Modification Form for Permit BIO-RRI-0028

Permit Holder: J.G. Pickering

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

~~Faran Vafaie~~

Oula Akawi

~~Theo Small~~

Caroline Oneil

Zengxuan Nong

~~Matt Frontini~~

Alanna Watson

Additional Personnel
(Please list additional personnel here)

Brittany Balint
Giovanni-Michael Arpino
Paul Comartin
Hao Yin

	Please stroke out any approved Biohazards to be removed below	**Write additional Biohazards for approval below. Give the full name - do not abbreviate.**
Approved Microorganisms	E.coli dh5 alpha competent cells, AdMax: adenovirus type 5	
Approved Primary and Established Cells	[Primary](Human): Primary smooth muscle cells. (Rodent): fibroblast. [Established] (Human): HEK 293, HAEC, Hela, HT-1080, Fibroblast (transofmred). (Rodent): Renca, 3T3L1, 3T3-Swiss albino, C3H/10T 1/2. (Non-	
Approved Use of Human Source Material	Primary smooth muscle cells	
Approved Genetic Modifications (Plasmids/Vectors)	[Plasmid] - pEGFP-N3, pIRES2-EGFP, pLNCX2, pQCXIN, pQCXIP. [Vectors] - Adeno E1A, Admax vectors (pD311, pDC411, pDC511).	lentiviral cells injected in mice pLKO.1
Approved Use of Animals	Rats, mice	
Approved Biological Toxin(s)		

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

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: J. S. Richardson

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

Date of Last Biohazardous Agents Registry Form: Mar 3, 2010

Date of Last Modification (if applicable): _____

BioSafety Officer(s): Ronald Roseboom November 9, 2010

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

Research Summary

The purpose of this study is to ascertain molecular details of processes that contribute to the formation of new blood vessels. Smooth muscle cells, fibroblasts and endothelial cells are prominent constituents of the new vessels but how they integrate to form vessels in the adult organism is unknown. We will specifically examine human smooth muscle cells and progenitors and their ability to dynamically organize and stabilize the new artery wall. Genes expressing fluorescent proteins will be inserted into self-inactivating lentivirus and infected into cells in culture. The shRNA-expressing lentivirus (Mission lentiplex) will be removed after 3 days and the infected cells will be incubated for 7 days for expression. The subcutaneous injection of matrigel, together with cultured cells stably over-expressing the gene of interest will be injected into the mice subcutaneously and the mice will be sent for experimental analysis 7 – 10 days following injection.

All lentiviral work will be performed in a biosafety level 2 facility in Dr. Husain's lab at the McEwen Centre for Regenerative Medicine at the University of Toronto. The mice will be shipped to the University of Western Ontario and the mice will be housed in a level 2 facility at ACVS for 72 hours (ACVS Modification Form submitted Nov.8/10). Intravital microscopy will be utilized to determine the extent of angiogenesis, cell interactions and vasoreactivity. This procedure will be performed in Dr. Ellis's biosafety level 2 lab in the Medical Sciences Building in Room M498A. This procedure will be done with universal precautions and will use PPE where appropriate. Following the experiment the mice will be sacrificed and the site of angiogenesis will be harvested and analyzed histologically.

Use of an animal system enables the real time analysis of blood vessel formation, so that a thorough analysis can be performed. This proposal aims to elucidate the key steps which may be critical to angiogenesis.

SIGMA-ALDRICH

MATERIAL SAFETY DATA SHEET

Date Printed: 11/08/2010
Date Updated: 12/05/2008
Version 1.2

Section 1 - Product and Company Information

Product Name	MISSION LENTIPLEX POOLED TRC LIBRARY, HUMAN
Product Number	SHPH01
Brand	SIGMA
Company	Sigma-Aldrich Canada, Ltd
Address	2149 Winston Park Drive Oakville ON L6H 6J8 CA
Technical Phone:	9058299500
Fax:	9058299292
Emergency Phone:	800-424-9300

Section 2 - Composition/Information on Ingredient

Substance Name	CAS #	SARA 313
MISSION LENTIPLEX POOLED TRC LIBRARY, HUMAN	None	No

Ingredient Name	CAS #	Percent	SARA 313
FETAL BOVINE SERUM	None	10	No
L-GLUTAMINE	56-85-9	0.06	No
PYRUVIC ACID SODIUM SALT	113-24-6	0.85	No
STREPTOMYCIN	57-92-1	0.01	No
D-BENZYLPENICILLENIC ACID	61-33-6	0.001	No
GLUCOSE	50-99-7	0.405	No
PHENOL RED	143-74-8	<= 0.9	No
SODIUM CHLORIDE	7647-14-5	0.58	No
WATER	7732-18-5	85.6	No
MEM NON-ESSENTIAL AMINO ACIDS SOLUTION 100X	None	<= 1	No
SODIUM BICARBONATE	144-55-8	0.33	No

Section 3 - Hazards Identification

HMIS RATING
HEALTH: 0
FLAMMABILITY: 0
REACTIVITY: 0

NFPA RATING
HEALTH: 0
FLAMMABILITY: 0
REACTIVITY: 0

For additional information on toxicity, please refer to Section 11.

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 breathing becomes difficult, call a physician.

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

EYE EXPOSURE
In case of contact with eyes, flush 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

FLASH POINT
N/A

AUTOIGNITION TEMP
N/A

FLAMMABILITY
N/A

EXTINGUISHING MEDIA
Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.

FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Specific Hazard(s): Emits toxic fumes under fire conditions.

Section 6 - Accidental Release Measures

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.

Section 7 - Handling and Storage

HANDLING
User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE
Suitable: Keep tightly closed.
Store at -70°C

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Safety shower and eye bath. Mechanical exhaust required.

PERSONAL PROTECTIVE EQUIPMENT
Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Respiratory protection is not required. Where protection is desired, use multi-purpose combination (US) or type ABEK (EN

14387) respirator cartridges.
Hand: Protective gloves.
Eye: Chemical safety goggles.

GENERAL HYGIENE MEASURES
Wash thoroughly after handling.

Section 9 - Physical/Chemical Properties

Appearance — Physical State: Liquid

Property	Value	At Temperature or Pressure
pH	N/A	
BP/BP Range	N/A	
MP/MP Range	N/A	
Freezing Point	N/A	
Vapor Pressure	N/A	
Vapor Density	N/A	
Saturated Vapor Conc.	N/A	
Bulk Density	N/A	
Odor Threshold	N/A	
Volatile%	N/A	
VOC Content	N/A	
Water Content	N/A	
Solvent Content	N/A	
Evaporation Rate	N/A	
Viscosity	N/A	
Surface Tension	N/A	
Partition Coefficient	N/A	
Decomposition Temp.	N/A	
Flash Point	N/A	
Explosion Limits	N/A	
Flammability	N/A	
Autoignition Temp	N/A	
Refractive Index	N/A	
Optical Rotation	N/A	
Miscellaneous Data	N/A	
Solubility	N/A	

N/A = not available

Section 10 - Stability and Reactivity

STABILITY
Stable: Stable.
Materials to Avoid: Strong oxidizing agents.

HAZARDOUS DECOMPOSITION PRODUCTS
Hazardous Decomposition Products: Carbon monoxide, Carbon dioxide.

HAZARDOUS POLYMERIZATION
Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

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

Ingestion: May be harmful if swallowed.

