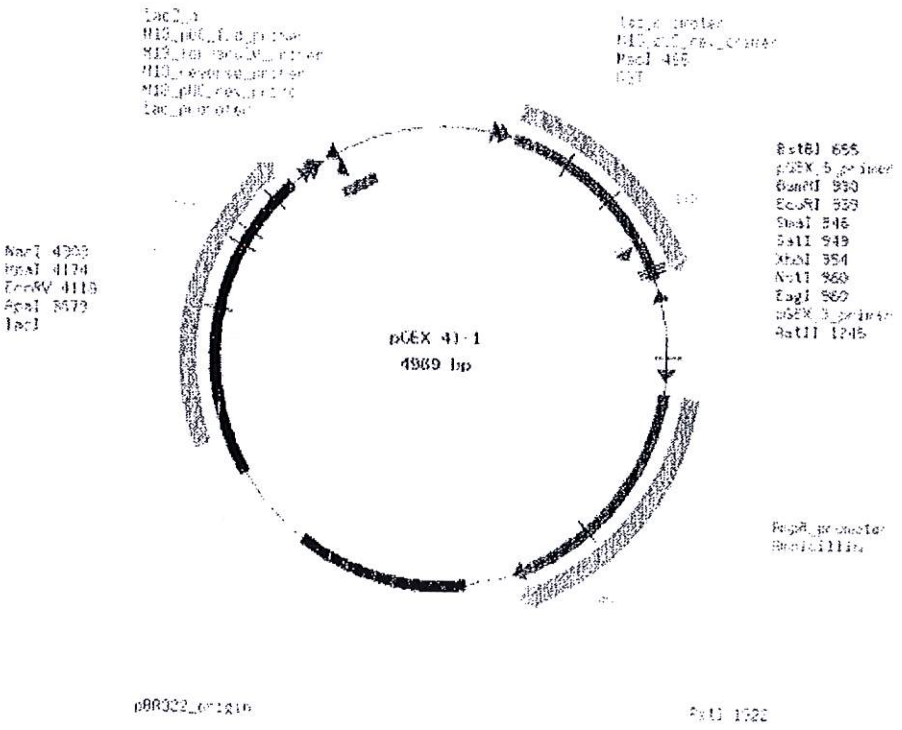


Vector Backbone: pGEX-4T-1

Vendor:	Amersham
Vector Type:	Bacterial
Viral/Non-viral:	Nonviral
Stable/Transient:	Transient
Constitutive/Inducible:	Constitutive
Promoter:	tac
Expression Level:	High (activate with IPTG)
Backbone Size (bp):	4900
Sequencing Primer:	pGEX Fwd
Sequencing Primer Sequence:	5'd[GGGCTGGCAAGCCACGTTTGGTG]3'
Tag:	GST (Nterm)
Bacteria Resistance:	Ampicillin
Mammalian Selection:	N/a
Catalog Number:	27-4580-01
Sequence and Map:	Sequence (Click to see features and cutters)

Comments: Thrombin cleavage site; can directly insert cDNA from lambda gt11 libraries

Click on map to enlarge



pGEX Vectors (GST Gene Fusion System)

- A tac promoter for chemically inducible, high-level expression.
- An internal lac I^q gene for use in any E. coli host.
- Very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on antigenicity and functional activity.
- PreScission™, thrombin, or Factor Xa protease recognition sites for cleaving the desired protein from the fusion product.

Page down for more information

Order Information				
Product	Pack size	Product Code	Price	Qty
Glutathione S-transferase Gene Fusion Vectors*				
PGEX-5X-3	1 EA	27-4586-01	CAN$630.00	

* All vectors include E. coli BL21 cells. Additional informat on about vectors is found at www.gelifesciences.com/pGEX.
* All vectors include E. coli BL21 cells.

All of the GST gene fusion vectors offer:

Map of the glutathione S-transferase fusion vectors showing the reading frames and main features. Even though stop codons in all three frames are not depicted in this map, all thirteen vectors have stop codons in all three frames downstream from the multiple cloning site.

pGEX Vectors (GST Gene Fusion System)

Technical Information

Thirteen pGEX vectors are available (see figure). Nine of the vectors have an expanded multiple cloning site (MCS) that contains six restriction sites. The expanded MCS facilitates the unidirectional cloning of cDNA inserts obtained from libraries constructed using many available lambda vectors. pGEX-6P-1, pGEX-6P-2, and pGEX-6P-3 each encode the recognition sequence for site-specific cleavage by PreScission™ Protease. (see PreScission Protease) between the GST domain and the multiple cloning site. pGEX-4T-1, pGEX-4T-2, and pGEX-4T-3 are derived from pGEX-2T and contain a thrombin protease recognition site. pGEX-5X-1, pGEX-5X-2, and pGEX5X-3 are derivatives of pGEX-3X and possess a factor Xa protease recognition site.

Download the pGEX sequence map in PDF format. For ASCII format please scoll down.

pGEX-2TK is uniquely designed to allow the detection of expressed proteins by directly labeling the fusion products *in vitro* (1). This vector contains the recognition sequence for the catalytic subunit of cAMP-dependent protein kinase obtained from heart muscle. The protein kinase site is located between the GST domain and the MCS. Expressed proteins can be directly labeled using protein kinase and [γ-^{32}P]ATP and readily detected using standard radiometric or autoradiographic techniques. pGEX-2TK is a derivative of pGEX-2T; its fusion proteins can be cleaved with thrombin.

Cleavage of pGEX-6P GST fusion proteins occurs between the Gln and Gly residues of the recognition sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro (2). Low temperature (5°C) digestion minimizes the degradation of the protein of interest. Because PreScission™ Protease has been engineered with a GST-tag, it can also be removed from the cleavage mixture simultaneously with the GST portion of the fusion protein. The pGEX-6P Expression Vectors permit convenient site-specific cleavage and simultaneous purification on Glutathione Sepharose™. The pGEX-6P series provides all three translational reading frames linked between the GST coding region and the multiple cloning site.

Collectively, the pGEX vectors provide all three translational reading frames beginning with the EcoR I restriction site. pGEX-1λT, pGEX-6P-1, pGEX-4T-1, and pGEX-5X-1 can directly accept and express cDNA inserts isolated from λ gt11 libraries.

Vector	Unformatted	Formatted	GenBank Accession No.	
pGEX-1 lambda T, 27-4805-01	ASCII	PDF	U13849	
pGEX-2T, 27-4801-01	ASCII	PDF	U13850	
pGEX-2TK, 27-4587-01	ASCII	PDF	U13851	
pGEX-3X, 27-4803-01	ASCII	PDF	U13852	
pGEX-4T-1, 27-4580-01	ASCII	PDF	U13853	
pGEX-4T-2, 27-4581-01	ASCII	PDF	PDF	U13855

pGEX-5X-1, 27-4584-01	ASCII	PDF	U13856
pGEX-5X-2, 27-4585-01	ASCII	PDF	U13857
pGEX-5X-3, 27-4586-01	ASCII	PDF	U13858
pGEX-6P-1, 27-4597-01	ASCII	PDF	U78872
pGEX-6P-2, 27-4598-01	ASCII	PDF	U78873
pGEX-6P-3, 27-4599-01	ASCII	PDF	U78874

Click on "ASCII" to download an unformatted sequence for use by a sequence analysis program. Click on "PDF" to download a formatted sequence and restriction site table. If you prefer accessing the sequence in *GenBank*, refer to the right-hand column for the GenBank accession number:

- **Expression:** Proteins are expressed as fusion proteins with the 26 kDa glutathione S-transferase (GST). The GST gene contains an ATG and ribosome-binding site, and is under control of the tac promoter. A translation terminator is provided in each reading frame. The resulting fusion protein may be purified using the GST Purification Module (27-4570-01, -02; see GST Purification Modules.)
- **Enzymatic cleavage with PreScission™ Protease:** pGEX-6P-1, -2, -3 allow for removal of the GST carrier protein from the fusion protein by enzymatic cleavage with PreScission™ Protease. Because PreScission™ Protease has been engineered with a GST-tag, it can also be removed simultaneously with the GST portion of the fusion protein.
- **Enzymatic cleavage with thrombin:** pGEX-1 lambda T, pGEX-2T, pGEX-2TK, pGEX-4T-1, -2, -3 allow for removal of the GST carrier protein from the fusion protein by enzymatic cleavage with thrombin.
- **Enzymatic cleavage with factor Xa:** pGEX-3X, pGEX-5X-1, -2, -3 allow for removal of the GST carrier protein from the fusion protein by enzymatic cleavage with factor Xa.
- **Direct labeling *in vitro*:** pGEX-2TK allows for direct labeling of fusion proteins in vitro with 32P using the catalytic subunit of cAMP-dependent protein kinase.
- **Host(s):** *E. coli*. The plasmid provides lac Iq repressor.
- **Selectable marker(s):** Plasmid confers resistance to 100 µg/ml ampicillin.
- **Amplification:** Recommended.

Properties of pGEX Vectors • Induction: tac promoter inducible with 1-5 mM IPTG.

• pGEX-1 Lambda T Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211: -35: 183-188: *lac* operator: 217-237: Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
- MCS: 930-944
- Beta-lactamase gene region: Promoter: -10: 1308-1313; -35: 1285-1290; Start codon (ATG): 1355; Stop codon (TAA): 2213
- *lacIq* gene region: Start codon (GTG): 3296; Stop codon (TGA): 4376
- Plasmid replication region: Site of replication initiation: 2973; Region necessary for replication: 2280-2976
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1019-997

• pGEX-2T Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211: -35: 183-188: *lac* operator: 217-237: Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
- MCS: 930-945
- Beta-lactamase gene region: Promoter: -10: 1309-1314; -35: 1286-1291; Start codon (ATG): 1356; Stop codon (TAA): 2214
- *lacIq* gene region: Start codon (GTG): 3297; Stop codon (TGA): 4377
- Plasmid replication region: Site of replication initiation: 2974; Region necessary for replication: 2281-2977
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1020-998

• pGEX-2TK Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211: -35: 183-188: *lac* operator: 217-237: Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935:
- Coding for kinase recognition site: 936-950
- MCS: 951-966
- Beta-lactamase gene region: Promoter: -10: 1330-1335; -35: 1307-1312: Start codon (ATG): 1377; Stop codon (TAA): 2235
- *lacIq* gene region: Start codon (GTG): 3318; Stop codon (TGA): 4398
- Plasmid replication region: Site of replication initiation: 2995: Region necessary for replication: 2302-2998

Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1041-1019

• pGEX-3X Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211: -35: 183-188: *lac* operator: 217-237: Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258: Coding region for Factor Xa cleavage: 921-932
- MCS: 934-949
- Beta-lactamase gene region: Promoter: -10: 1313-1318: -35: 1290-1295: Start codon (ATG): 1360; Stop codon (TAA): 2218
- *lacIq* gene region: Start codon (GTG): 3301; Stop codon (TGA): 4381
- Plasmid replication region: Site of replication initiation: 2978: Region necessary for replication: 2285-2981
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1024-1002

• pGEX-4T-1 Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211: -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
- MCS: 930-966
- Beta-lactamase gene region: Promoter: -10: 1330-1335: -35: 1307-1312: Start codon (ATG): 1377; Stop codon (TAA): 2235
- *lacIq* gene region: Start codon (GTG): 3318: Stop codon (TGA): 4398
- Plasmid replication region: Site of replication initiation: 2995; Region necessary for replication: 2302-2998
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1041-1019

• pGEX-4T-2 Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
- MCS: 930-967
- Beta-lactamase gene region: Promoter: -10: 1331-1336: -35: 1308-1313; Start codon (ATG): 1378; Stop codon (TAA): 2236
- *lacIq* gene region: Start codon (GTG): 3319; Stop codon (TGA): 4399
- Plasmid replication region: Site of replication initiation: 2996; Region necessary for replication: 2303-2959
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1042-1020

• pGEX-4T-3 Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211; -35: 183-188: *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
- MCS: 930-965
- Beta-lactamase gene region: Promoter: -10: 1329-1334; -35: 1306-1311: Start codon (ATG): 1376; Stop codon (TAA): 2234
- *lacIq* gene region: Start codon (GTG): 3317: Stop codon (TGA): 4397
- Plasmid replication region: Site of replication initiation: 2994: Region necessary for replication: 2301-2997
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1040-1018

• pGEX-5X-1 Control Regions:
- Glutathione S-transferase gene region: *lac* promoter: -10: 205-211; -35 183-188: *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921-932
- MCS: 934-969
- Beta-lactamase gene region: Promoter: -10: 1333-1338: -35: 1310-1315; Start codon (ATG): 1380; Stop codon (TAA): 2238
- *lacIq* gene region: Start codon (GTG): 3321: Stop codon (TGA): 4401
- Plasmid replication region: Site of replication initiation: 2998: Region necessary for replication: 2305-3001
- Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1044-1022

- **pGEX-5X-2 Control Regions:**
  - Glutathione S-transferase gene region: *lac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921-932
  - MCS: 934-970
  - Beta-lactamase gene region: Promoter: -10: 1334-1339; -35: 1311-1316; Start codon (ATG): 1381; Stop codon (TAA): 2239
  - *lacIq* gene region: Start codon (GTG): 3322; Stop codon (TGA): 4402
  - Plasmid replication region: Site of replication initiation: 2999; Region necessary for replication: 2306-3002
  - Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1045-1023

- **pGEX-5X-3 Control Regions:**
  - Glutathione S-transferase gene region: *lac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921-932
  - MCS: 934-971
  - Beta-lactamase gene region: Promoter: -10: 1335-1340; -35: 1312-1317; Start codon (ATG): 1382; Stop codon (TAA): 2240
  - *lacIq* gene region: Start codon (GTG): 3323; Stop codon (TGA): 4403
  - Plasmid replication region: Site of replication initiation: 3000; Region necessary for replication: 2307-3003
  - Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1046-1024

References

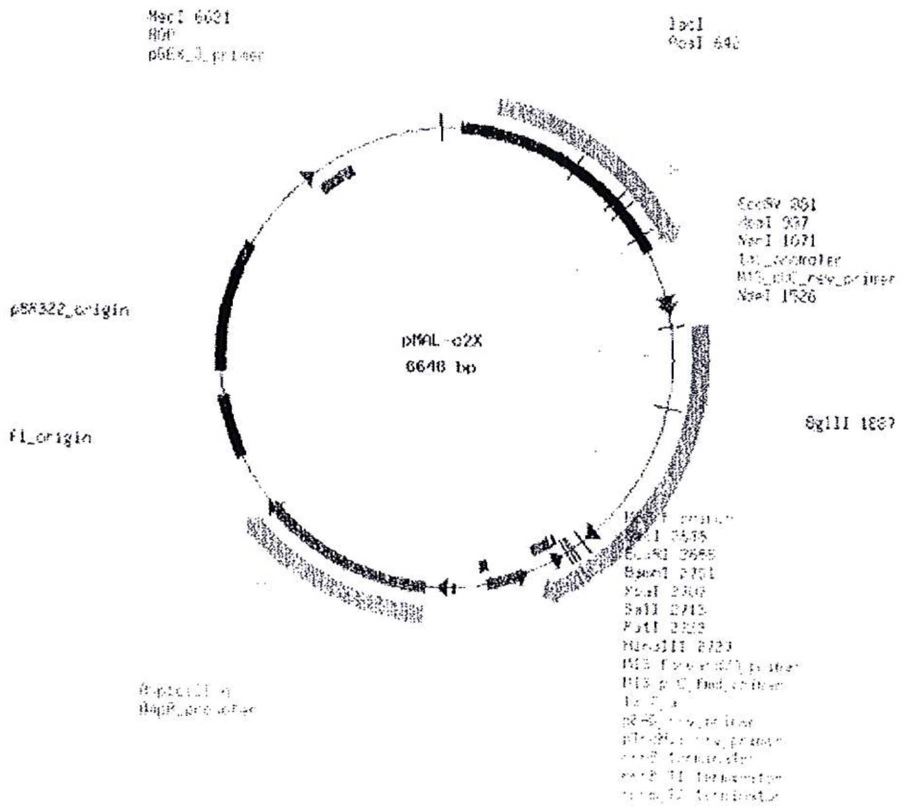
1. Kaelin, W.G. *et al* *Cell 70*, 351 (1992).

Vector Backbone: pMAL-c2X

Vendor: New England Biolabs
Vector Type: Bacterial
Promoter: P-lac
Backbone Size (bp): 6700
Tag: Maltose-binding protein, MBP (Nterm)
Bacteria Resistance: Ampicillin
Catalog Number: N8077S
Sequence and Map: Sequence (Click to see features and cutters)

Comments: Maltose-binding protein fusion cleaved by Factor Xa. Goes to periplasm.

Click on map to enlarge



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BioLabs Inc.

New England Biolabs
240 Country Road
Ipswich, MA 01938

MATERIAL SAFETY DATA SHEET

Telephone: (978)927-5054
Toll free: 1-800-632-5227
Fax: (978)921-1350
e-mail: info@neb.com

Msds Revision Date: 5/08

Vector
#N8076

SECTION 1 - PRODUCT

Product Name: pMAL-c2X

SECTION 2– CHEMICAL INFORMATION

1.	Tris-HCl	< 1%	Cas.	#77-86-1
2.	EDTA	< 1%	Cas.	#60-00-4

SECTION 3–HAZARDOUS IDENTIFICATION

NAME OF CHEMICAL: Factor X from Bovine Plasma Cas No. #9001-29-0 SARA 313: NO

HMIS Rating
Health: 0
Flammability: 0
Reactivity: 0

NFPA Rating
Health: 0
Flammability: 0
Reactivity: 0

SECTION 4 –FIRST AID MEASURES

ORAL EXPOSURE: If swallowed, wash out mouth with water provided person is conscious. Call a physician.

INHALATION EXPOSURE: If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.

DERMAL EXPOSURE: In case of contact, immediately wash skin with soap and copious amounts of water.

EYE EXPOSURE: In case of contact, immediately flush eyes with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

SECTION 5–FIRE FIGHTING MEASURES

Extinguishing Media: Water spray.
Carbon Dioxide, dry chemical powder or appropriate foam.

Special Firefighting Procedures: Wear self contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

Unusual Fire and Explosions Hazard (s):
Emits toxic fumes under fire conditions.

Flash point: N/A

Flammability: N/A

Autoignition Temp: N/A

SECTION 6 –ACCIDENTAL RELEASE MEASURES

PROCEDURE(S) OF PERSONAL PRECAUTION(S):

Exercise appropriate precautions to minimize direct contact with skin or eyes and prevent inhalation.

METHODS FOR CLEANING UP:

Absorb on sand or vermiculite and place in closed containers for disposal. Ventilate area and wash spill site after material pickup is complete.

Flush spill area with copious amounts of water.

SECTION 7– HANDLING AND STORAGE

Handling:

User Exposure: Avoid Inhalation.

Avoid contact with eyes, skin and clothing.

Avoid prolonged or repeated exposure.

Storage:

Keep tightly closed.

SECTION 8 – EXPOSURE CONTROLS/PPE

Engineering Controls: Safety shower and eye bath. Mechanical exhaust required.

Personal Protective Equipment:

Respiratory

Hand:
Compatible chemical-resistant gloves.

Eye:
Chemical safety goggles.

General Hygiene Measures:

Wash hands thoroughly after handling.

Wash contaminated clothing before use.

SECTION 9 - PHYSICAL AND CHEMICAL PROPERTIES

Appearance: **Physical State: Liquid**

N/A = not available

Property	Value
Molecular Weight:	NA
pH:	NA
BP/BP Range:	NA
MP/MP Range:	NA
Freezing Point:	NA
Vapor Pressure:	NA
Vapor Density:	NA
Saturated Vapor:	NA
SG/Density:	NA
Bulk Density:	NA
Odor Threshold:	NA
Volatile %:	NA
Voc Content:	NA
Water Content:	NA
Solvent Content:	NA
Evaporation Rate:	NA
Viscosity:	NA
Surface Tension:	NA
Partition Coefficient:	NA
Decomposition Temp:	NA
Flash Point:	NA
Explosion Limits:	NA
Flammability:	NA
Autoignition Temp:	NA
Refraction Index:	NA
Optical Rotation:	NA
Miscellaneous Data:	NA
Sollubility in Water:	NA

SECTION 10 - STABILITY AND REACTIVITY

Stability: Stable

Materials to avoid: Strong oxidizing agents

Hazardous Polymerization: Will not occur

Hazardous Decomposition Products:
Nature of decomposition products not known.

SECTION 11- TOXICOLOGICAL INFORMATION

Route of Exposure:

Skin Absorption: May be harmful if absorbed through the skin.
Skin Contact: May cause skin irritation.
Eye Contact: May cause eye irritation.
Inhalation: May be harmful if inhaled.
Material may be irritating to mucous membranes and upper respiratory tract.
Ingestion: May be harmful if swallowed.

Sign and Symptoms of Exposure:
To the best of our knowledge, the chemical, physical and toxicological properties have not been thoroughly investigated.

SECTION 12- ECOLOGICAL INFORMATION

Data Not yet Available

SECTION 13- DISPOSAL CONSIDERATIONS

Appropriate Method of Disposal of Substance or Preparation

Contact a licensed professional waste disposal service to dispose of this material.

Dissolve or mix the material with a combustable solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

Observe all federal, state and local environmental regulations.

SECTION 14- TRANSPORT INFORMATION

DOT

Proper Shipping Name: None

Non-hazardous for Transport: This substance is considered to be non-hazardous for transport

IATA

Non-Hazardous for Transport: Non-hazardous for air transport.

SECTION 15–REGULATORY INFORMATION

DISCLAIMER: For R&D use only. Not for drug, household or other uses.
WARRANTY: The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Inc., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

SECTION 16–OTHER INFORMATION

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide.

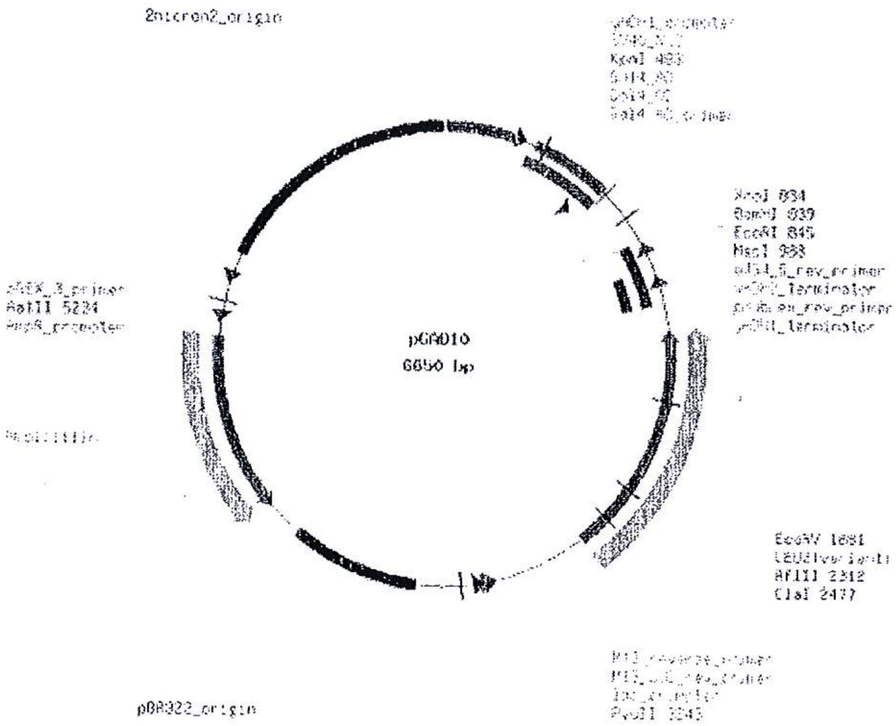
New England Biolabs shall not be held liable for any damage resulting from handling or from contact with the above product

Vector Backbone: pGAD10

Backbone Size (bp): 6650
GenBank Accession Number: U13188
Sequence and Map: Sequence (Click to see features and cutters)

Comments: NCBI gi: 532698 Hosts: E.coli. (Information source: VectorDB.)

Click on map to enlarge



Vector Backbone: pAS2

Vendor: ATCC
Backbone Size (bp): 8500
Catalog Number: 87008

Comments: Restriction digests of the clone give the following sizes (kb): BamHI--8.6; SalI--8.6; SmaI--8.6. (ATCC staff) Shuttle expression vector used to create fusion proteins consisting of the nuclear localization sequence from SV40 T antigen, the GAL4 DNA-binding domain (aa 1-147), and a HA (hemagglutinin) epitope tag in frame with the activation domain. [1] The order of the major features in this plasmid is: ADC1 (ADH) promoter -> - GAL4 DNA binding domain - HA - NdeI/MCS/SalI - ADC1 terminator - pMB1 ori - ampR - 2 micron ori - TRP1 -> - f1 ori - <- CYH2. [2] Growth: LB plus ampicillin (ATCC medium number 1227) 37C Deposited by: Elledge S.J. Hosts: E.coli, yeast, Saccharomyces cerevisiae. (Information source: VectorDB.)

	cDNA	**Vector**
1	B-arrestin1	pRK5
2	B-arrestin1-GFP	pEGFP-N3
3	B-arrestin1-YFP	pEYFP-N3
4	B-arrestin2-Flag	pcDNA3 zeo (+)
5	B-arrestin 2 -GFP	pGFP-N3
6	B-arrestin2-YFP	pEYFP-N3
7	Barr1-Rluc	pRluc-N2
8	Barr2-Rluc	pRluc-N2
9	Barr1-EGFP	pEGFP-N3
10	Barr2-EGFP	pEGFP-N3
11	B2AR-GFP	pEGFP-N1
12	Flag-B2AR	pcDNA1-amp
13	CRF1α	pcDNA3.1+
14	HA-CRF1α	pcDNA3.1+
15	CRF1α-CFP	pECFP-NI
16	CRF1α-GFP	pEGFP-N1
17	CRFIα-YFP	pEYFP-NI
18	CRF1α-Rluc	pRluc-N3
19	FLAG-5HT2a	?
20	FLAG-AT1R	pcDNA3.1+
21	GRK2	pcDNA3-amp
22	Ral	pcDNA3
23	Ral G23V	pcDNA3
24	Ral S38N	pcDNA3
25	PLCd1-GFP	pEGFP-C1
26	Rab5	pcDNA1-amp
27	Rab5-S34N	pcDNA1-amp
28	Rab5-Q79L	pcDNA1-amp
29	GFP-Rab5	pEGFP-C2
30	GFP-Rab5-S34N	pEGFP-C2
31	GFP-Rab5-Q79L	pEGFP-C2
32	GFP-Rab4	pEGFP-C2
33	GFP-Rab4-N121I	pEGFP-C2
34	GFP-Rab4-Q67L	pEGFPC2L2
35	GFP-Rab4-S22N	pEGFPC2L2
36	GFP-Rab7	pEGFP-C2-L2
37	GFP-Rab7-N125I	pEGFP-C1
38	GFP-Rab7-Q67L	pEGFP-C1
39	GFP-Rab11	pEGFP-C2link2
40	GFP-Rab11-S25N	pEGFP-C2link2
41	GFP-Rab11-Q70L	pEGFP-C2link2
42	GFP-Rab11-N124I	pEGFP-C2link2
43	mGluR1a-Flag	pcDNA1-amp
44	mGluR1b	pcDNA1
45	mGluR5-Flag	pcDNA1-amp
46	spinophilin-GFP	pEGFP-N3

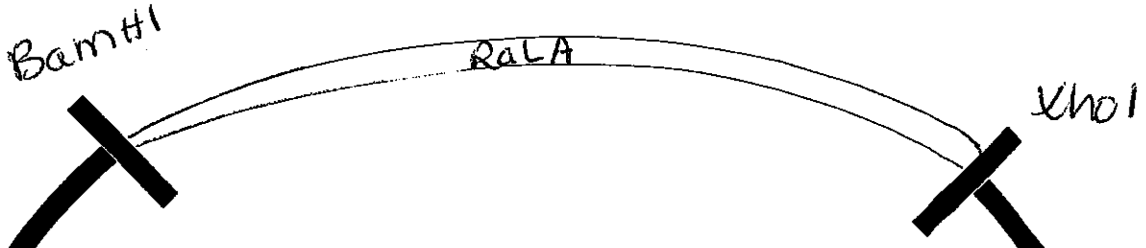
cDNA Inventory Data Sheet

CONSTRUCT: Ral pcdna 3 # 1

Species: human

Cloning Sites: 5' BamH1 Date: Oct 2000

3' xho1



Plasmid

Vector: pcdna3

Expression: euk

Selection: Amp Insert Size: 621bp

Epitope Tag: — Mutation: —

Source: Invitrogen (vector)

Modifications:

STORAGE

Working Stock:

Frozen Stock:

Glycerol Stock:

Open Reading Frame:

5' _

3' _

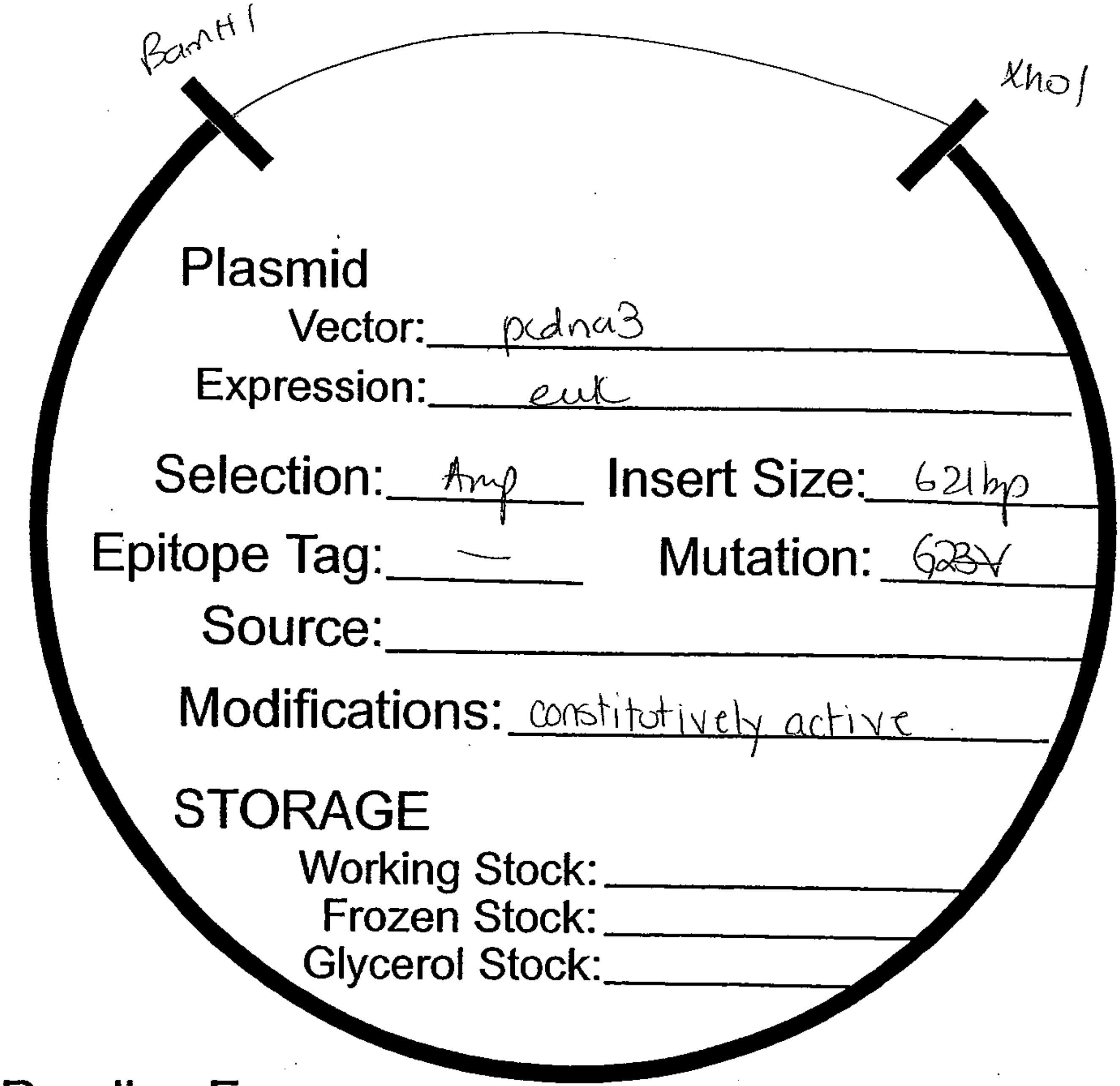
cDNA Inventory Data Sheet

CONSTRUCT: G23V Ral pcdna3 # 2

Species: human

Cloning Sites: 5' BamH1 Date: Oct 2000

3' Xho1



Plasmid

Vector: pcdna3

Expression: euk

Selection: Amp Insert Size: 621bp

Epitope Tag: — Mutation: G23V

Source:

Modifications: constitutively active

STORAGE

Working Stock:

Frozen Stock:

Glycerol Stock:

Open Reading Frame:

5' _

3' _

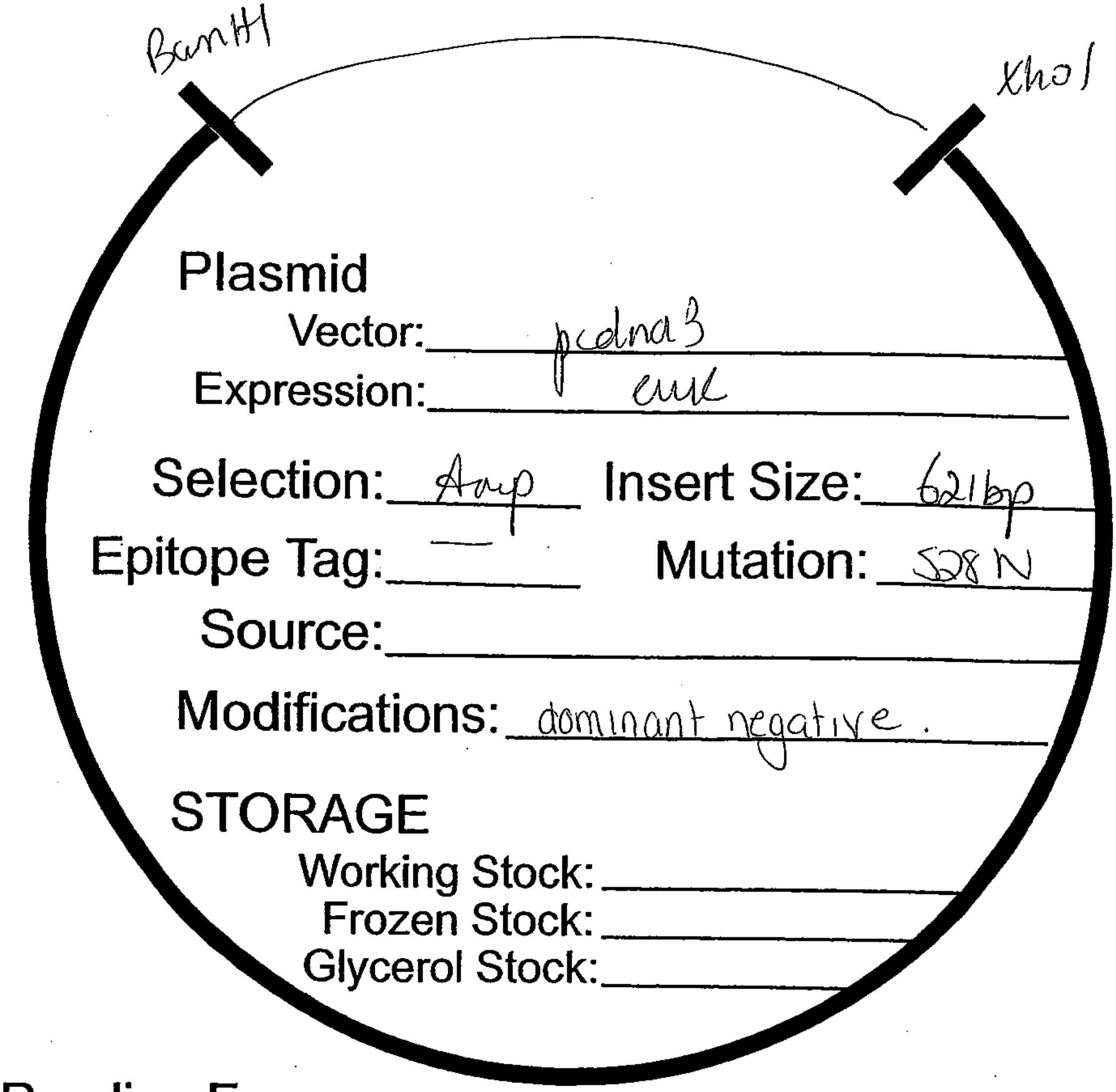
cDNA Inventory Data Sheet

CONSTRUCT: S28N pcdna3 # 3

Species: human

Cloning Sites: 5' BamH1 Date: Oct/2000

3' XhoI



Plasmid

Vector: pcdna3

Expression: cmv

Selection: Amp Insert Size: 621bp

Epitope Tag: — Mutation: S28N

Source:

Modifications: dominant negative.

STORAGE

Working Stock:

Frozen Stock:

Glycerol Stock:

Open Reading Frame:

5' -

3' -

cDNA Inventory Data Sheet #3

cDNA Information:

Name: GFP-Rab4-wt

Species: Human

Cloning Sites: 5' - HindIII
3' -

Insert Size: ~760bp

Mutation(s):

Epitope Tag: GFP

cDNA Source: ?

Prepared by: F. Ribeiro

Vector Information:

Name: pEGFP-C2

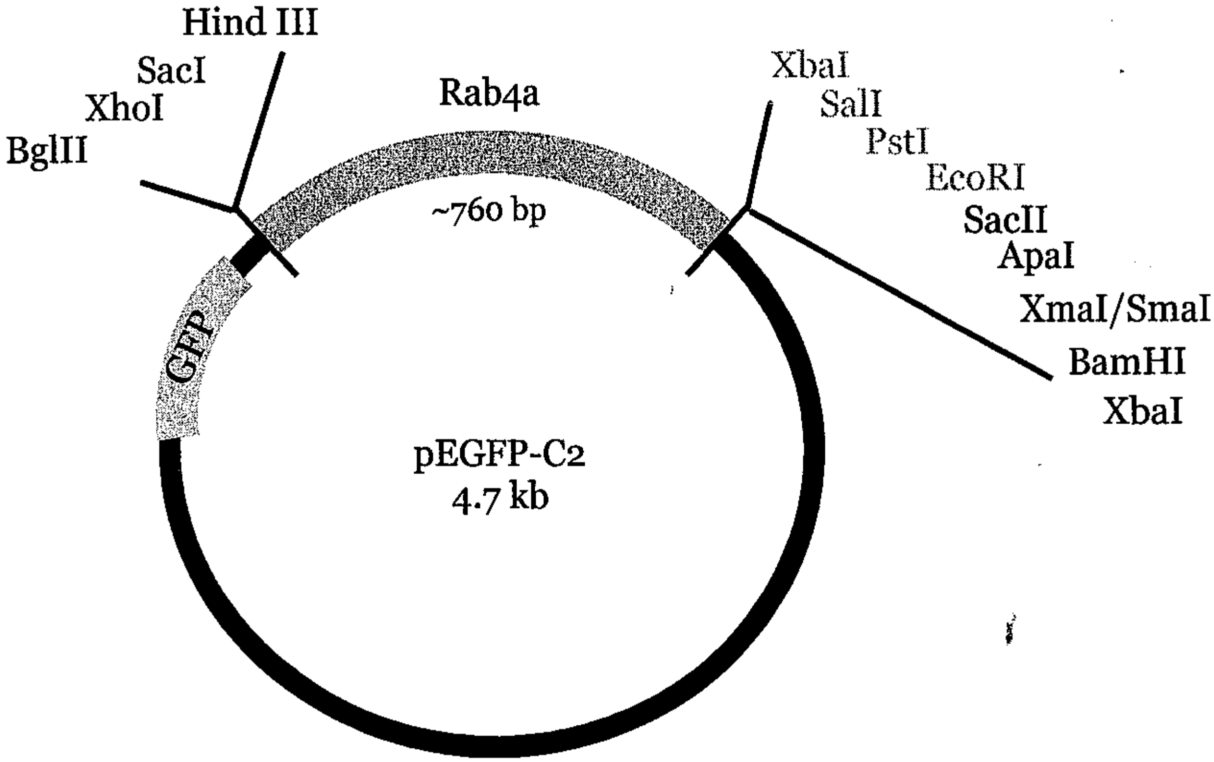
Vector Source: Clontech

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information: 1st 5 amino acids of Rab4 missing



cDNA Inventory Data Sheet #4

cDNA Information:

Name: GFP-Rab4-N121I

Species: Human

Cloning Sites: 5' - HindIII
3' -

Insert Size: ~850bp

Mutation(s): N121I

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2

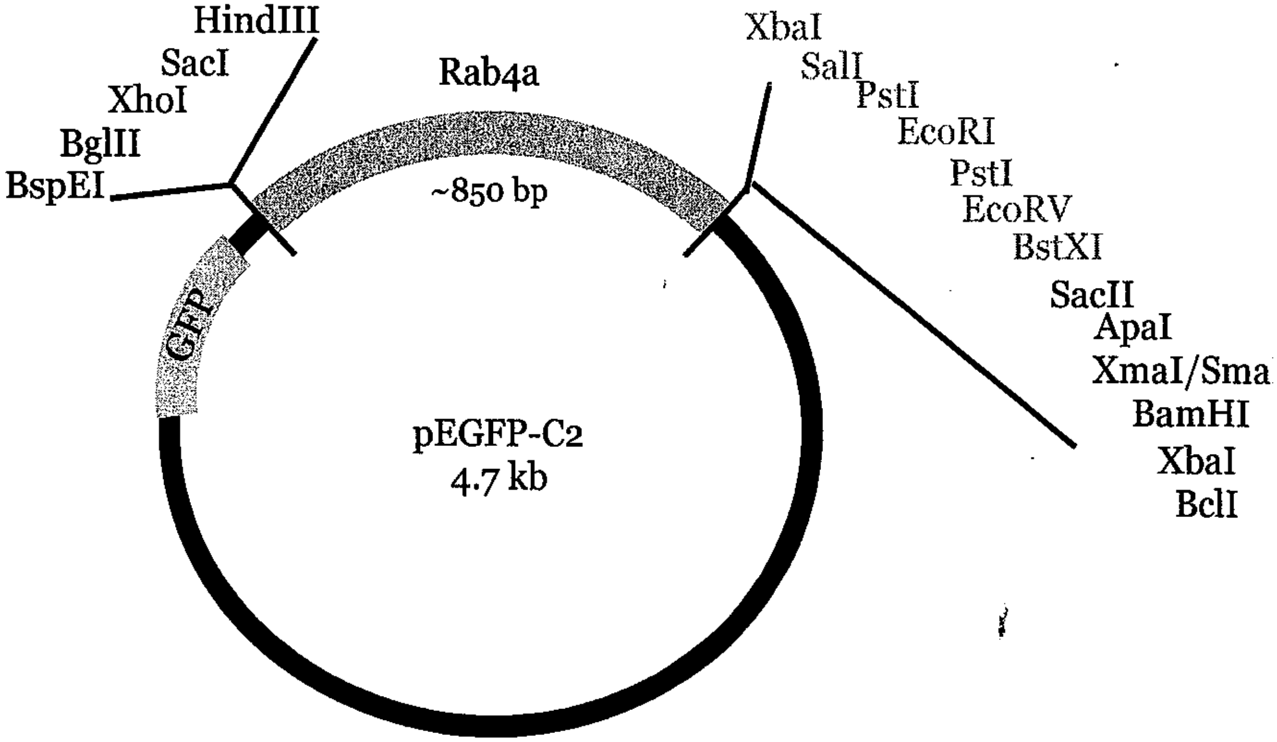
Vector Source: Clontech

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information: 1st 5 amino acids of Rab4 missing



cDNA Inventory Data Sheet

#5

cDNA Information:

Name: GFP-Rab4-Q67L

Species: Human

Cloning Sites: 5' - BamHI/BglII
3' -NotI

Insert Size: ~760bp

Mutation(s): Q67L

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2-Link2

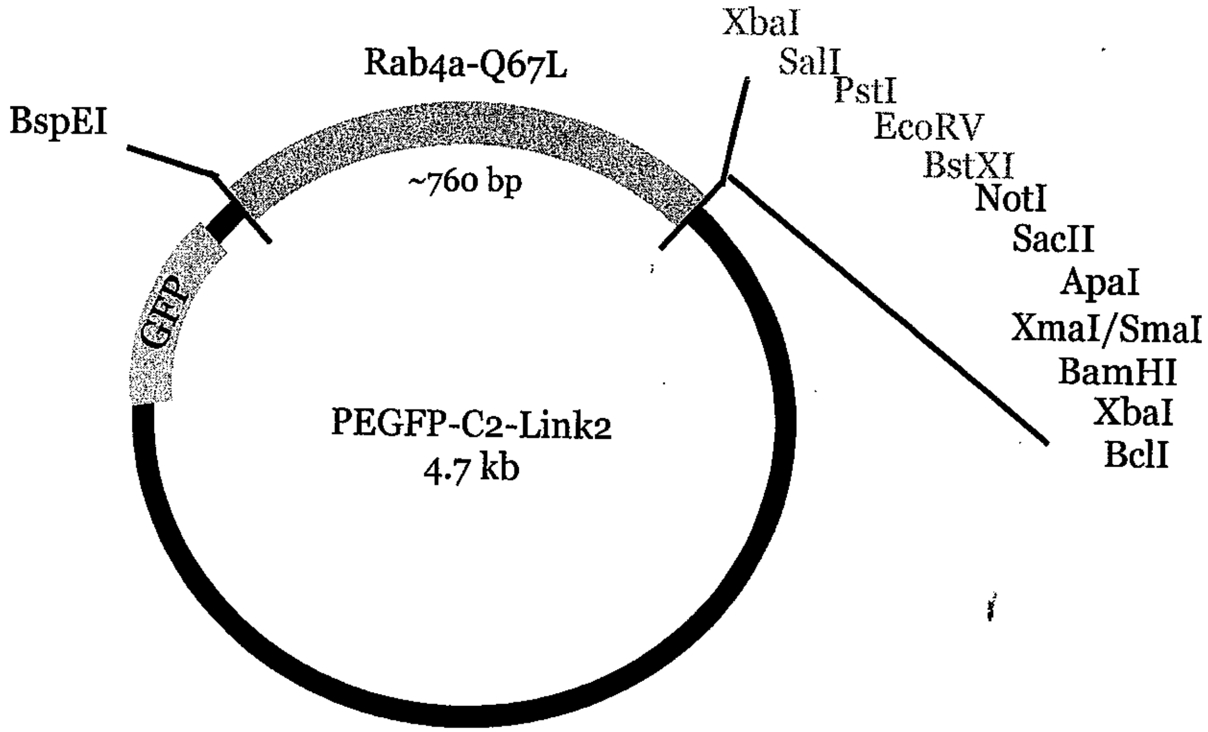
Vector Source: Clontech/Modified by P.Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: **5'** - EGFP-C **3'** -

Additional Information: 1st 5 amino acids of Rab4 missing



cDNA Inventory Data Sheet #6

cDNA Information:

Name: GFP-Rab4-S22N

Species: Human

Cloning Sites: 5' - BamHI/BglII
3' - NotI

Insert Size: ~820bp

Mutation(s): S22N

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2-L2

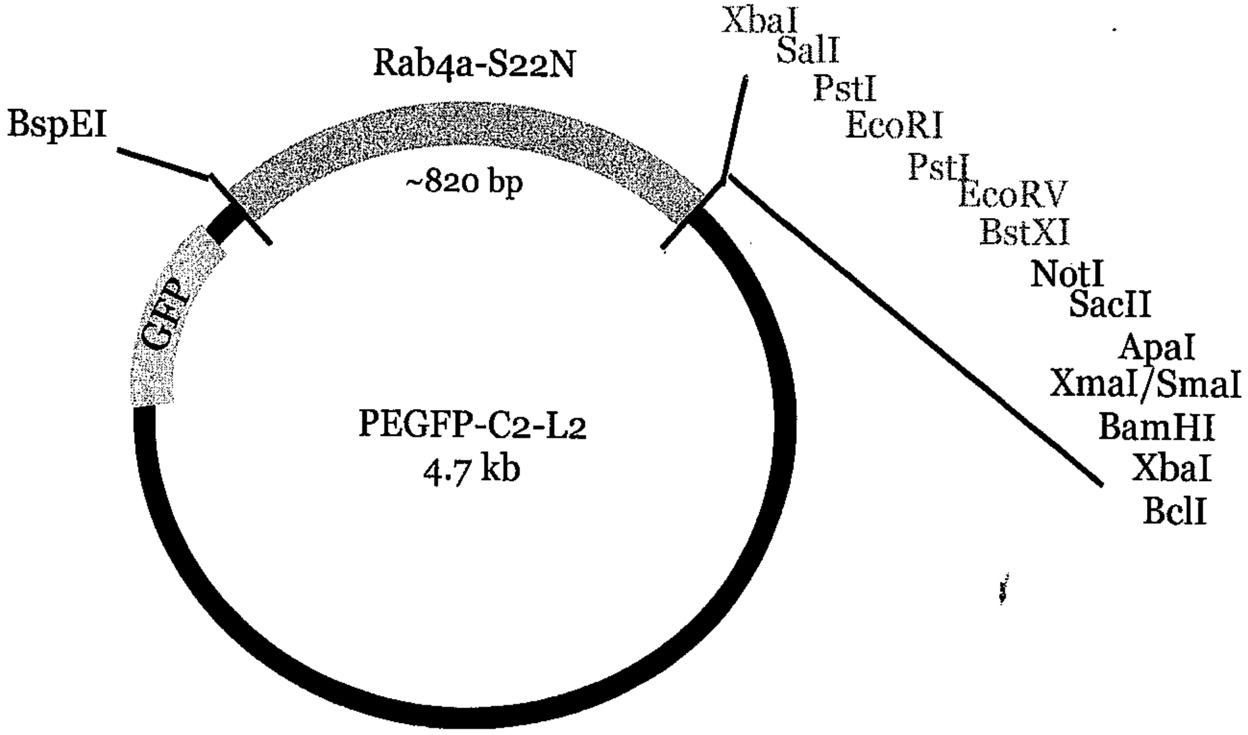
Vector Source: Clontech/P. Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information: 1st 5 amino acids of Rab4 missing



cDNA Inventory Data Sheet #1

cDNA Information:

Name: Rab5-wt

Species: Dog

Cloning Sites: 5' - EcoRI
3' - XhoI ?

Insert Size: ~650bp + 3'UTR

Mutation(s):

Epitope Tag:

cDNA Source: ?

Prepared by: P.Anborgh

Vector Information:

Name: pcDNA1

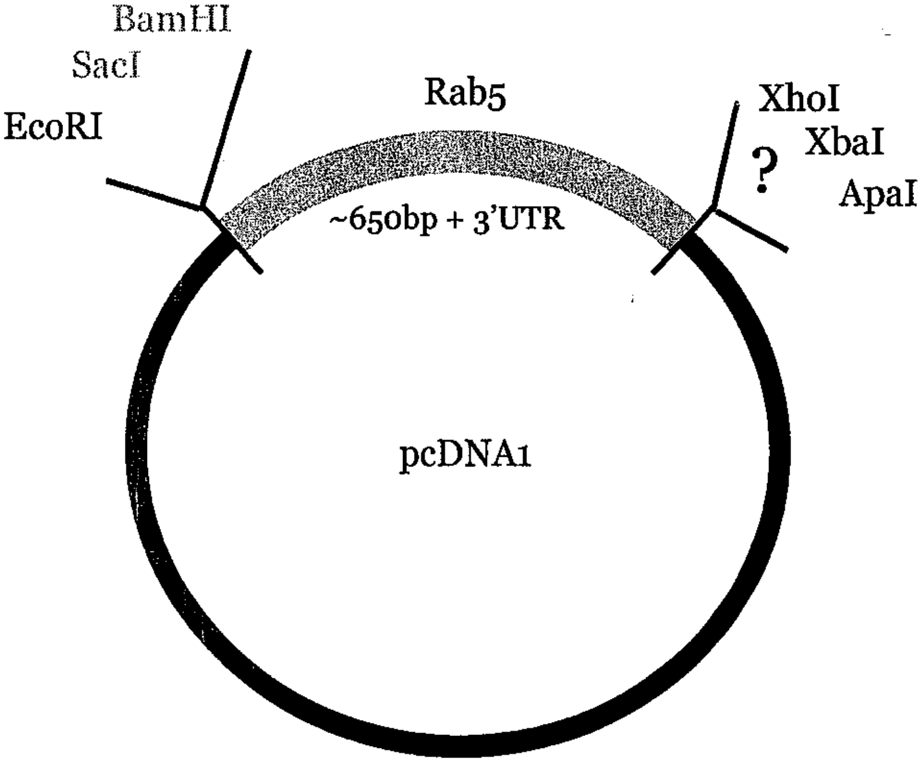
Vector Source: Invitrogen

Selection: amp

Expression: mammalian

Sequencing Primers: **5'** -T7 **3'** - SP6

Additional Information: large 3'UTR (~700bp)



cDNA Inventory Data Sheet #2

cDNA Information:

Name: Rab5-S34N

Species: Dog

Cloning Sites: 5' - EcoRI
3' - XhoI ?

Insert Size: ~650bp + 3'UTR

Mutation(s): S34N

Epitope Tag:

cDNA Source: ?

Prepared by: P.Anborgh

Vector Information:

Name: pcDNA1

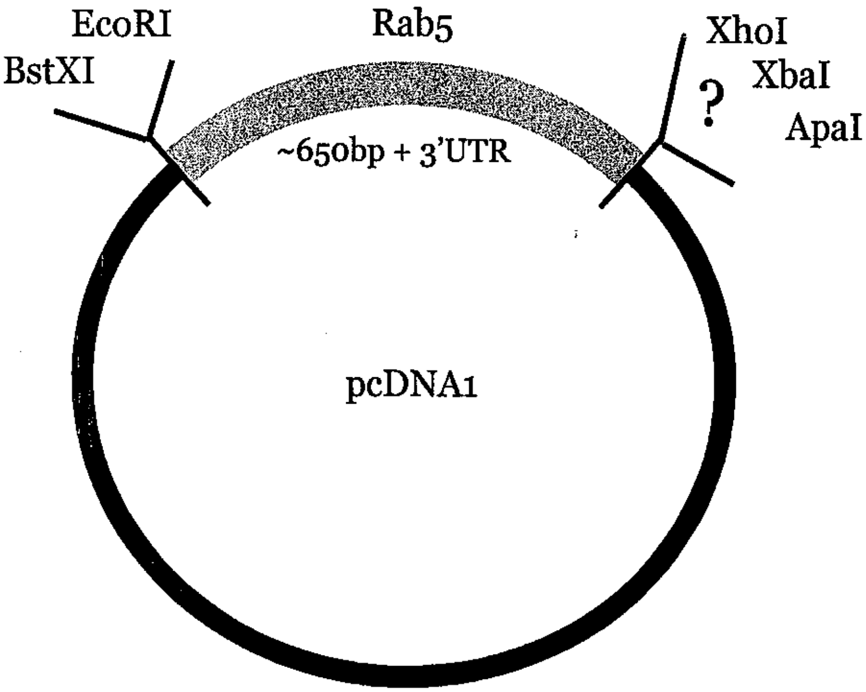
Vector Source: Invitrogen

Selection:amp

Expression: mammalian

Sequencing Primers: 5' -T7 3' - SP6

Additional Information: large 3'UTR (~700bp)



cDNA Inventory Data Sheet #3

cDNA Information:

Name: Rab5-Q79L

Species: Dog

Cloning Sites: 5' - EcoRI
3' - XhoI ?

Insert Size: ~650bp + 3'UTR

Mutation(s): Q79L

Epitope Tag:

cDNA Source: ?

Prepared by: P.Anborgh

Vector Information:

Name: pcDNA1

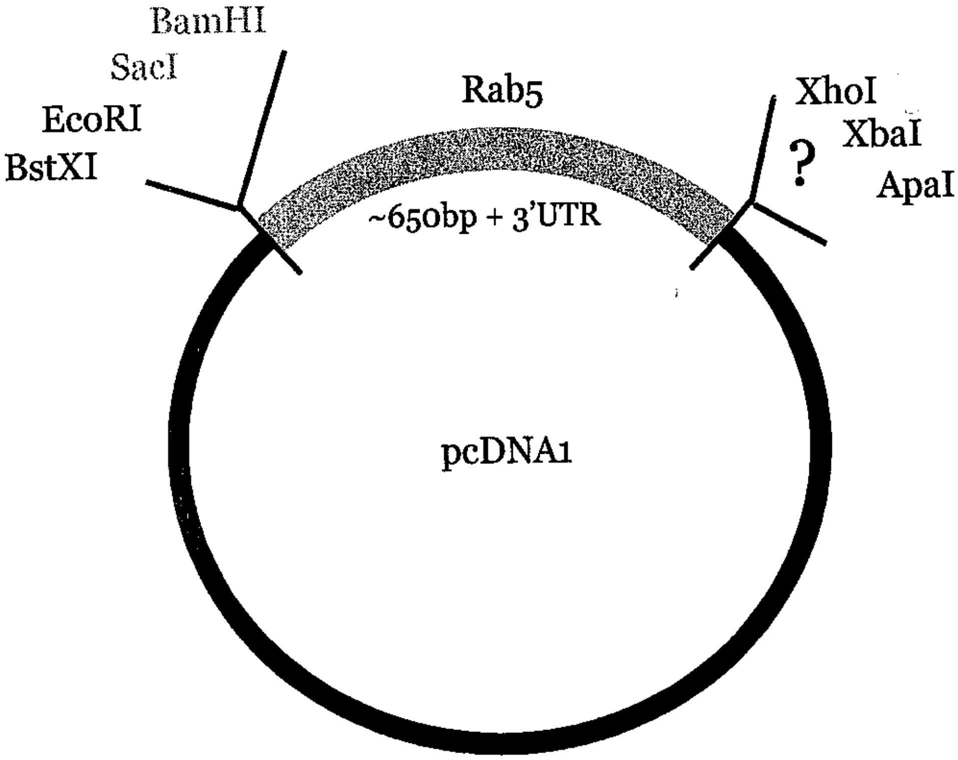
Vector Source: Invitrogen

Selection:amp

Expression: mammalian

Sequencing Primers: 5' -T7 3' - SP6

Additional Information: large 3'UTR (~700bp)



cDNA Inventory Data Sheet #4

cDNA Information:

Name: GFP-Rab5a-wt

Species: Dog

Cloning Sites: 5' - HindIII
3' - Sal I/XhoI

Insert Size: 1355bp
(~650bp Rab5 + 3' UTR)

Mutation(s):

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2

Vector Source:

Selection: kan

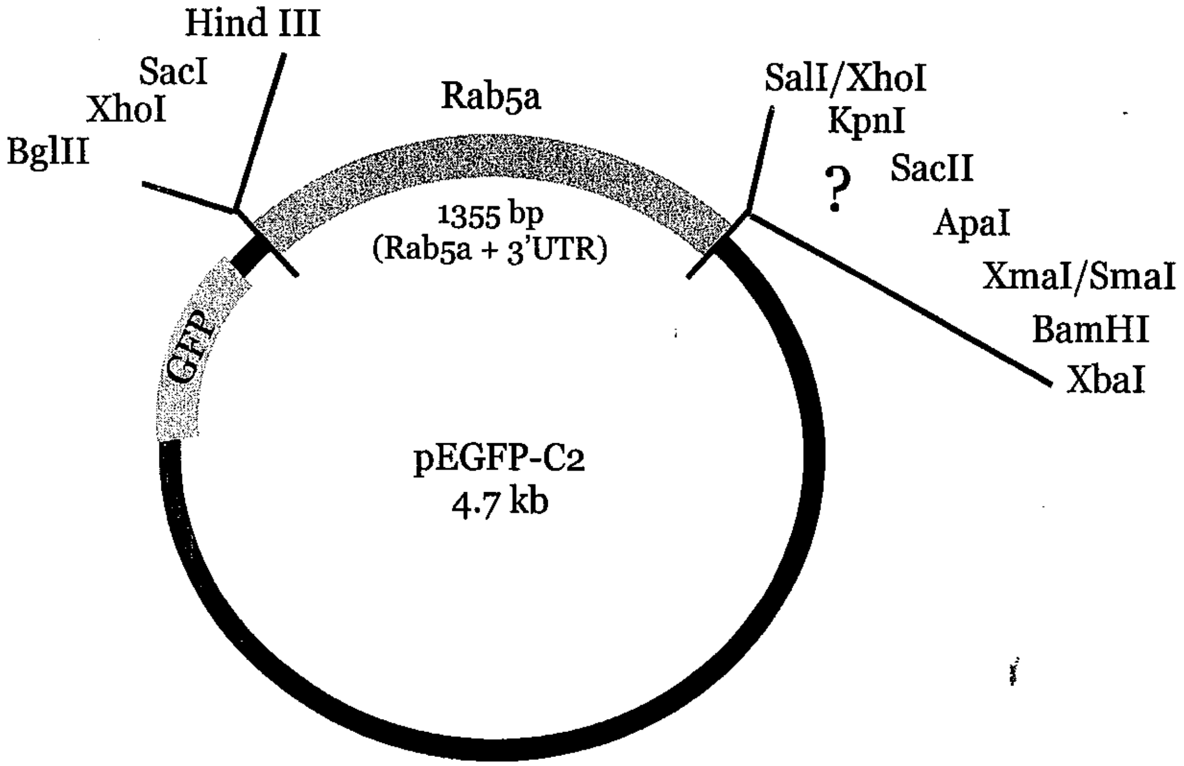
Expression: ma

Sequencing Primers: 5' - EGFP-C

JSOS Primer did not reach 3' cloning site

*the sites in the map are assumed to be present based on info from old sheet + pEGFP-C2 MCS