SIGNS 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

No data 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 combustible 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 Air Transport: Non-hazardous for air transport.

Section 15 - Regulatory Information

UNITED STATES REGULATORY INFORMATION
SARA LISTED: No

CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: Yes
NDSL: No

Section 16 - Other 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.

SIGMA-ALDRICH

MATERIAL SAFETY DATA SHEET

Date Printed: 11/08/2010
Date Updated: 07/07/2009
Version 1.2

Section 1 - Product and Company Information

Product Name	MISSION TRANSDUCTION CONTROL, DNA, PLKO.1-PURO CMV-TAGCFP
Product Number	SHC010
Brand	SIGMA
Company	Sigma-Aldrich Canada, Ltd
Address	2149 Winston Park Drive Oakville ON L6H 6J8 CA
Technical Phone:	9058299500
Fax:	9058299292
Emergency Phone:	800-424-9300

Section 2 - Composition/Information on Ingredient

Substance Name	CAS #		SARA 313
MISSION® HUMAN CONTROL VECTOR	None		No
Ingredient Name	**CAS #**	**Percent**	**SARA 313**
The hazards identified with this product are those associated with the following component(s):	None		
TRIS-EDTA BUFFER 100X CONCENTRATE	None	1	No

Section 3 - Hazards Identification

EMERGENCY OVERVIEW

Irritant.
Irritating to eyes, respiratory system and skin.

HMIS RATING

HEALTH: 2
FLAMMABILITY: 0
REACTIVITY: 0

NFPA RATING

HEALTH: 2
FLAMMABILITY: 0
REACTIVITY: 0

For additional information on toxicity, please refer to Section 11.

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.

Section 5 - Fire Fighting Measures

FLASH POINT
N/A

AUTOIGNITION TEMP
N/A

FLAMMABILITY
N/A

EXTINGUISHING MEDIA
Suitable: Water spray. Carbon dioxide, dry chemical powder, or appropriate foam.

FIREFIGHTING
Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Specific Hazard(s): Emits toxic fumes under fire conditions.

Section 6 - Accidental Release Measures

PROCEDURE(S) OF PERSONAL PRECAUTION(S)
Wear respirator, chemical safety goggles, rubber boots, and heavy rubber gloves.

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.

Section 7 - Handling and Storage

HANDLING
User Exposure: Do not breathe vapor. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE
Store at -20°C

Section 8 - Exposure Controls / PPE

ENGINEERING CONTROLS
Mechanical exhaust required. Safety shower and eye bath.

PERSONAL PROTECTIVE EQUIPMENT
Respiratory: Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Where risk assessment shows air-purifying respirators are appropriate use a full-face respirator with multi-purpose combination (US) or type ABEK (EN 14387) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator.
Hand: Compatible chemical-resistant gloves.

Eye: Chemical safety goggles.

GENERAL HYGIENE MEASURES
Wash thoroughly after handling.

Section 9 - Physical/Chemical Properties

Appearance: Physical State: Liquid

Property	Value	At Temperature or Pressure
pH	N/A	
BP/BP Range	N/A	
MP/MP Range	N/A	
Freezing Point	N/A	
Vapor Pressure	N/A	
Vapor Density	N/A	
Saturated Vapor Conc.	N/A	
Bulk Density	N/A	
Odor Threshold	N/A	
Volatile%	N/A	
VOC Content	N/A	
Water Content	N/A	
Solvent Content	N/A	
Evaporation Rate	N/A	
Viscosity	N/A	
Surface Tension	N/A	
Partition Coefficient	N/A	
Decomposition Temp.	N/A	
Flash Point	N/A	
Explosion Limits	N/A	
Flammability	N/A	
Autoignition Temp	N/A	
Refractive Index	N/A	
Optical Rotation	N/A	
Miscellaneous Data	N/A	
Solubility	N/A	

N/A = not available

Section 10 - Stability and Reactivity

STABILITY
Stable: Stable.
Materials to Avoid: Strong oxidizing agents.

HAZARDOUS DECOMPOSITION PRODUCTS
Hazardous Decomposition Products: Nature of decomposition products not known.

HAZARDOUS POLYMERIZATION
Hazardous Polymerization: Will not occur

Section 11 - Toxicological Information

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

SIGNS 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

No data 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 combustible 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 Air Transport: Non-hazardous for air transport.

Section 15 - Regulatory Information

US CLASSIFICATION AND LABEL TEXT
Indication of Danger: Irritant.
Risk Statements: Irritating to eyes, respiratory system and skin.
Safety Statements: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

UNITED STATES REGULATORY INFORMATION
SARA LISTED: No

CANADA REGULATORY INFORMATION
WHMIS Classification: This product has been classified in accordance with the hazard criteria of the CPR, and the MSDS contains all the information required by the CPR.
DSL: No
NDSL: No

Section 16 - Other 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.

Cell

A Lentiviral RNAi Library for Human and Mouse Genes Applied to an Arrayed Viral High-Content Screen

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DOI 10.1016/j.cell.2006.01.040

SUMMARY

To enable arrayed or pooled loss-of-function screens in a wide range of mammalian cell types, including primary and nondividing cells, we are developing lentiviral short hairpin RNA (shRNA) libraries targeting the human and murine genomes. The libraries currently contain 104,000 vectors, targeting each of 22,000 human and mouse genes with multiple sequence-verified constructs. To test the utility of the library for arrayed screens, we developed a screen based on high-content imaging to identify genes required for mitotic progression in human cancer cells and applied it to an arrayed set of 5,000 unique shRNA-expressing lentiviruses that target 1,028 human genes. The screen identified several known and ~100 candidate regulators of mitotic progression and proliferation; the availability of multiple shRNAs targeting the same gene facilitated functional validation of putative hits. This work provides a widely applicable resource for loss-of-function screens, as well as a roadmap for its application to biological discovery.

INTRODUCTION

The information available from genome sequencing efforts has transformed the nature of biological inquiry and has led to an increased need for tools that enable genome-scale functional studies. Sequencing the *Saccharomyces cerevisiae* genome fundamentally altered experimental approaches and led to the creation and widespread use of a yeast gene-deletion collection that has dramatically facilitated studies of gene function (Winzeler et al., 1999). Similarly, in model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, the recognition that RNA interference (RNAi) can be exploited to suppress gene expression (Fire et al., 1998; Kennerdell and Carthew, 1998) has led to the rapid identification of the genes underlying many biological processes through powerful loss-of-function screens (Bettencourt-Dias et al., 2004; Boutros et al., 2004; Fraser et al., 2000; Kamath et al., 2003; Kiger et al., 2003; Lum et al., 2003). Although powerful genetic tools already existed for both *D. melanogaster* and *C. elegans*, the availability of genome-scale libraries of RNAi reagents has facilitated comprehensive and, at the same time, increasingly complex loss-of-function screens.

RNAi also suppresses gene expression in mammalian cells (Elbashir et al., 2001), and chemically synthesized siRNAs have become essential tools for biological studies. Indeed, screens in human cells using commercially available libraries of synthetic siRNAs targeting defined gene families have identified modulators of TRAIL-induced apoptosis (Aza-Blanc et al., 2003) and cell survival (Mackeigan et al., 2005) as well as kinases required for clathrin- and caveolae-mediated endocytosis (Pelkmans et al., 2005). Unfortunately, many interesting mammalian cell types are resistant to the transfection methods needed to introduce synthetic siRNAs into cells.