Additional Information: large 3' UTR (~700 bp)



cDNA Inventory Data Sheet #5

cDNA Information:

Name: GFP-Rab5a-S34N

Species: Dog

Cloning Sites: 5' - HindIII
3' - Sal I/XhoI

Insert Size: 1355bp
(~650bp Rab5 + 3' UTR)

Mutation(s): S34N

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2

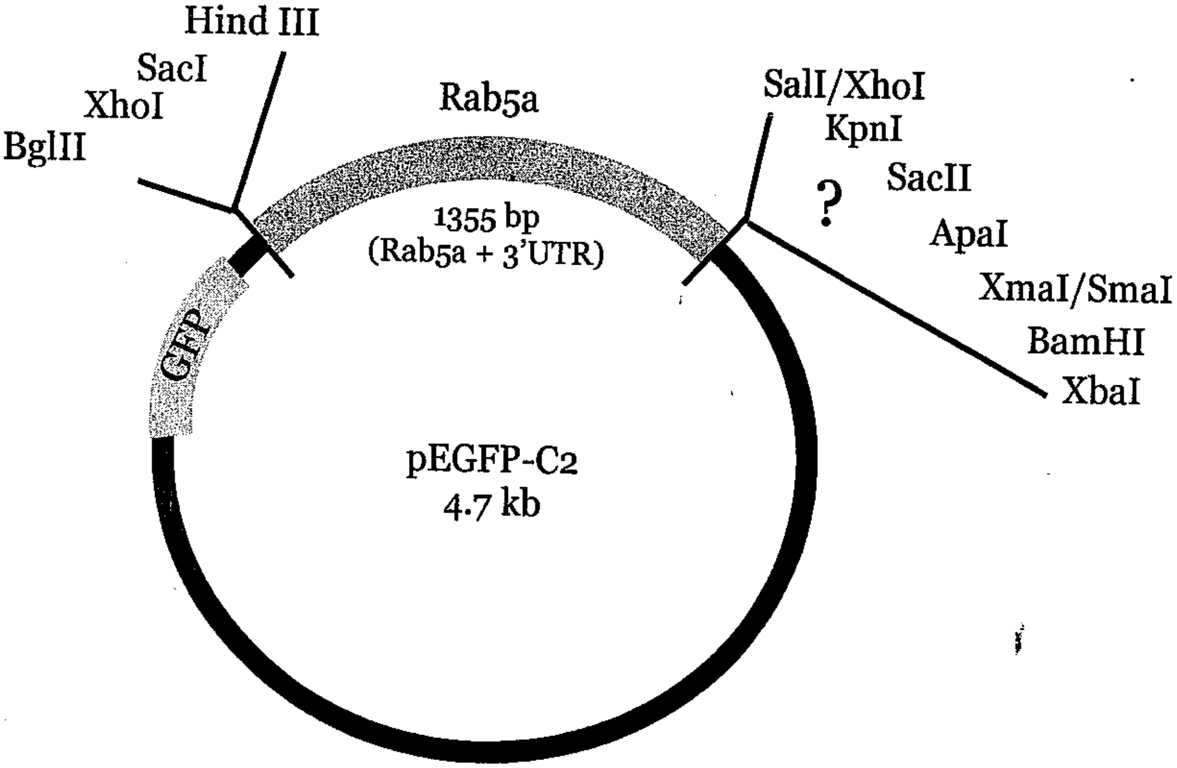
Vector Source: P. Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information: large 3'UTR (~700bp)



cDNA Inventory Data Sheet #6

cDNA Information:

Name: GFP-Rab5a-Q79L (F48S)

Species: Dog

Cloning Sites: 5' - HindIII
3' - Sal I/XhoI

Insert Size: 1355bp
(~650bp Rab5 + 3' UTR)

Mutation(s): F48S/Q79L

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2

Vector Source: P. Anborgh

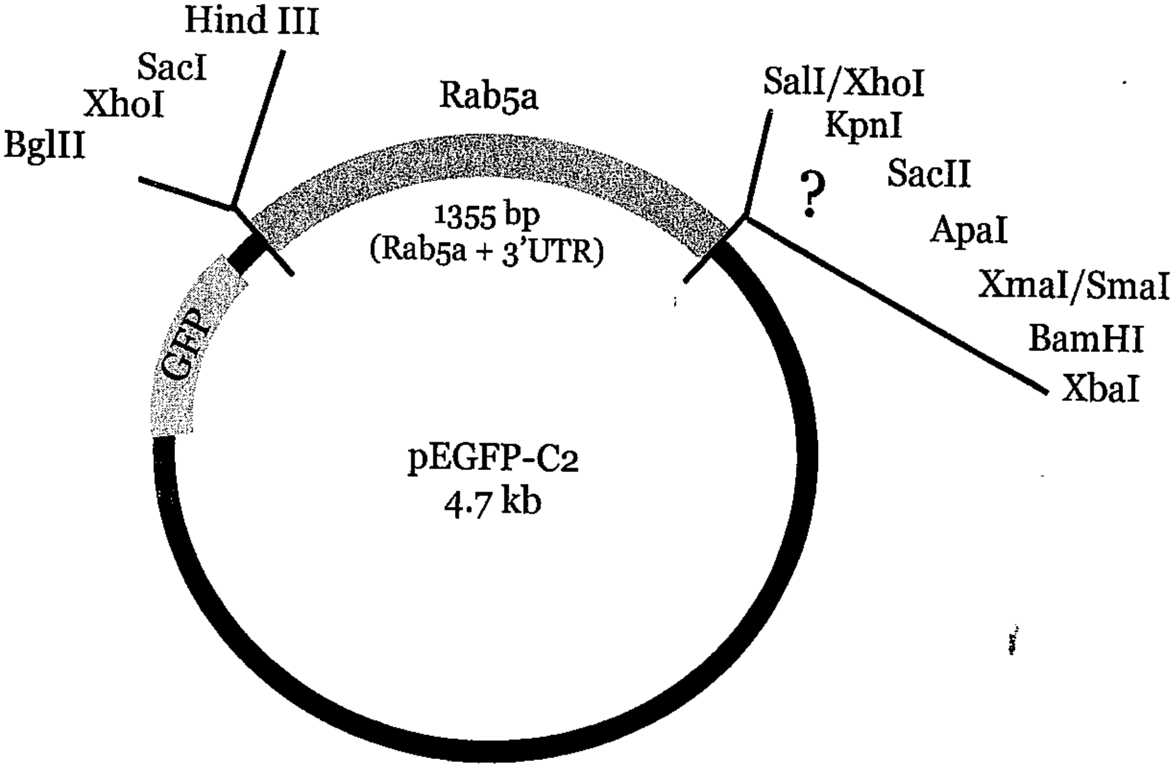
Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information:

Additional mutation F48S
Large 3' UTR (~700bp)



cDNA Inventory Data Sheet #6

cDNA Information:

Name: GFP-Rab7-Q67L

Species: Dog

Cloning Sites: 5' - SmaI
3' -

Insert Size: ~600 bp

Mutation(s):

Epitope Tag: GFP

cDNA Source:Q67L

Prepared by: Dr. B. Van Deurs

Vector Information:

Name: pEGFP-C1

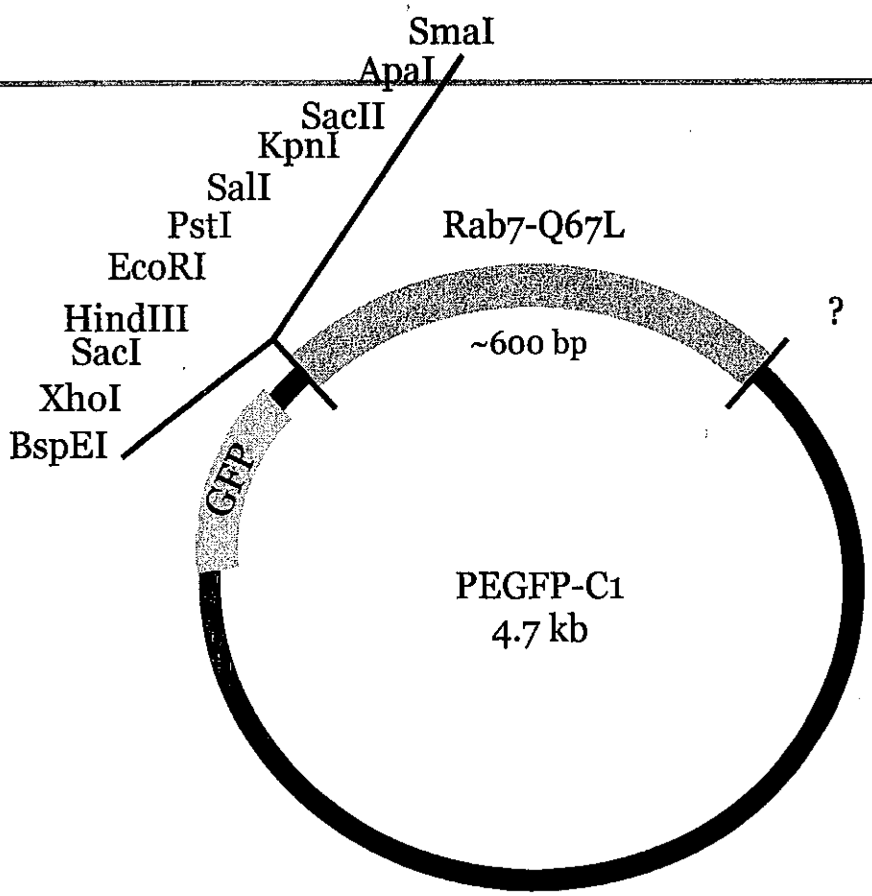
Vector Source: Clontech

Selection: kan

Expression: mammalian

Sequencing Primers: **5'** - EGFP-C **3'** -

Additional Information:



cDNA Inventory Data Sheet #4

cDNA Information:

Name: GFP-Rab7-wt **Species:** Human

Cloning Sites: 5' - BamHI/BglII
3' -NotI
Insert Size: ~625 bp

Mutation(s): **Epitope Tag:** GFP

cDNA Source: ? **Prepared by:** P. Anborgh

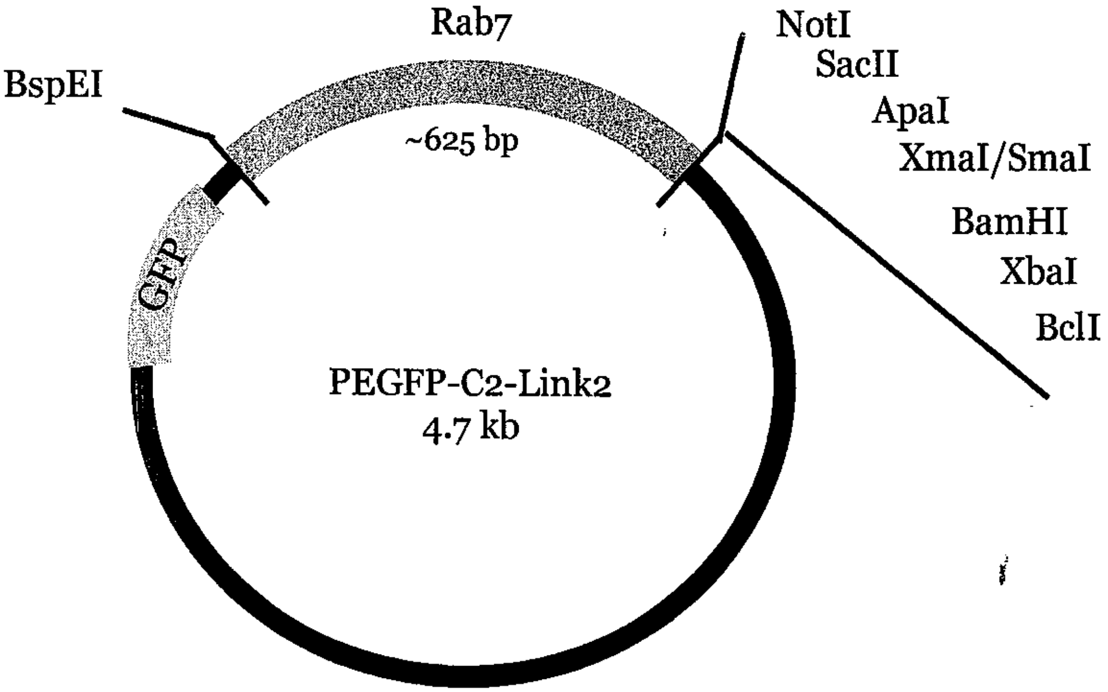
Vector Information:

Name: pEGFP-C2-Link2 **Vector Source:** Clontech/Modified by P.Anborgh

Selection: kan **Expression:** mammalian

Sequencing Primers: **5' -** EGFP-C **3'-**

Additional Information: aligned with human not dog as original inventory sheet indicated



cDNA Inventory Data Sheet #5

cDNA Information:

Name: GFP-Rab7-N125I **Species:** Dog

Cloning Sites: 5' - SmaI
3' -

Insert Size: ~600 bp

Mutation(s): N125I **Epitope Tag:** GFP

cDNA Source:

Prepared by: Dr. B. Van Deurs

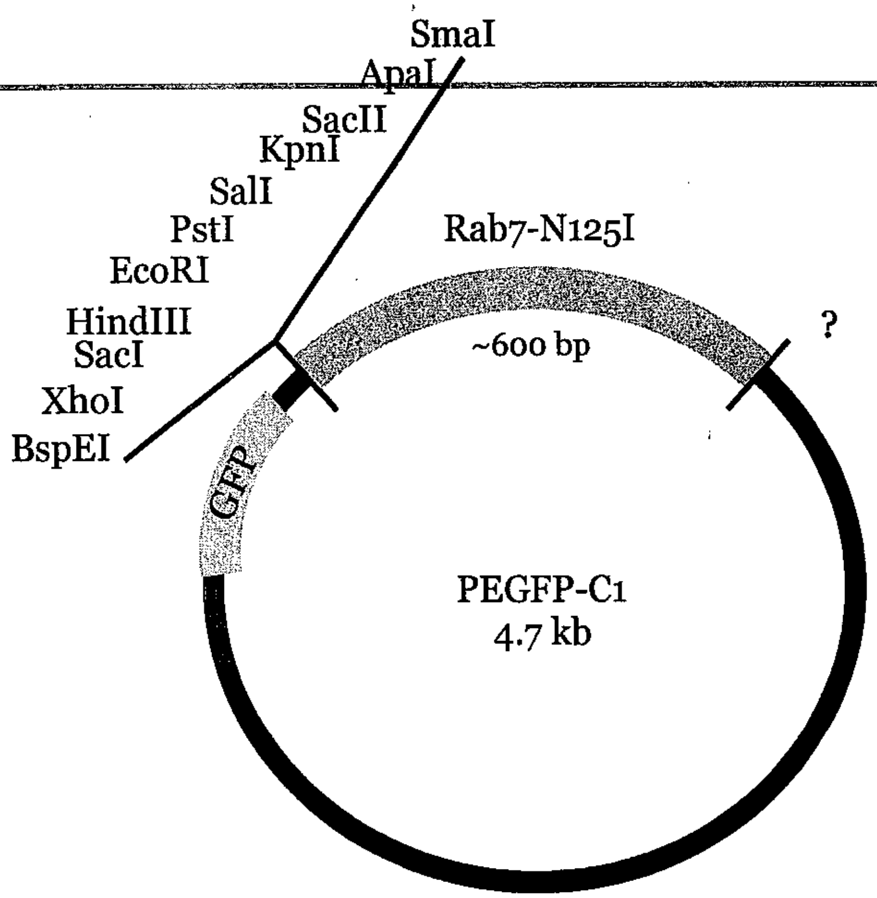
Vector Information:

Name: pEFGP-C1 **Vector Source:** Clontech

Selection: kan **Expression:** mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information:



cDNA Inventory Data Sheet #7

cDNA Information:

Name: GFP-Rab7-T22N

Species: Dog

Cloning Sites: 5' - SmaI (?)
3' -

Insert Size: ~600 bp

Mutation(s): T22N

Epitope Tag: GFP

cDNA Source:

Prepared by: Dr. B. Van Deurs

Vector Information:

Name: pEFGP-C1

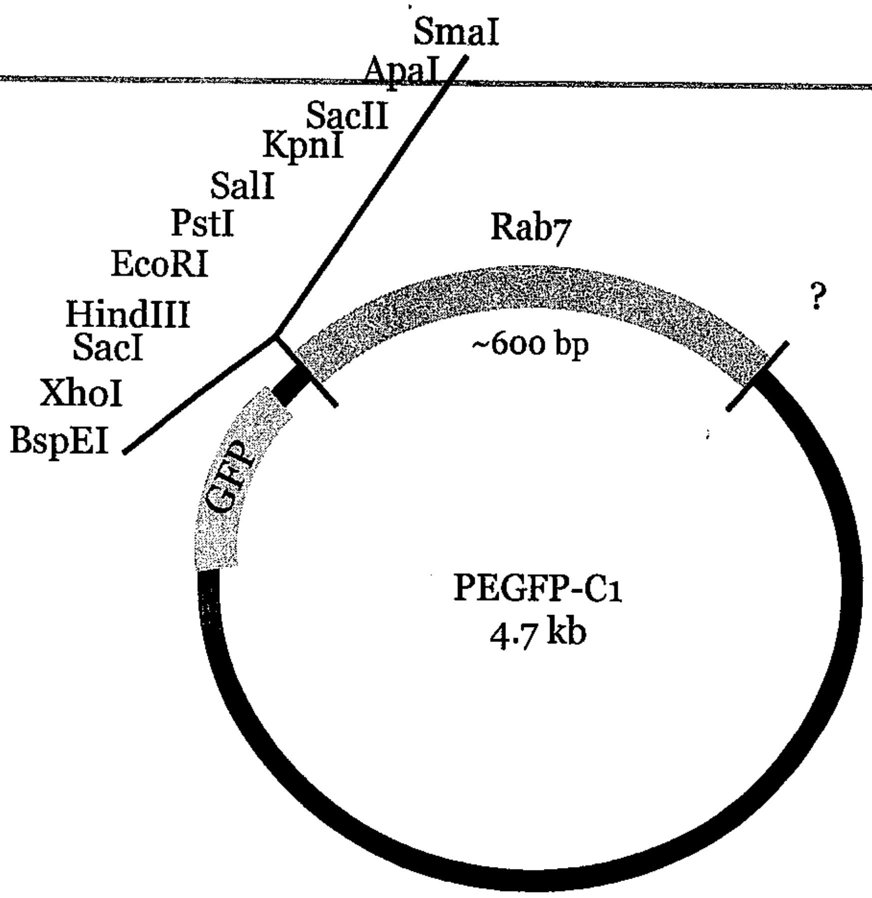
Vector Source: Clontech

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information:



cDNA Inventory Data Sheet #8

cDNA Information:

Name: GFP-Rab11-N124I

Species: Human

Cloning Sites: 5' - BamHI/BglII
3' -NotI

Insert Size: ~650 bp

Mutation(s): N124I

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2-Link2

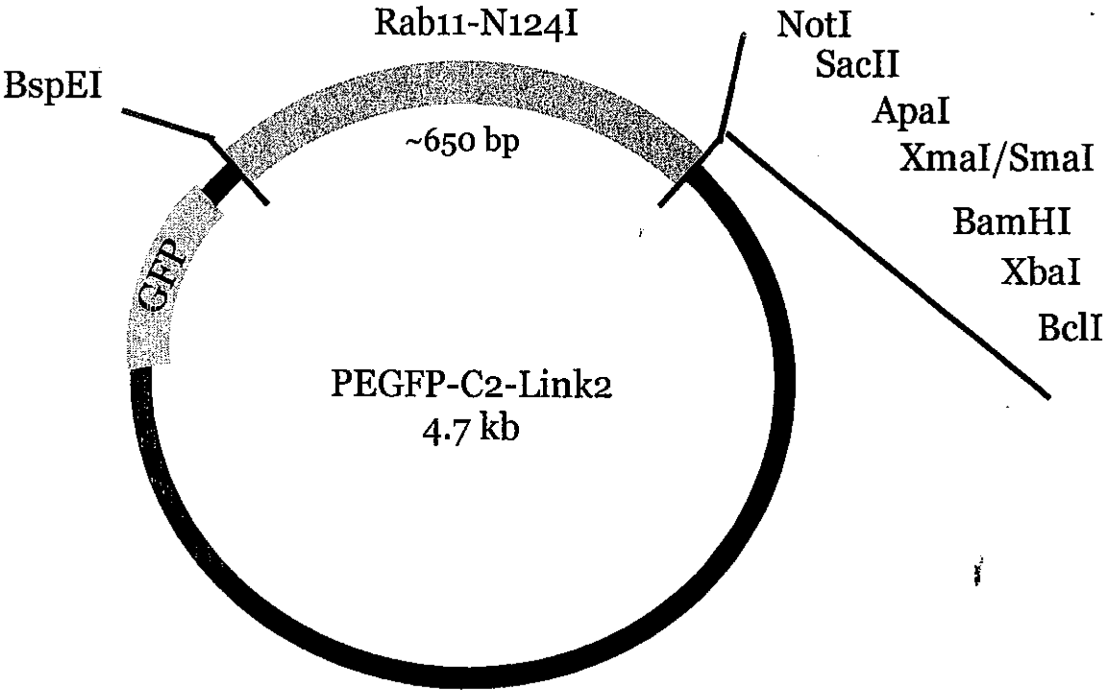
Vector Source: Clontech/Modified by P.Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information:



cDNA Inventory Data Sheet #5

cDNA Information:

Name: GFP-Rab11-wt

Species: Human

Cloning Sites: 5' - BamHI/BglII
3' -NotI

Insert Size: ~650 bp

Mutation(s):

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2-Link2

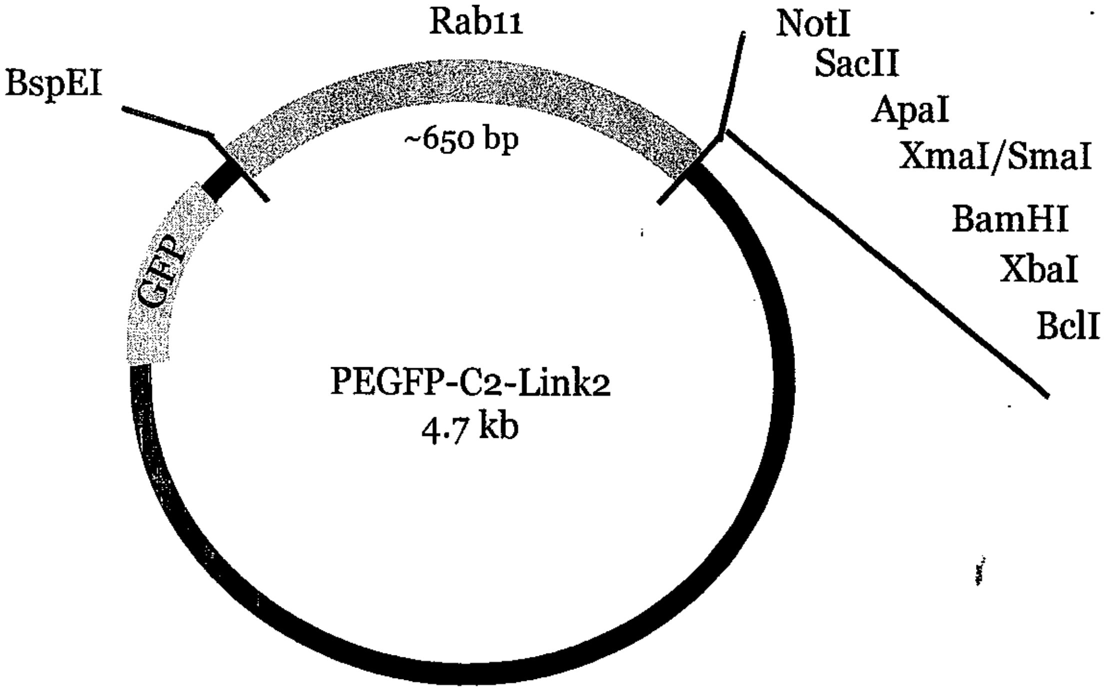
Vector Source: Clontech/Modified by P.Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: **5'** - EGFP-C **3' -**

Additional Information:



cDNA Inventory Data Sheet #7

cDNA Information:

Name: GFP-Rab11-Q70L

Species: Human

Cloning Sites: 5' - BamHI/BglII
3' -NotI

Insert Size: ~650 bp

Mutation(s): Q70L

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2-Link2

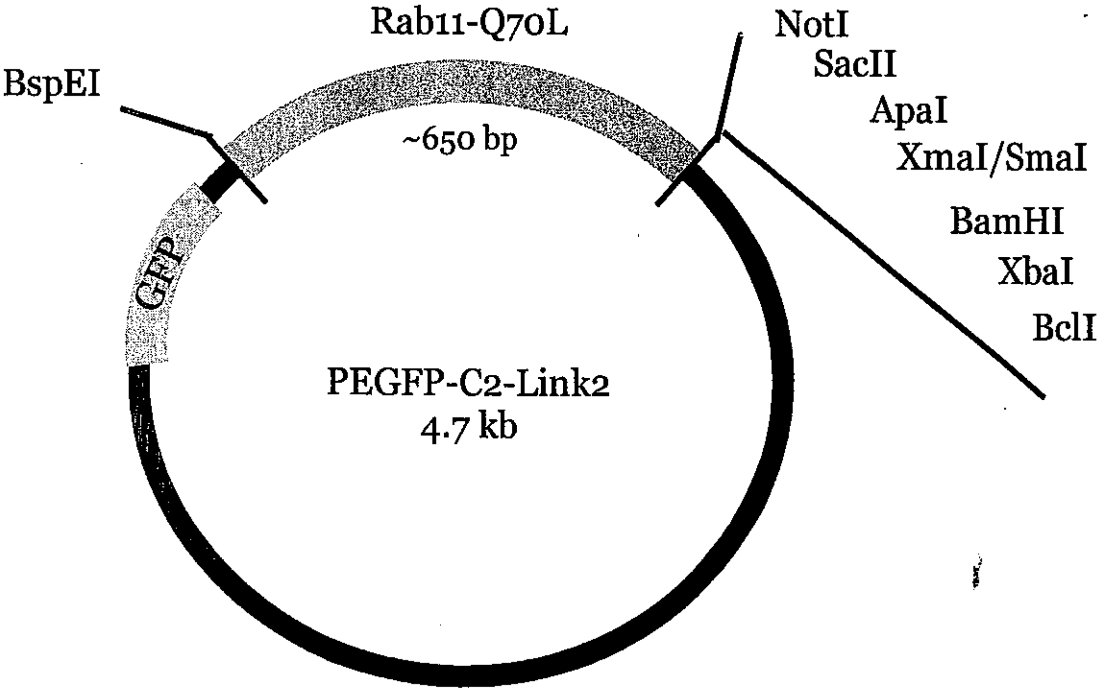
Vector Source: Clontech/Modified by P.Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: 5' - EGFP-C 3' -

Additional Information:



cDNA Inventory Data Sheet

#6

cDNA Information:

Name: GFP-Rab11-S25N

Species: Human

Cloning Sites: 5' - BamHI/BglII
3' -NotI

Insert Size: ~650 bp

Mutation(s): S25N

Epitope Tag: GFP

cDNA Source: ?