An alternative approach is to transduce mammalian cells with viruses carrying expression cassettes that encode short hairpin RNAs (shRNAs) to generate gene-specific siRNAs within cells; this approach can achieve stable and highly effective gene suppression in a variety of mammalian cell types (Abbas-Terki et al., 2002; Brummelkamp et al., 2002; Paddison et al., 2002; Stewart et al., 2003). Using large libraries of shRNA-expressing retroviral vectors, one group screened pools of retroviruses and identified components of the p53 pathway (Berns et al., 2004). Another group screened by transfecting cells with shRNA-expressing retroviral plasmids and identified genes involved in proteasome function (Paddison et al., 2004; Silva et al., 2005). Recently, the same two libraries were used to identify two novel tumor-suppressor genes (Kolfschoten et al., 2005; Westbrook et al., 2005). While these reports establish the precedent that shRNA libraries can be employed to perform loss-of-function screens in mammalian cells, it is clear that further exploration of the performance characteristics and limitations of such approaches is necessary before such large-scale applications become routine.

The ideal resource for mammalian genetics would consist of a widely available shRNA library that contains effective suppressors of all ~20,000 human and mouse genes in a format that permits transduction of a wide range of cell types, including nondividing cells and primary cells in both "pooled" and "arrayed" formats. Arrayed screens, in which each shRNA is tested in an individual well, allow the study of biologically subtle and complex phenotypes—for example, by high-content imaging of cells in individual wells. This requires the development of protocols for efficient production of a high-titer viral stock for each shRNA. Such a resource would allow biomedical researchers to perform comprehensive and reliable loss-of-function screens to identify all genes that affect a wide range of cellular processes.

We formed The RNAi Consortium (TRC) with the goals of generating genome-scale shRNA libraries in viral vectors and developing efficient protocols for arrayed viral screens. The TRC library is designed to target most human and mouse genes, with multiple distinct constructs targeting each gene. The lentiviral vectors in this library, unlike their oncoretroviral counterparts, can infect nondividing cells, a crucial asset for suppressing gene expression in tissues or cell lines refractory to transfection (Federico, 2003).

Here, we describe the creation of the initial portion of the TRC lentiviral shRNA library and characterize its properties. The library (designated TRC1) currently contains constructs targeting 22,000 human and mouse genes, with ~5 distinct shRNA constructs per gene. We address several significant challenges for efficient RNAi screening, including the variable effectiveness of different shRNA constructs, the potential for off-target effects, and the technical requirements for producing the high-titer viruses needed for arrayed screens. In addition, we have applied a subset of the TRC1 library in an arrayed virus-mediated shRNA screen to identify candidate regulators of mitotic progression in human colon cancer cells, using high-content imaging. We characterized the performance of the library in the context of this screen and identified 100 genes for which at least two independent shRNAs produce substantial and consistent changes in mitotic index. These genes represent a collection of candidate regulators of mitosis that merit further cell biological study. The TRC1 library offers a new resource for somatic-cell genetics, and its application in this study provides insights into the use of shRNA reagents in loss-of-function screens in mammalian cells.

RESULTS

shRNA Library Production

To generate the TRC lentiviral library, we adapted the Lentihair vector (Stewart et al., 2003) to create pLKO.1, which carries the puromycin-resistance gene and drives shRNA expression from a human *U6* promoter (Figure 1A). Although significant levels of recombination are often observed in retroviral vectors maintained in bacteria, we found that pLKO.1 undergoes very low levels of recombination during the cloning and plasmid-purification manipulations necessary for library construction. Specifically, diagnostic restriction digests of plasmid DNA from 244 library clones showed no evidence of recombination even after 10 rounds of sequential copying and regrowth (see Figure S1 in the Supplemental Data available with this article online), confirming that shRNA-containing pLKO.1 vectors remain structurally stable in bacteria.

We created a production pipeline (Figure 1B) to generate a library of sequence-verified shRNAs in pLKO.1. For each shRNA, we designed stem sequences matching a 21-base region of the target transcript with an intervening 6-base "loop" consisting of an XhoI site (Figure 1A). The 21-mer stem sequences were selected using previously described criteria for siRNA construction that attempt to maximize knockdown (Khvorova et al., 2003; Schwarz et al., 2003) and minimize off-target effects, as well as to ensure that most genes in the library contain shRNAs that target both the 3′ untranslated region (UTR) and coding sequence (CDS) of their transcripts (see Supplemental Data). Oligonucleotide pairs for 90 hairpin sequences were annealed separately and ligated into pLKO.1, and the ligations were transformed into competent bacteria in a 96-well microtiter plate. The 90 transformations in each plate were then pooled and plated onto a large agar plate. A total of 672 colonies were selected robotically for growth, plasmid purification, and sequencing. This process yields 94% of the designed clones (Figure S2); each gene has an average of 4.7 unique shRNA constructs, and 96% of the genes have four or more different constructs.

The TRC1 library currently includes over 100,000 vectors, targeting 12,000 human and 10,000 mouse genes. We continue to generate ~4,500 additional constructs per month. Detailed information on genes targeted in the

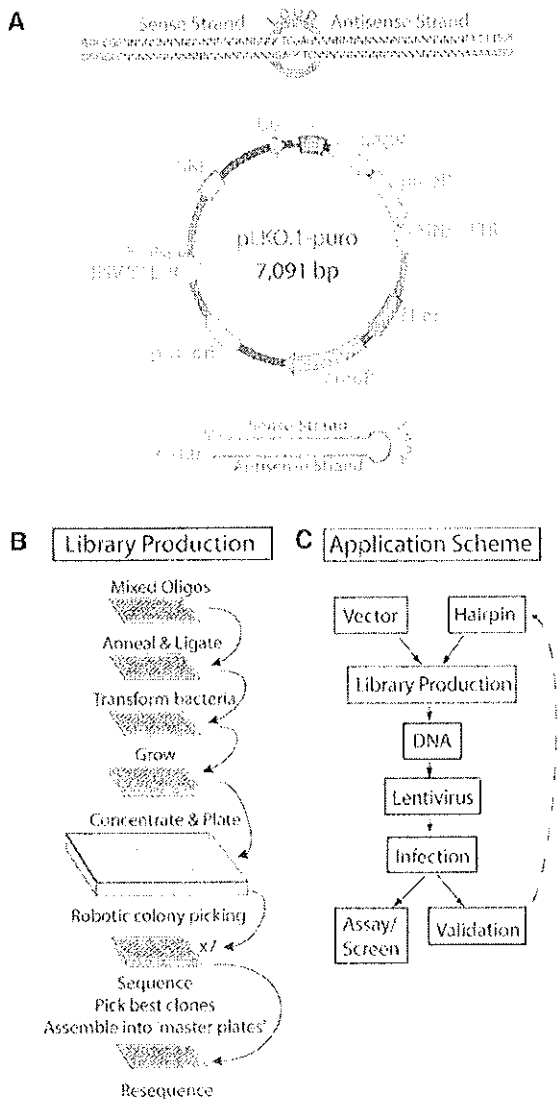

Figure 1. Vector Features, Library Production, and Application

(A) pLKO.1.

(B) Bacterial glycerol stock production method.

(C) Scheme for library production and use.

library can be found at http://www.broad.mit.edu/genome_bio/trc/rnai.html.

High-Throughput Lentivirus Production

To exploit this library, we developed a high-throughput (HT) method to generate high-titer lentiviruses (Figure 1C). Specifically, we optimized a semiautomated procedure in 96-well plates in which HEK293T cells were transfected with library and packaging plasmids in a three-plasmid lentivirus packaging system (Naldini et al., 1996; Zufferey et al., 1997). We collected 300 μl of transfected cell supernatants containing VSV-G pseudotyped lentiviruses over 36–60 hr and aliquoted and stored these lentivirus-containing supernatants at −80°C. As described below, a typical screen was performed in 384-well plates and used only ~3 μl of lentiviral supernatant per well. Thus, the procedure above yields sufficient volumes of lentiviral supernatants from a single 96-well plate for ~100 screens.

To monitor the infection efficiency of lentiviruses generated by this HT method, we measured the proportion of cells that acquire resistance to puromycin treatment following infection. We infected A549 lung cancer cells with an arrayed set of ~1,500 distinct shRNA-expressing lentiviruses. The experiment was performed in duplicate, with puromycin added to one replicate and the other replicate left untreated. We calculated the ratio of cell numbers in paired wells (with and without puromycin treatment) after 4 days. Wells were designated as successfully infected if this ratio exceeded 0.25. By this criterion, 87% of the ~1,500 lentiviruses yielded successful infections (Figure S3), consistent with the viral titers measured for a random sampling of library lentiviruses of 2×10^6–2×10^7 cfu/ml (data not shown). These data indicate that this HT process generates lentiviral stocks of sufficiently high titer to infect target cells without the need to normalize titers among wells or to concentrate the lentiviral stocks.

One attractive feature of this lentiviral library is its ability to transduce a wide range of cell types, including primary and nondividing cells. We successfully infected several primary cell types, including mouse embryonic stem cells, mouse embryonic fibroblasts, and rat neonatal cardiomyocytes as well as extremely slow-growing or nondividing cells, including HCN-1A human cortical neurons, with pLKO.1-based lentiviruses (Figure S4). We and others have now successfully used pLKO.1 and its derivatives to infect many cell types (Table S1), confirming that this library can be used to study a wide range of mammalian cells.

We next asked whether the viral titers generated by our HT process are sufficient to suppress gene expression. Using quantitative RT-PCR (qRT-PCR), we determined whether shRNAs specific for 12 tyrosine kinases affected the mRNA levels of these targets in A549 lung cells infected in duplicate experiments. For all 12 genes, at least one of the shRNA viruses reproducibly decreased target transcript levels (Figure 2A), and, for 10 out of 12 genes, the shRNA virus that produced the best knockdown reduced mRNA levels greater than 4-fold. Overall, 31% of the 54 lentiviruses reduced transcript levels by greater than 4-fold (Figure 2B). We observed similar patterns and levels of knockdown when infecting HT29 colon carcinoma cells with the same lentiviruses (data not shown). Thus, lentiviruses produced by this HT process reproducibly and strongly suppress gene expression, suggesting that virus-containing supernatants can be used directly for primary arrayed screens.

For some screening applications, the use of viral pools is advantageous; however, this format requires that infection of a cell with a single copy of an shRNA-expressing

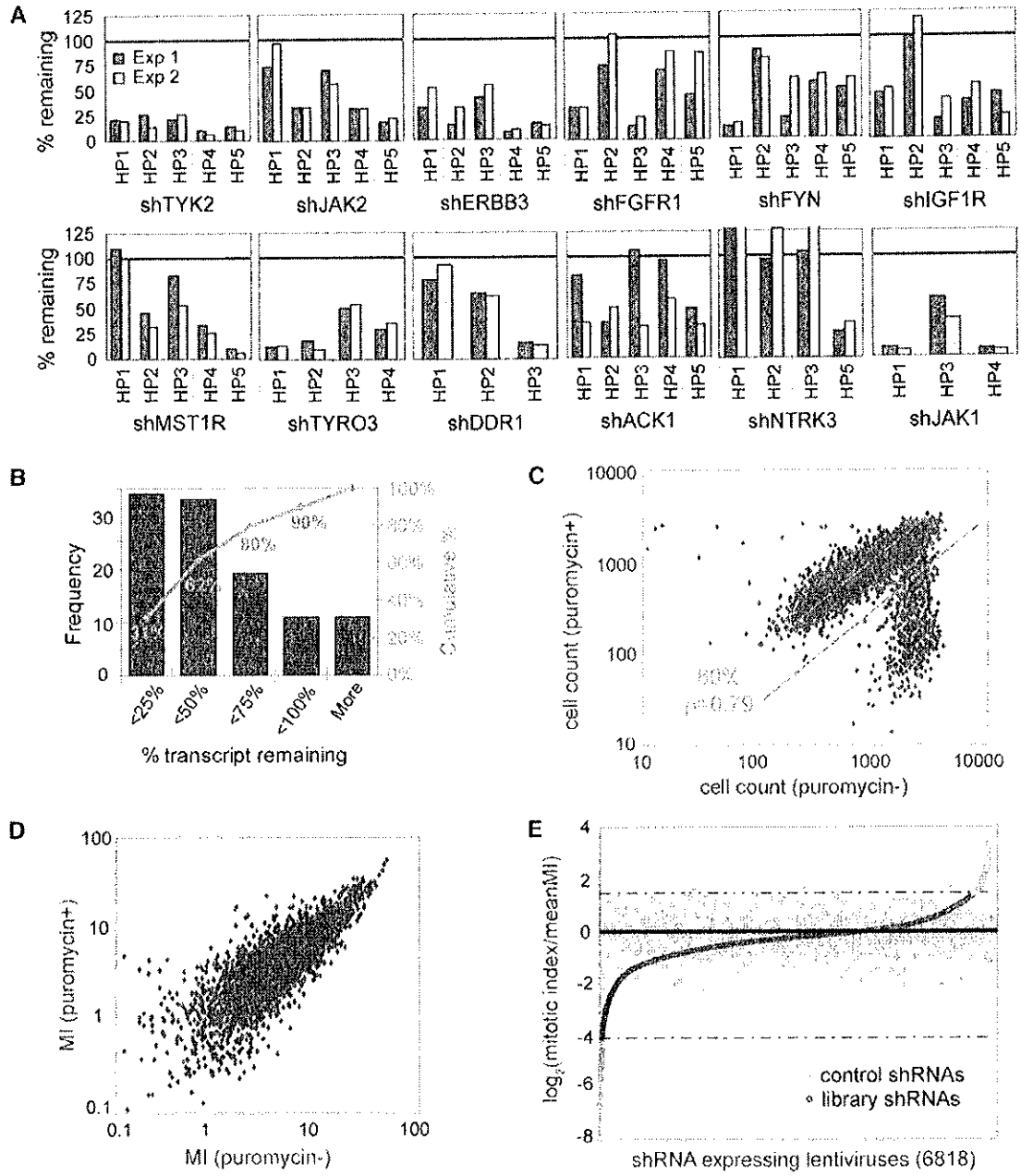

Figure 2. Knockdown Performance of HT-Generated Lentivirus in A549 Cells and Mitotic-Index Screen in HT29 Cells

(A) Knockdown performance of lentiviruses representing 54 shRNAs targeting 12 different tyrosine kinases. Transcript levels for duplicate experiments were measured by qRT-PCR and are reported for each shRNA hairpin relative to average transcript levels for two control infections (i.e., an shRNA targeting either lamin A/C or scrambled sequence). Knockdown for the first set of infections is shown by dark blue bars and the second set of infections by light blue bars.