Prepared by: P. Anborgh

Vector Information:

Name: pEGFP-C2-Link2

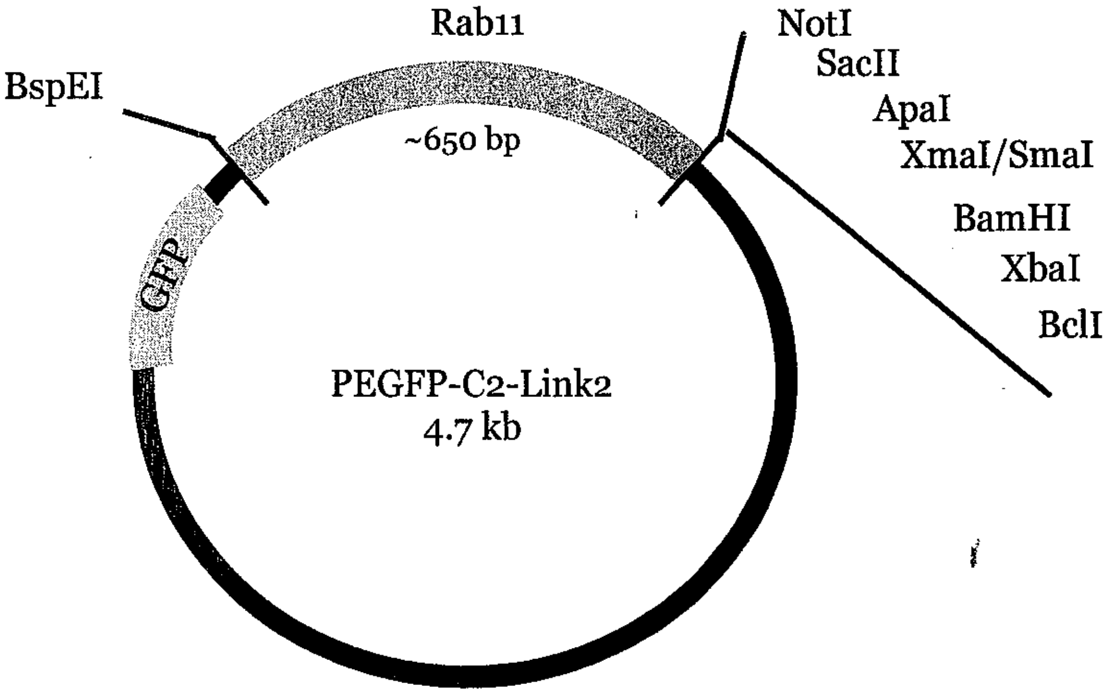
Vector Source: Clontech/Modified by P.Anborgh

Selection: kan

Expression: mammalian

Sequencing Primers: **5'** - EGFP-C **3'** -

Additional Information:



#2

DNA INVENTORY

NAME: GRK2 (βark1)

INSERT: **NAME:** GRK2 (βark1)

SPECIES: bovine

CLONING SITES: **5'**

3'

SIZE:

SOURCE:

MODIFICATIONS:

VECTOR: **NAME:** pcDNA1-amp

SELECTION: ampicillin

SIZE: 4.8 Kb

SOURCE: Invitrogen

MODIFICATIONS:

STORAGE:

WORKING STOCK: 4°C, GRK rack

FROZEN STOCK: -20°C, GRK box

GLYCEROL STOCK: -80°C, GRK box

cDNA Inventory Data Sheet

CONSTRUCT: βarrestin 1-YFP # 7

Species: ____

loning Sites: 5' ? Date: Feb 2003

3' ____

- have sequences

Plasmid

Vector: pEYFP-N (Clontech)

Expression: euk.

Selection: kana. Insert Size: ____

Epitope Tag: ____ Mutation: ____

Source: Dr. Stéphane Laporte.

Modifications: ____

STORAGE

Working Stock: ____

Frozen Stock: ____

Glycerol Stock: ____

pen Reading Frame:

5' _

3' _

cDNA Inventory Data Sheet

CONSTRUCT: B-arrestin1-GFP # 6

Species:

Cloning Sites: 5'

Date:

3'

Plasmid

Vector:

Expression:

Selection:

Insert Size:

Epitope Tag:

Mutation:

Source: Stephane Laporte - no information sent c̄ DNA.

Modifications:

STORAGE

Working Stock:

Frozen Stock:

Glycerol Stock:

Open Reading Frame:

5'

3'

cDNA Inventory Data Sheet

DNA Information:

Name: Barr1-CFP

Species: Rat (NM012910)

Cloning Sites: 5' - HindIII
3' - KpnI

Insert Size: ~1200bp

Mutation(s):

Epitope Tag: CFP

cDNA Source:

Prepared by: L.Dale

Vector Information:

Name: pECFP-N3

Vector Source:

Selection: kan

Expression: mam lian

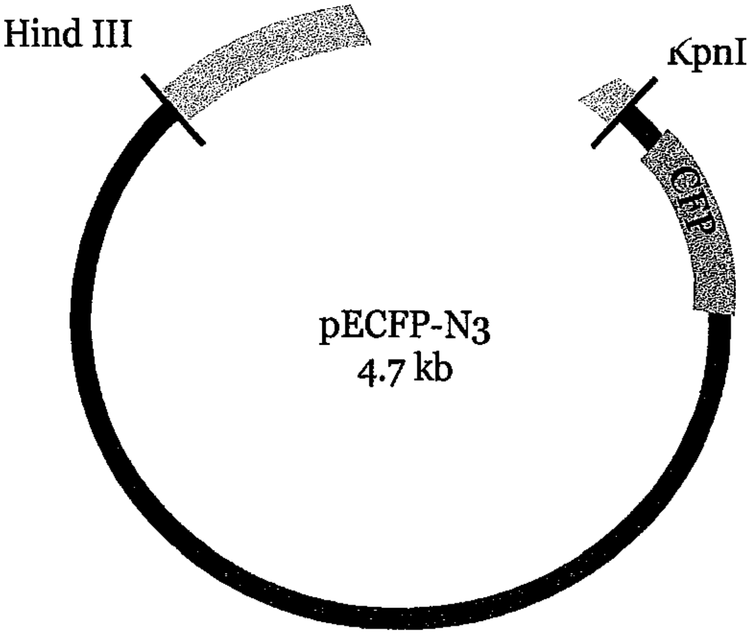
Sequencing Primers: 5' -

Additional Information:

- subclned Barr1 fro n)

- WORKS??? NEEDS T

Needs to be tested



DNA INVENTORY

12

NAME: β-arrestin2-cGFP

INSERT: **NAME:** β-arrestin2-cGFP

SPECIES: rat

CLONING SITES: **5'**

3'

SIZE:

SOURCE:

MODIFICATIONS: stop codon replaced to create fusion protein at C-terminal end of β-arrestin with GFP

VECTOR: **NAME:** pGFP-N3

SELECTION: Kanamycin

SIZE: 4.7 Kb

SOURCE: Clonetech

MODIFICATIONS:

STORAGE:

WORKING STOCK: 4°C, β-arrestin rack

FROZEN STOCK: -20°C, β-arrestin box

GLYCEROL STOCK: -80°C, β-arrestin box

#9

DNA INVENTORY

NAME: β-arrestin 2 C-Flag

INSERT: **NAME:** β-arrestin 2 with flag epitope tag (C-terminal end)

SPECIES: rat

CLONING SITES: **5'**

3'

SIZE:

SOURCE:

MODIFICATIONS:

VECTOR: **NAME:** pcDNA3-~~amp~~ (zeo)

SELECTION: ampicillin

SIZE: 5.5 Kb

SOURCE: Invitrogen

MODIFICATIONS:

STORAGE:

WORKING STOCK: 4°C, β-arrestin rack

FROZEN STOCK: -20°C, β-arrestin box

GLYCEROL STOCK: -80°C, β-arrestin box

cDNA Inventory Data Sheet

CONSTRUCT: βarrestin 2-YFP # B

Species:

loning Sites: 5' ? Date: Feb2003.

3'

-have sequence.

Plasmid

Vector: pEYFP-

Expression: euk.

Selection: kana Insert Size:

Epitope Tag: Mutation:

Source: gift from Dr. Stéphane Laporte.

Modifications:

STORAGE

Working Stock:

Frozen Stock:

Glycerol Stock:

pen Reading Frame:

5'

3'

cDNA Inventory Data Sheet

CONSTRUCT: β-arrestin 2-CFP (SL) #35

Species: ____________________

Cloning Sites: 5' ____________ Date: ______

3' ____________

Plasmid

Vector: ____________________

Expression: ____________________

Selection: ________ Insert Size: ________

Epitope Tag: ________ Mutation: ________

Source: Stephane Laporte

Modifications: ____________________

STORAGE

Working Stock: ____________

Frozen Stock: ____________

Glycerol Stock: ____________

en Reading Frame:

5' _

3' _

cDNA Inventory Data Sheet

DNA Information:

Name: Fl-mGluR5 **Species:** Rat

Cloning Sites: 5' - HindIII
3' - XbaI **Insert Size:** ~3530bp + ~700bp

Mutation(s): **Epitope Tag:** Flag

cDNA Source: Nakanishi **Prepared by:** L.Dale

Vector Information:

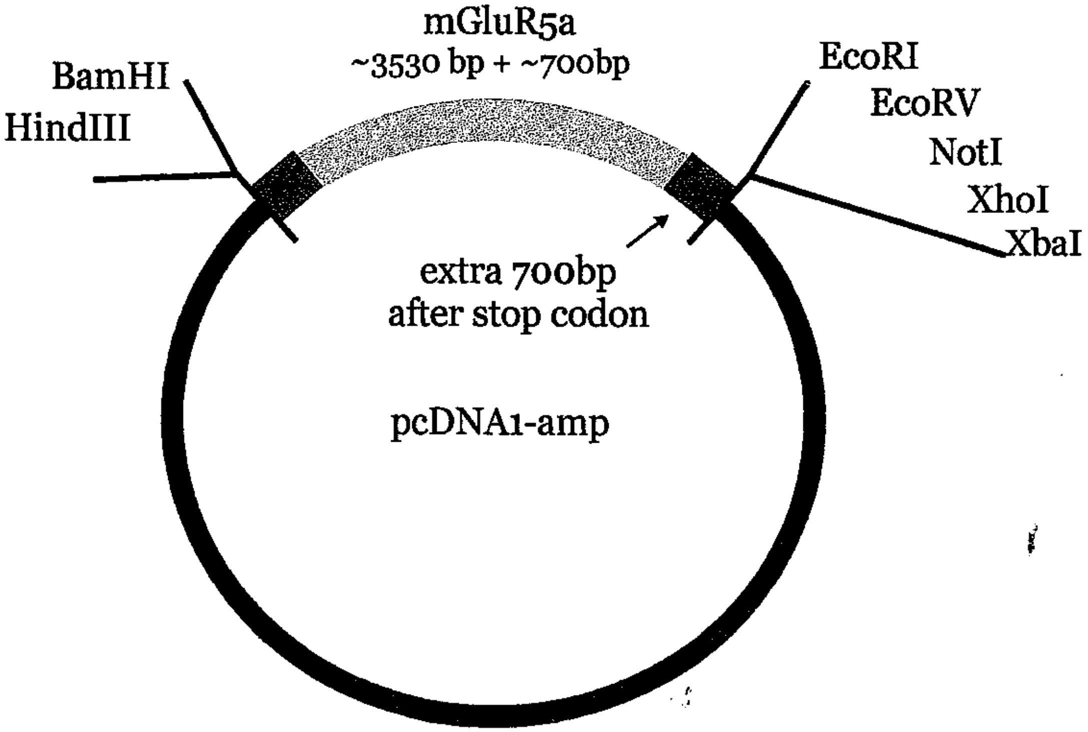
Name: pcDNA1-amp **Vector Source:** Invitrogen

Selection: Amp **Expression:** Eukaryotic

Sequencing Primers: 5' - T7 3' - SP6

Additional Information:

- HindIII, BamHI, Kozak, signal sequence and Flag epitope introduced by PCR



2

DNA INVENTORY

NAME: Flag Angiotensin $_{1A}$ Receptor (AT$_{1A}$R)

INSERT: **NAME:** angiotensin $_{1A}$ receptor with Flag epitope tag

SPECIES: rat

CLONING SITES: 5' HindIII

3' XbaI

SIZE:

SOURCE:

MODIFICATIONS:

VECTOR: **NAME:** pcDNA1-amp

SELECTION: ampicillin

SIZE: 4.8 Kb

SOURCE: Invitrogen

MODIFICATIONS:

STORAGE:

WORKING STOCK: 4°C, receptor rack

FROZEN STOCK: -20°C, receptor box #1

GLYCEROL STOCK: -80°C, receptor box #1

cDNA Inventory Data Sheet

DNA Information:

Name: Fl-mGluR1a

Species: Rat

Cloning Sites: 5' - BamHI
3' - XbaI

Insert Size: ~3610bp

Mutation(s):

Epitope Tag: Flag

cDNA Source: Nakanishi

Prepared by: L.Dale

Vector Information:

Name: pcDNA1-amp

Vector Source: Invitrogen

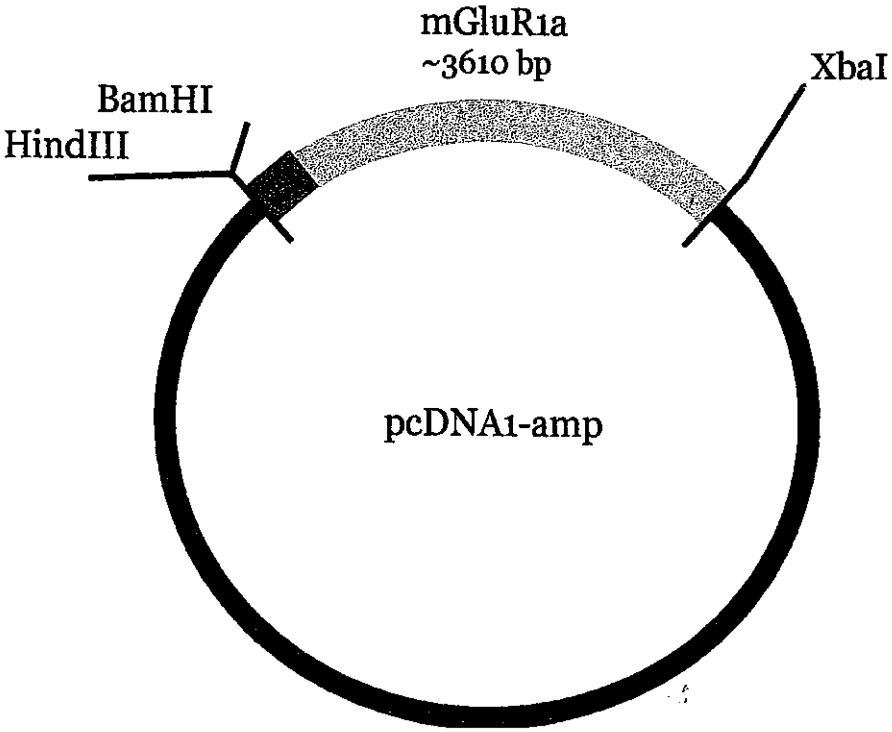
Selection: Amp

Expression: Eukaryotic

Sequencing Primers: **5' - T7** **3' - SP6**

Additional Information:

- BamHI, Kozak, signal sequence and Flag epitope introduced by PCR



cDNA Inventory Data Sheet

CONSTRUCT: mGluR1b #

Species: Rat

Cloning Sites: 5' SmaI Date: June 15/01

3' XbaI

Plasmid

Vector: pcDNA-1-amp

Expression:

Selection: amp Insert Size:

Epitope Tag: Flag Mutation:

Source:

Modifications:

STORAGE

Working Stock:

Frozen Stock:

Glycerol Stock:

Open Reading Frame:

5' _

3' _

Attachment 5:

MSDS for hormones used in the lab, Angiotensin II, Corticotropin releasing factor (CRF), Serotonin (5HT)

Material Safety Data Sheet

Version 3.4
Revision Date 11/05/2011
Print Date 08/30/2012

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : Angiotensin II human

Product Number : A9525
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA

Manufacturer : Sigma-Aldrich Corporation
3050 Spruce St.
St. Louis, Missouri 63103
USA

Telephone : +1 9058299500
Fax : +1 9058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300
Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

Target Organs

Heart, Adrenal cortex.

WHMIS Classification

Not WHMIS controlled. Not WHMIS controlled.

Not a dangerous substance according to GHS.

HMIS Classification

Health hazard:	0
Chronic Health Hazard:	*
Flammability:	0
Physical hazards:	0

Potential Health Effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Skin	May be harmful if absorbed through skin. May cause skin irritation.
Eyes	May cause eye irritation.
Ingestion	May be harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Formula : $C_{50}H_{71}N_{13}O_{12}$
Molecular Weight : 1,046.18 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
Angiotensin II human			
4474-91-3	-	-	-

4. FIRST AID MEASURES

General advice
Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration.