(B) Summary of knockdown levels for the duplicate infections of the 54 shRNA viruses from (A).

(C and D) Cell counts (C) and MI scores (D) following infection of HT29 cells with TRC1 as determined by automated image analysis with, versus without, puromycin selection.

(E) Distribution of MI scores for all shRNA infections. MI scores for library shRNAs are sorted in order of increasing MI and are marked by red (low), blue (normal), and green (high) diamonds. MI scores for 700 control shRNAs are displayed in gray in random order to indicate the background range of MI. High and low MI thresholds for selection of MI hits are marked by the dashed lines.

virus suffices to cause a phenotype. We observed that lentiviruses expressing shRNAs targeting *FASTK* or *AKT3* (two essential genes) kill HT29 cells even at concentrations where the cells are infected by a single lentivirus (Figure S5).

High-Content Screen for Regulators of Mitosis

Mitotic-Index Assay

We next sought to characterize the utility of the shRNA library in an arrayed screen with high-content imaging. We chose to focus on the regulation of mitosis in human HT29 colon cancer cells, a cell line that has been widely used for the study of many normal and neoplastic processes. We selected a subset of the TRC1 library consisting of 4,903 unique shRNA-expressing lentiviruses targeting 1,028 human genes (Table S2) with a single, distinct shRNA-expressing lentivirus in each well. The targeted genes included 476 protein kinases and 180 phosphatases that represent 88% and 80%, respectively, of known NCBI reference sequences assigned to these gene classes (Manning et al., 2002). The remaining 372 genes included nonprotein kinases, tumor suppressors, and DNA binding and modification enzymes.

To detect cells in mitosis, we used automated fluorescence microscopy and image analysis to identify the cells in each well that contain histone H3 phosphorylated on serine 10 (pH3), a well-established marker for mitotic cells. Substantial evidence indicates that pH3 levels also correlate with proliferation rate and that the intracellular pattern of pH3 staining differentiates between stages of mitosis (Gasparri et al., 2004; Hendzel et al., 1997). In addition, we visualized all cells with a DNA binding dye (Hoechst) to identify nuclei and measure DNA content and an actin stain (phalloidin) to detect cytoplasmic size and shape. We calculated the fraction of cells in mitosis (mitotic index, or MI) by dividing the number of pH3-positive cells by total cell number. As a second independent measure of the effect of gene suppression on mitosis, we extracted histograms of DNA content from the Hoechst images.

A test of viral doses showed that the addition of 0.5–4.0 μl of lentiviral stocks per well of a 384-well plate yielded high rates of infection in HT29 cells without reductions in cell counts from toxicity (Figures S6A and S6B). To screen for mitotic regulators, we used 3 μl of library lentiviruses to infect HT29 cells in 384-well plates and cultured duplicate sets in the presence or absence of puromycin. This dose corresponded to an average moi of ~5. Four days after infection, cells were fixed; stained for pH3, DNA, and actin; and imaged using an automated fluorescence microscope. The MI was determined by automated image analysis. We determined that MI did not depend on viral dose for a number of control and MI-altering shRNAs (Figure S6C). The accuracy of the automated analysis was verified by direct visual inspection of ~9% of the 13,551 composite images produced in the screen.

We successfully screened 4,903 distinct shRNAs. Based on the same puromycin-selection test used for the A549 infections, 80% of lentiviruses successfully infected HT29 target cells; the correlation coefficient between cell numbers in puromycin-treated wells and untreated wells was $\rho = 0.79$ (Figure 2C). As expected based on the high rate of infection, mitotic indices obtained with and without puromycin selection were in good agreement for each lentivirus (Figure 2D), and we therefore averaged these measurements for subsequent analyses. The average MI for all infected HT29 cells was 5.1. The data approximately fit a Poisson distribution in its central regions, but with wider tails representing significant outliers in cell-cycle distribution (Figure 2E).

Based on visual inspection of 1,185 fluorescent images, we found that images from wells with MI > 9 or MI < 1 show intensities and patterns of pH3 staining that are distinct from typical wells (MI ~ 5). Moreover, the MI values and visually observed morphological changes were consistent across repeat infections.

Analysis of Known Mitotic Regulators

We first examined whether shRNAs targeting genes known to play important roles in regulating the cell cycle induced changes in MI. For example, inhibition of *CDC2/CDK1*, the canonical cyclin-dependent kinase that controls progression through G2/M (Harborth et al., 2001), was expected to cause a G2/M arrest with faint punctate staining of the pH3 mitotic marker in our assay. A lentivirus targeting *CDC2* (shCDC2-820) induced a uniform faint punctate pH3 staining pattern characteristic of G2/M phase arrest (Figure 3A). Image analysis computed an MI of 9.7, and visual examination of the images revealed that, in fact, a majority of cells exhibited pH3 staining. DNA content analysis confirmed that shCDC2-820 caused a dramatic G2/M shift (Figure 3A, right). Additional experiments confirmed that shCDC2-820 suppressed the expression of the Cdc2 protein and, as expected, caused decreases in cyclin B levels without affecting levels of Cdk2 or α-tubulin (Figure 3B).

We next examined shRNAs targeting aurora B (*AURKB*), a kinase that directly phosphorylates serine 10 of histone H3 during mitosis (Keen and Taylor, 2004). Three distinct shRNAs targeting *AURKB* (shAURKB-1185, shAURKB-468, and shAURKB-558) reproducibly induced low MIs and characteristic multinucleate phenotypes in infected cells (Figure 3C). Moreover, an obvious shift toward the G2/M (shAURKB-1185) or polyploid state (shAURKB-468 and shAURKB-558) was observed in DNA content histograms extracted from the primary screening images (Figure 3C, bottom). In immunoblot analyses, these shRNAs strongly reduced AurkB expression and pH3 levels without affecting the expression of the closely related aurora A gene (*AURKA*) (Figure 3D). We note that the lentiviruses carrying shAURKB-468 and shAURKB-558 that induced a more complete knockdown of AurkB also resulted in more severe polyploidy.

A number of additional genes known to regulate the cell cycle and mitotic progression showed high (>14) or low (<0.3) MIs in the screen (Tables S3A and S3B). For example, shRNAs targeting the cell-cycle effectors *PLK1* (shPLK1-513) and *CDK2* (shCDK2-923) caused large

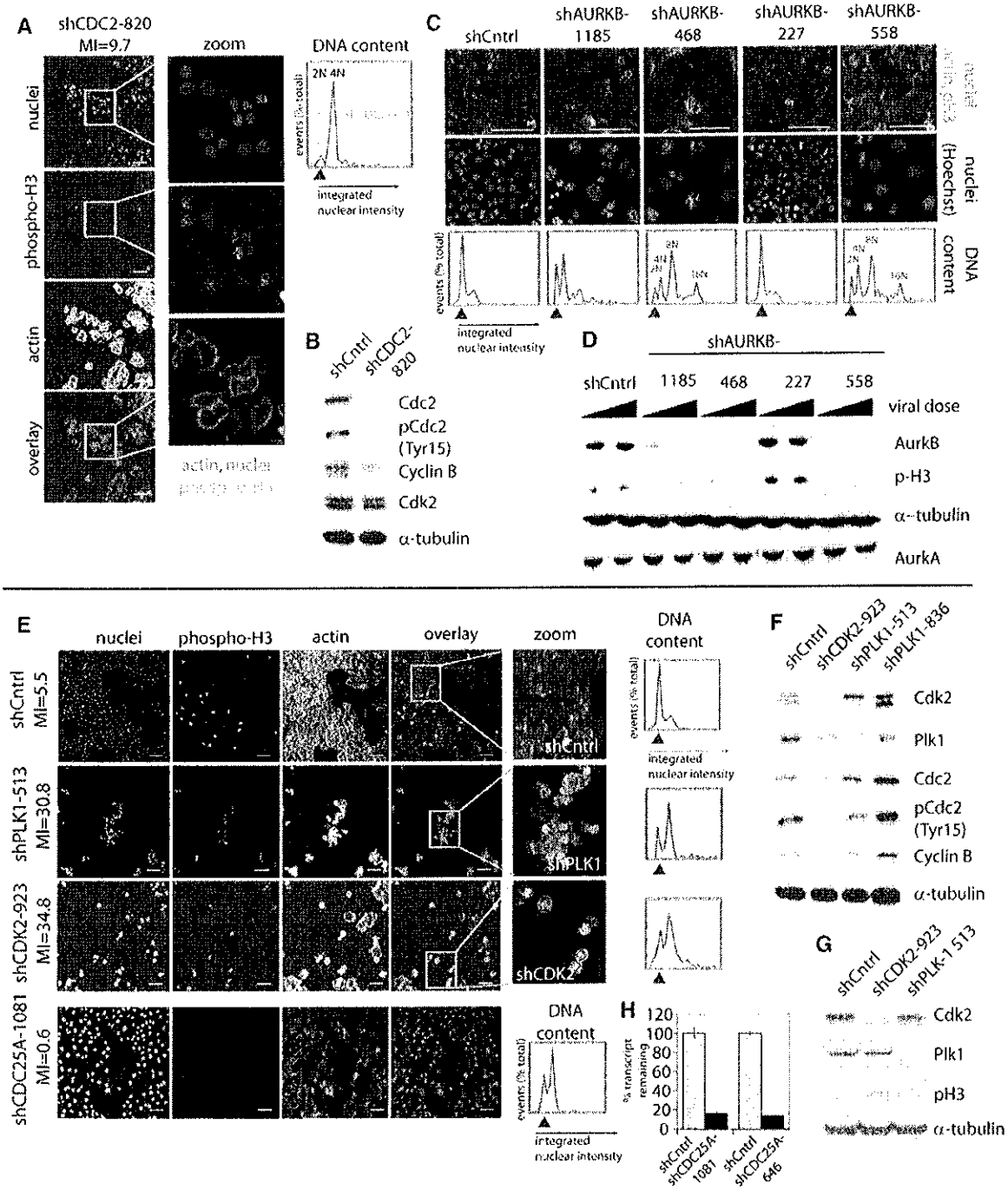

Figure 3. Identification and Target Verification of Known Regulators of Mitosis

(A) Images of HT29 cells following shRNA-induced knockdown of *CDC2* (shCDC2-820) that gave an elevated MI = 9.7 from the primary screen (all channels for the same field are shown). DNA content histograms are shown to the right for shCDC2-820-induced knockdown of *CDC2* (blue line) and shRNA control (shCntrl) infections (gray line). The percentage of total events is shown on the vertical axis and the integrated nuclear intensity on the horizontal axis. The control histogram is the average for ten images taken from control infections. The black solid triangle indicates the normal G1 DNA content peak for HT29 cells.

(B) Immunoblot analysis of Cdc2, tyrosine 15-phosphorylated Cdc2, cyclin B, Cdk2, and α-tubulin protein levels following shRNA knockdown with either shCntrl (targeting *GFP*) or shCDC2-820 (targeting *CDC2*) in HT29 cells. Cdk2 and α-tubulin were included as loading controls.

(C) Knockdown of aurora B in HT29 cells. Images are of aurora B (AURKB) knockdown cells from four distinct shRNAs targeting *AURKB* (shAURKB-1185, shAURKB-468, shAURKB-227, and shAURKB-558) as well as a control infection (shCntrl). Top panels show overlays (blue = nuclei, green = pH3,

increases in MI (to 30 and 35, respectively), and images from the primary screen show a concomitant drop in cell numbers for both (Figure 3E). The shRNA shPLK1-513 caused a dramatic G2/M shift, and shCDK2-923 induced an increase in S phase and G2/M phase cells (Figure 3E). We confirmed that shPLK1-513 decreased Plk1 expression without significantly affecting Cdc2 or cyclin B (Figure 3F), whereas shCDK2-923 decreased Cdk2 expression and led to the expected drop in cyclin B and phospho-Tyr-Cdc2 (Figure 3F). A second shRNA targeting *PLK1* (shPLK1-836) that failed to affect MI did not decrease Plk1 protein levels (Figure 3F).

To determine whether these shRNAs induced a similar phenotype in another cell type, we infected BJ-hTERT fibroblasts (Hahn et al., 1999) with shPLK1-513- or shCDK2-923-expressing lentiviruses. As expected, shPLK1-513 and shCDK2-923 efficiently knocked down Plk1 and Cdk2, respectively, in BJ-hTERT cells (Figure 3G). Suppression of Cdk2 in BJ-hTERT cells led to increased pH3 levels (Figure 3G), as was observed in HT29 cells (Figure 3E). In contrast, knockdown of Plk1 in these cells did not affect pH3 levels (Figure 3G), corroborating a report showing that small-molecule inhibition of Plk1 in human fibroblasts fails to cause a G2/M arrest (Gumireddy et al., 2005).

Finally, two distinct shRNAs targeting *CDC25A*, a phosphatase required for dephosphorylation of tyrosine 15 of Cdc2 and progression from G2 to M, induced low MI in the primary screen, and we verified that these shRNAs decrease *CDC25A* transcript levels (Figure 3H). The DNA content histogram for cells expressing shCDC25A-1081 showed a significant G2 shift (Figure 3E, bottom). In addition, shRNAs targeting other genes with known roles in cell-cycle regulation, including *PLK2*, *PLK4*, *CHEK1*, *SMAD4*, and *BUB1*, also caused altered MI values (Tables S3A and S3B). These findings demonstrate that our mitotic screening assay responds to suppression of known cell-cycle regulators and thus is a sensitive tool to identify additional mitotic regulators.

Novel Regulators of Mitosis

We then examined the results of the screen to identify potential novel mitotic regulators. A gene was defined as a "hit" if at least two independent shRNAs targeting the gene showed notably high or low MI values. We required that two independent shRNAs produce consistent phenotypes to reduce the chance that genes identified in this screen were due to off-target effects of shRNAs (Jackson and Linsley, 2004). We required that both shRNAs exceed the threshold noted above (MI > 9 or < 1) and that at least one exceed a more stringent threshold (MI > 14 or < 0.3) (Tables S3A and S3B). In addition, we measured changes in the expression levels of three genes known to be induced by interferon (*INFB1*, *OAS1*, and *OAS2*) after infection of cells with a selection of shRNAs that scored in our screen. None of these shRNAs induced the interferon pathway (Figure S7).