In case of skin contact
Wash off with soap and plenty of water.

In case of eye contact
Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water.

5. FIREFIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for firefighters
Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Avoid dust formation. Avoid breathing vapors, mist or gas.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Provide appropriate exhaust ventilation at places where dust is formed.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: -20 °C

Keep in a dry place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Respiratory protection is not required. Where protection from nuisance levels of dusts are desired, use type N95 (US) or type P1 (EN 143) dust masks. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Choose body protection in relation to its type, to the concentration and amount of dangerous substances, and to the specific work-place., The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
General industrial hygiene practice.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	powder
Colour	white

Safety data

pH	no data available
Melting point/freezing point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	ca.25 g/l
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong acids, Strong bases

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)
Other decomposition products - no data available

---

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
LD50 Intravenous - rat - 17.4 mg/kg

LD50 Intravenous - mouse - 30.8 mg/kg

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC:	No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.
ACGIH:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity
Reproductive toxicity - rat - Intravenous
Effects on Newborn: Growth statistics (e.g., reduced weight gain).

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion	May be harmful if swallowed.
Skin	May be harmful if absorbed through skin. May cause skin irritation.

Eyes May cause eye irritation.

Signs and Symptoms of Exposure
Angiotensin is a potent vasoconstrictor. It acts directly on the adrenal gland to stimulate the release of aldosterone, which in turn affects metabolism of electrolytes and water.

Synergistic effects
no data available

Additional Information
RTECS: BW2165000

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

WHMIS Classification

Not WHMIS controlled.	Not WHMIS controlled.

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

TOCRIS
bioscience

Version 2.0 SDS Revision Date: Jul 18th 2011
SDS Print Date: May 18th 2012

Safety Data Sheet

www.tocris.com

1. IDENTIFICATION OF THE SUBSTANCE/MIXTURE AND OF THE COMPANY/UNDERTAKING

1.1 Product identifiers

Product Name: CRF (human, rat)
Catalog Number: 1151
CAS Number: 86784-80-7

1.2 Relevant identified uses of the substance or mixture and uses advised against

Identified Uses: Laboratory chemicals, Manufacture of substances

1.3 Details of the supplier of the safety data sheet

Company: Tocris Bioscience
Tocris House, IO Centre
Moorend Farm Avenue
Bristol, BS11 0QL, UK

Telephone: + 44 (0)117 916-3333
Fax: + 44 (0)117 916-3344
Internet: www.tocris.com
E-mail address: customerservice@tocris.co.uk

1.4 Emergency Telephone number

Emergency Tel: + 44 (0)117 916-3333 (09.00 - 17.00 GMT)

2. HAZARDS IDENTIFICATION

2.1 Classification of the substance or mixture

This substance does not meet the classification criteria of the EC Directives 67/548/EEC, 1999/45/EC or 1272/2008.

2.2 Label elements

The product does not need to be labelled in accordance with EC directives or respective national laws.

2.3 Other hazards - none

3. COMPOSITION/INFORMATION ON INGREDIENTS

3.1 Substances

Product Name: CRF (human, rat)
Synonyms: Corticotropin-Releasing Factor (human, rat)
Formula: $C_{208}H_{344}N_{60}O_{63}S_2$
Molecular Weight: 4758
CAS Number: 86784-80-7

4. FIRST AID MEASURES

4.1 Description of first aid measures

General advice
Consult a doctor and show this safety data sheet.

If inhaled
Remove to fresh air and monitor breathing. If breathing becomes difficult, give oxygen. If breathing stops, give artificial respiration. Consult a doctor.

In case of skin contact
Immediately wash skin with copious amounts of soap and water for at least 15 minutes. Remove contaminated clothing and shoes and wash before reuse. Consult a doctor.

In case of eye contact
Flush with copious amounts of water for at least 15 minutes. Consult a doctor.

If swallowed
Rinse mouth with water. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Consult a doctor.

4.2 Most important symptoms and effects, both acute and delayed

To the best of our knowledge, the chemical, physical and toxicological properties have not been thoroughly investigated.

4.3 Indication of immediate medical attention and special treatment needed

Show this safety data sheet to the doctor in attendance. Immediate medical attention is required.

5. FIRE-FIGHTING MEASURES

5.1 Extinguishing media

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

5.2 Special hazards arising from the substance or mixture

In combustion, may emit toxic fumes.

5.3 Precautions for fire-fighters

Wear suitable protective clothing to prevent contact with skin and eyes and self-contained breathing apparatus.

6. ACCIDENTIAL RELEASE MEASURES

6.1 Personal precautions, protective equipment and emergency procedures

Do not take action without suitable protective clothing - see section 8 of SDS. Evacuate personnel to safe areas. Ensure adequate ventilation. Avoid breathing vapors, mist, dust or gas.

6.2 Environmental precautions

Do not let product enter drains.

6.3 Methods and materials for containment and cleaning up

Cover spillage with suitable absorbent material. Using non-spark tools, sweep up material and place in an appropriate container. Decontaminate spill site with 10% caustic solution and ventilate area until after disposal is complete. Hold all material for appropriate disposal as described under section 13 of SDS.

6.4 Reference to other sections

For required PPE see section 8. For disposal see section 13.

7. HANDLING AND STORAGE

7.1 Precautions for safe handling

Use in a chemical fume hood, with air supplied by an independent system. Avoid inhalation, contact with eyes, skin and clothing. Avoid the formation of dust and aerosols. Use in a well-ventilated area. Keep away from sources of ignition. Avoid prolonged or repeated exposure.

7.2 Conditions for safe storage, including any incompatibilities.

Store in cool, well-ventilated area. Keep away from direct sunlight. Keep container tightly sealed until ready for use. Recommended storage temperature: Desiccate at -20°C

7.3 Specific end uses

Use in a laboratory fume hood where possible. Refer to employer's COSHH risk assessment.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Control parameters

Components with workplace control parameters
Contains no substances with occupational exposure limit values.

8.2 Exposure controls

Appropriate engineering controls
Use in a fume hood where applicable. Ensure all engineering measures described under section 7 of SDS are in place. Ensure laboratory is equipped with a safety shower and eye wash station.

Personal protective equipment

Eye/face protection
Use appropriate safety glasses.

Skin protection
Use appropriate chemical resistant gloves (minimum requirement use standard BS EN 374:2003). Gloves should be inspected before use. Wash and dry hands thoroughly after handling.

Body protection
Wear appropriate protective clothing.

Respiratory protection
If risk assessment indicates necessary, use a suitable respirator.

9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 Information on basic physical and chemical properties

Appearance	White lyophilised solid	Vapor pressure	No data available
Odor	No data available	Vapor density	No data available
Odor threshold	No data available	Relative density	No data available
pH	No data available	Solubility(ies)	Soluble to 1.10 mg/ml in water
Melting / freezing point	No data available	Partition coefficient	No data available
Boiling point / range	No data available	Auto-ignition temperature	No data available
Flash point	No data available	Decomposition temperature	No data available
Evaporation rate	No data available	Viscosity	No data available
Flammability (solid, gas)	No data available	Explosive properties	No data available
Upper / lower flammability or explosive limits	No data available	Oxidising properties	No data available

9.2 Other safety information

No data available

10. STABILITY AND REACTIVITY

10.1 Reactivity

Stable under recommended transport or storage conditions.

10.2 Chemical stability

Stable under recommended storage conditions.

10.3 Possibility of hazardous reactions

Hazardous reactions will not occur under normal transport or storage conditions. Decomposition may occur on exposure to conditions or materials listed below.

10.4 Conditions to avoid

Heat, moisture.

10.5 Incompatible materials

Strong acids/alkalis, strong oxidising/reducing agents.

10.6 Hazardous decomposition products

In combustion may emit toxic fumes. No known decomposition information.

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11. TOXICOLOGICAL INFORMATION

11.1 Information on toxicological effects

Acute Toxicity
IVN-RAT TDLo: 1148µg/kg; IVN-RAT TDLo: 3111µg/kg; IVN-RAT TDLo: 2700µg/kg; IVN-RAT LD50: >1mg/kg; IVN-DOG LD50 >1mg/kg; IVN-RAT TDLo: 560µg/kg; IVN-DOG TDLo: 840µg/kg

Skin corrosion/irritation
Classification criteria are not met based on available data

Serious eye damage/irritation
Classification criteria are not met based on available data

Respiratory or skin sensitization
Classification criteria are not met based on available data

Germ cell mutagenicity
Classification criteria are not met based on available data

Carcinogenicity
Classification criteria are not met based on available data

Reproductive toxicity
Classification criteria are not met based on available data

Specific target organ toxicity - single exposure
Classification criteria are not met based on available data

Specific target organ toxicity - repeated exposure
Classification criteria are not met based on available data

Aspiration hazard
Classification criteria are not met based on available data

Symptoms / Routes of exposure

Inhalation:	There may be irritation of the throat with a feeling of tightness in the chest.
Ingestion:	There may be irritation of the throat.
Skin:	There may be mild irritation at the site of contact.
Eyes:	There may be irritation and redness.

Delayed / Immediate Effects: No known symptoms.

Additional Information
RTECS No: GM7925000
RTECS Substance category: Reproductive effector Exposure may cause irritation to eyes, mucous membranes, upper respiratory tract and skin; Exposure may also cause the following: Effect fertility and/or unborn and breastfed infants; Somnolence; Gastrointestinal disturbances; Ptosis; Ataxia; Endocrine and Lymphatic system changes; Blood composition changes; Uterine weight change.
To the best of our knowledge, the chemical, physical and toxicological properties have not been fully investigated.

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12. ECOLOGICAL INFORMATION

12.1 Toxicity

No data available

12.2 Persistence and degradability

No data available

12.3 Bioaccumlative potential

No data available

12.4 Mobility in soil

No data available

12.5 Results of PBT and vPvB assessment

No data available

12.6 Other adverse effects

May be harmful to the aquatic environment.

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13. DISPOSAL CONSIDERATIONS

13.1 Waste treatment methods

Product
Transfer to a suitable container and arrange for collection by specialized disposal company in accordance with National legislation.

Contaminated packaging
Dispose of in a regulated landfill site or other method for hazardous or toxic wastes in accordance with National legislation.

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14. TRANSPORT INFORMATION

Classified according to the criteria of the UN Model Regulations as reflected in the IMDG Code, ADR, RID and IATA.

14.1 UN-Number

Does not meet the criteria for classification as hazardous for transport.

14.2 UN proper shipping name

Does not meet the criteria for classification as hazardous for transport.

14.3 Transport hazard class(es)

Does not meet the criteria for classification as hazardous for transport.

14.4 Packaging group

Does not meet the criteria for classification as hazardous for transport.

14.5 Environmental hazards

This product is not classified as environmentally hazardous according to the UN Model Regulations, nor a marine pollutant according to the IMDG Code.

14.6 Special precautions for users

No data available

15. REGULATORY INFORMATION

This safety datasheet complies with the requirements of Regulation (EC) No. 453/2010.

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

No data available

15.2 Chemical safety assessment

A Chemical Safety Assessment has not been made for this product.

16. OTHER INFORMATION

Further Information

Copyright 2011 Tocris Bioscience. This company shall not be held liable for any damage resulting from handling or from contact with the above product. This material must only be handled by suitably qualified experienced scientists in appropriately equipped and authorized facilities. The above information is believed to be correct but does not purport to be all inclusive and should be used as a guide only for experienced personnel. Always consult your safety advisor and follow appropriate local and national safety legislature. The absence of warning must not, under any circumstance, be taken to mean that no hazard exists.

End of safety data sheet

SIGMA-ALDRICH

sigma-aldrich.com

Material Safety Data Sheet

Version 4.6
Revision Date 04/26/2012
Print Date 08/30/2012

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : Serotonin hydrochloride

Product Number : H9523
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier	: Sigma-Aldrich Canada, Ltd 2149 Winston Park Drive OAKVILLE ON L6H 6J8 CANADA	Manufacturer	: Sigma-Aldrich Corporation 3050 Spruce St. St. Louis, Missouri 63103 USA

Telephone : +1 9058299500
Fax : +1 9058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300
Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

Target Organs

Nerves., Smooth muscle., Vascular system., Endocrine system., Liver, Damage to the heart.Nerves., Smooth muscle., Vascular system., Endocrine system., Liver, Damage to the heart.

WHMIS Classification

D2A	Very Toxic Material Causing Other Toxic Effects	Reproductive hazard
D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant
		Moderate respiratory irritant
		Moderate eye irritant

GHS Classification

Acute toxicity, Oral (Category 4)
Acute toxicity, Inhalation (Category 4)
Acute toxicity, Dermal (Category 4)
Skin irritation (Category 2)
Eye irritation (Category 2A)
Specific target organ toxicity - single exposure (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word: Warning

Hazard statement(s)

H302 + H312	Harmful if swallowed or in contact with skin
H315	Causes skin irritation.
H319	Causes serious eye irritation.
H332	Harmful if inhaled.
H335	May cause respiratory irritation.

Precautionary statement(s)

P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P280	Wear protective gloves/ protective clothing.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

HMIS Classification

Health hazard:	2
Chronic Health Hazard:	*
Flammability:	0
Physical hazards:	0

Potential Health Effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.
Ingestion	May be harmful if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : 5-HT
3-(2-Aminoethyl)-5-hydroxyindolehydrochloride
5-Hydroxytryptaminehydrochloride

Formula : $C_{10}H_{12}N_2O \cdot HCl$

Molecular Weight : 212.68 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
3-(2-Aminoethyl)-indol-5-ol hydrochloride			
153-98-0	-	-	-

4. FIRST AID MEASURES

General advice

Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled

If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact

Wash off with soap and plenty of water. Consult a physician.

In case of eye contact

Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed

Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIREFIGHTING MEASURES

Conditions of flammability

Not flammable or combustible.

Suitable extinguishing media

Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for firefighters

Wear self contained breathing apparatus for fire fighting if necessary.

Hazardous combustion products

Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen chloride gas

Explosion data - sensitivity to mechanical impact

no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

Light sensitive. Keep in a dry place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator.For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Safety glasses with side-shields conforming to EN166 Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	powder
Colour	light grey

Safety data

pH	no data available
Melting point/freezing point	Melting point/range: 149 - 154 °C (300 - 309 °F) - lit.
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
Light.

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen chloride gas
Other decomposition products - no data available

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation

no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity

no data available

Carcinogenicity

IARC:	No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.
ACGIH:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

Overexposure may cause reproductive disorder(s) based on tests with laboratory animals.

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
Inhalation - May cause respiratory irritation.

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	May be harmful if swallowed.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Signs and Symptoms of Exposure
Damage to the heart.

Synergistic effects
no data available

Additional Information
RTECS: NM2571000

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product

Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material.

Contaminated packaging

Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)

Not dangerous goods

IMDG

Not dangerous goods

IATA

Not dangerous goods

15. REGULATORY INFORMATION

WHMIS Classification

D2A	Very Toxic Material Causing Other Toxic Effects	Reproductive hazard
D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant
		Moderate respiratory irritant
		Moderate eye irritant

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Corporation and its Affiliates shall not be held liable for any damage resulting from handling or from contact with the above product. See www.sigma-aldrich.com and/or the reverse side of invoice or packing slip for additional terms and conditions of sale.