The screen yielded 87 genes associated with high MI (Table 1 and Table S4) and 15 genes associated with low MI (Table 2). To understand the cell-cycle effects caused by suppression of these genes, we reanalyzed primary screen images to assess changes in DNA content. Figure 4 and Figure 5A show images for two distinct shRNAs targeting genes from Table 1 and Table 2 and corresponding DNA histograms superimposed in yellow. These images reveal a rich panoply of morphological features that accompany the changes in mitotic index and cell-cycle distribution. Notably, some sets of genes show strikingly similar phenotypes, suggesting that they may function in the same pathway. For example, shRNAs targeted against *PAK7*, *FGR*, and *NTRK2* show high MI and common changes in morphology, including enlarged cell and nuclear sizes and intense actin staining on the cell periphery (Figure 4). Nearly all of the shRNAs yielding high MI also produced substantive changes in the DNA content distribution. The shRNAs targeting *PDGFRB* and *U2AF2* resulted in a particularly marked G2/M arrest (Figure 4). The low-MI hits showed an even greater diversity of cell morphologies, most accompanied by altered DNA content profiles. The shRNAs targeted against *GSK3β* and *SGK3* resulted in cells with extended processes and DNA content histograms with predominant G1 peaks, while those targeted against *BUB1B* and *PAK4* caused greatly enlarged cell and nuclear sizes and a G2/M arrest (Figure 5A).

We visually inspected the patterns of pH3 staining in the primary screening images to determine if the cell populations showed overrepresentation of specific phases of mitosis relative to controls. A number of genes did show

red = actin) and lower panels show Hoechst staining. Bottom panels show the corresponding DNA content histograms for each shRNA. The solid black triangle indicates the normal G1 DNA content peak for HT29 cells.

(D) Immunoblot analysis of AurkB, pH3, α-tubulin, and AurkA levels following shRNA knockdown with shCntrl virus (targeting *GFP*), shAURKB-1185, shAURKB-468, shAURKB-227, or shAURKB-558 viruses in HT29 cells. Each infection was done at two viral doses (1 and 4 μl). α-tubulin served as a loading control.

(E) Images of *PLK1*, *CDK2*, and *CDC25A* knockdown HT29 cells from the primary mitotic-index screen with high (shPLK1-513 and shCDK2-923) or low (shCDC25A-1081) MIs. Corresponding DNA content histograms are shown to the right. The solid black triangles indicate the normal G1 DNA content peak in HT29 cells.

(F) Immunoblot analysis of Cdk2, Plk1, Cdc2, tyrosine-phosphorylated Cdc2, cyclin B, and α-tubulin levels following shRNA knockdown targeting *CDK2* (shCDK2-923) and *PLK1* (shPLK1-513) in HT29 cells. shPLK1-836 served as a negative control for Plk1 knockdown.

(G) Immunoblot analysis of Cdk2, Plk1, pH3, and α-tubulin levels following shRNA knockdown targeting *CDK2* (shCDK2-923) and *PLK1* (shPLK1-513) in BJ-TERT fibroblasts.

(H) Quantitative RT-PCR analysis of *CDC25A* transcript levels following knockdown with two distinct shRNAs (shCDC25A-1081 and shCDC25A-646) in HT29 cells. Error bars indicate the standard error for three qPCR measurements. Scale bars = 50 μm.

Table 1. Subset of Gene Targets for which Two or More shRNAs Induced an Increase in MI

Gene ID	Symbol	Hairpin Name	Average MI	Description
7145	TNS	shTNS-6197	43.0	tensin
		shTNS-5263	13.4	
2268	FGR	shFGR-385	28.1	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
		shFGR-460	26.1	
		shFGR-339	9.7	
5159	PDGFRB	shPDGFRB-2371	30.6	platelet-derived growth factor receptor, beta polypeptide
		shPDGFRB-1985	16.1	
4915	NTRK2	shNTRK2-2123	34.1	neurotrophic tyrosine kinase, receptor, type 2
		shNTRK2-1968	15.6	
7525	YES1	shYES1-905	25.4	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
		shYES1-1338	19.8	
7075	TIE1	shTIE1-3795	24.5	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
		shTIE1-3206	12.4	
5571	PRKAG1	shPRKAG1-157	24.0	protein kinase, AMP-activated, gamma 1 noncatalytic subunit
		shPRKAG1-565	23.4	
		shPRKAG1-1269	15.4	
5997	RGS2	shRGS2-510	25.6	regulator of G protein signaling 2, 24 kDa
		shRGS2-508	20.9	
		shRGS2-158	13.6	
11183	MAP4K5	shMAP4K5-2826	23.6	mitogen-activated protein kinase kinase kinase kinase 5
		shMAP4K5-2158	13.5	
11338	U2AF2	shU2AF2-606	31.2	U2 (RNU2) small nuclear RNA auxiliary factor 2
		shU2AF2-289	12.9	
5563	PRKAA2	shPRKAA2-1028	31.0	protein kinase, AMP-activated, alpha 2 catalytic subunit
		shPRKAA2-2127	13.0	
5651	PRSS7	shPRSS7-1306	21.2	protease, serine, 7 (enterokinase)
		shPRSS7-2651	21.2	
7010	TEK	shTEK-520	27.0	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)
		shTEK-1275	11.2	
		shTEK-1276	9.6	
55137	FIGN	shFIGN-1661	21.3	fidgetin
		shFIGN-1450	18.6	
5922	RASA2	shRASA2-572	23.6	RAS p21 protein activator 2
		shRASA2-1607	14.0	
2869	GRK5	shGRK5-526	22.5	G protein-coupled receptor kinase 5
		shGRK5-356	14.5	
9156	EXO1	shEXO1-1586	25.2	exonuclease 1
		shEXO1-2736	10.9	
6197	RPS6KA3	shRPS6KA3-982	23.9	ribosomal protein S6 kinase, 90 kDa, polypeptide 3
		shRPS6KA3-2052	12.0	
10733	PLK4	shPLK4-433	18.8	polo-like kinase 4 (*Drosophila*)
		shPLK4-1377	15.6	

Table 1. Continued

Gene ID	Symbol	Hairpin Name	Average MI	Description
57144	PAK7	shPAK7-1918	19.8	p21(CDKN1A)-activated kinase 7
		shPAK7-616	14.4	
6725	SRMS	shSRMS-1235	14.6	src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites
		shSRMS-1231	12.6	
		shSRMS-814	12.0	

At least one shRNA induced an MI > 14, and at least one additional shRNA elicited an MI > 9. See Table S4 for a full list of genes meeting these criteria.

such a pattern, suggesting that these genes are involved in progression through the observed stage of mitosis (Figure 5B).

The genes identified here provide a rich starting point for the investigation of potential mitotic regulators. Each putative hit requires further study to confirm that the observed phenotype reflects knockdown of the targeted gene ("target confirmation") and to elucidate its biological role. We suggest the following criteria for target confirmation: (1) reproduction of the phenotype in multiple independent experiments, (2) verification that the shRNA decreases the expression level of the target gene, and (3) demonstration of a correlation between the observed phenotype and the extent of gene suppression across multiple shRNAs targeting the same gene.

We selected four genes (*YES1*, *TIE1*, *ROCK1*, and *MET*) for which multiple shRNAs produced high MI and that had not previously been implicated in the regulation of mitosis for follow-up experiments. For each shRNA, we confirmed the initial phenotype and measured target-gene knockdown. For the shRNAs targeting *YES1*, *TIE1*, and *ROCK1*, we found a strong correlation between knockdown level and increased MI as well as increased levels of pH3 (Figures 6A–6C). The shRNAs that induced greatest suppression of the target gene yielded the largest MI values, and shRNAs that produced slight or no increase in MI induced much weaker suppression of the target transcript. These results strongly suggest that the observed phenotypic effects are due to suppression of these target genes.

In contrast, the shRNAs targeting *MET* did not show a clear correlation between extent of gene knockdown and MI phenotype (Figure 6D). While the shRNA that produced the most elevated MI (shMET-1651) did cause a substantial knockdown of *MET*, another shRNA causing a strong knockdown (shMET-502) failed to increase MI. Additional work is needed to determine if changes in MET levels control the phenotypes observed in these cells.

We performed further biological characterization of *YES1*, *TIE1*, and *ROCK1*. First, we found that infection of immortalized BJ-TERT fibroblasts with shRNAs specific for *YES1*, *TIE1*, and *ROCK1* induced effective gene suppression (Figure 6E). In the case of *YES1* and *TIE1*, suppression of these genes in BJ-TERT cells induced pH3 as was observed in HT29 cells. Suppression of *ROCK1*, like *PLK1*, induced pH3 in HT29 cells but not in human fibroblasts. These findings show that some genes identified in this screen can regulate mitosis in both nonmalignant and malignant cells while others may exhibit specificity for cancer cells, suggesting possible cancer targets.

Second, we examined DNA content histograms for HT29 cells expressing the shRNA targeting *YES1*, *TIE1*, and *ROCK1* that induced the most striking MI phenotypes. A substantial percentage of cells expressing shYES1-1338, shYES1-905, and shTIE1-3795 were arrested in G2/M (Figures 6F–6H). Because deregulation of the cell cycle can lead to cell death (Golsteyn, 2005), we also checked whether suppression of any of these genes also induced apoptosis. We found that shRNAs that strongly suppressed *YES1* and *TIE1* also increased levels of the apoptotic marker cleaved PARP (Figures 6I and 6J), while those that target *ROCK1* did not induce apoptosis (data not shown).

Finally, we examined the list of genes identified in this screen to determine whether other genes obviously related to *YES1*, *TIE1*, and *ROCK1* were present. The *TIE1* receptor tyrosine kinase has roles in angiogenesis and development and is believed to function in a complex with the *TEK* receptor tyrosine kinase (Marron et al., 2000; Tsiamis et al., 2002). We found that three of the shRNAs that target *TEK* also cause substantial increases in MI (Table S3); we tested two of these shRNAs and verified that they decrease transcript levels of *TEK* (Figure 6K) but not of *TIE1* (data not shown). Furthermore, cells expressing shTEK-1275 and shTEK-520 also showed altered DNA content distribution, consistent with G2/M arrest (Figure 6L). These observations strongly suggest that the receptor complex that includes the products of *TIE1* and *TEK* plays a previously unknown role in the control of mitosis in cancer cells.

DISCUSSION

The discovery of RNAi has revolutionized the study of gene function in model organisms and promises to permit large-scale loss-of-function studies in mammals. Mammalian siRNA and shRNA libraries have now been used successfully (Berns et al., 2004; Kittler et al., 2004; Kolfschoten et al., 2005; Paddison et al., 2004; Pelkmans

Table 2. Gene Targets for which Two or More shRNAs Induced a Decrease in MI

Gene ID	Symbol	Hairpin Name	Average MI	Description
7535	ZAP70	shZAP70-2393	0.2	zeta-chain (TCR) associated protein kinase, 70 kDa
		shZAP70-1066	0.9	
1608	DGKG	shDGKG-1685	0.0	diacylglycerol kinase, gamma 90 kDa
		shDGKG-813	0.4	
10298	PAK4	shPAK4-285	0.0	p21(CDKN1A)-activated kinase 4
		shPAK4-1093	0.7	
2932	GSK3B	shGSK3B-867	0.1	glycogen synthase kinase 3 beta
		shGSK3B-1067	0.4	
		shGSK3B-562	1.0	
7525	YES1	shYES1-427	0.0	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
		shYES1-287	0.9	
701	BUB1B	shBUB1B-1822	0.0	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
		shBUB1B-3346	0.7	
		shBUB1B-521	1.0	
53904	MYO3A	shMYO3A-4214	0.1	myosin IIIA
		shMYO3A-1794	0.6	
23678	SGK3	shSGK3-1386	0.3	serum/glucocorticoid regulated kinase-like
		shSGK3-838	0.6	
3656	IRAK2	shIRAK2-1563	0.0	interleukin-1 receptor-associated kinase 2
		shIRAK2-540	0.9	
2585	GALK2	shGALK2-1330	0.2	galactokinase 2
		shGALK2-647	0.8	
51678	MPP6	shMPP6-617	0.0	membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6)
		shMPP6-527	1.0	
5502	PPP1R1A	shPPP1R1A-612	0.0	protein phosphatase 1, regulatory (inhibitor) subunit 1A
		shPPP1R1A-341	1.1	
1454	CSNK1E	shCSNK1E-766	0.2	casein kinase 1 epsilon
		shCSNK1E-462	0.9	
		shCSNK1E-583	0.9	
1859	DYRK1A	shDYRK1A-3947	0.2	dual-specificity tyrosine (Y) phosphorylation-regulated kinase 1A
		shDYRK1A-1033	1.0	
		shDYRK1A-2148	1.0	
8916	HERC3	shHERC3-1556	0.3	hect domain and RLD 3
		shHERC3-1348	1.0	

At least one shRNA induced an MI < 0.3, and at least one additional shRNA elicited an MI < 1.1.

et al., 2005; Silva et al., 2005; Westbrook et al., 2005), but many practical and theoretical challenges remain before such large-scale applications become routine. To create a resource that will enable high-throughput screening in mammalian cells, we formed the RNAi Consortium to generate genome-scale libraries that permit the delivery of siRNAs to a broad variety of cells at high efficiency. We focused initial efforts on enabling arrayed screening because this format offers some important advantages relative to pooled screens. Specifically, this format provides increased sensitivity in the initial assay, reduces the number of false negatives, directly identifies active shRNAs for follow-up without the need for postscreen deconvolution, and enables use of complex and information-rich assays

such as those involving cell-cell interactions and high-content imaging. Arrayed screens thus represent a powerful tool to reveal genes that are critical for many biological processes.

Here we describe a lentiviral shRNA library and its application to an arrayed screen in viral form. The features of this library and the methods for its application developed here enable effective arrayed screening in a wide range of cell types. The TRC1 library currently contains over 100,000 sequence-validated arrayed shRNA constructs targeting 12,000 human and 10,000 murine genes. We will continue to generate additional constructs until nearly all human and mouse genes are targeted. Methods for producing DNA and lentiviruses from this library are routine at a small scale, but many challenges exist in performing these manipulations at the scale necessary to perform HT studies. We report HT lentiviral production methods that constitute a relatively small part of the total cost of the screen when the reagents are distributed across many screens. This library can thus serve as a cost-effective, renewable, and scaleable RNAi-screening resource for the scientific community.

Quantitative assessment of library performance, measured on a sample of untitered library viruses, showed that 83% of genes tested had at least one shRNA virus that reduced transcript levels $\geq$4-fold. These results are likely to underestimate the intrinsic shRNA knockdown efficacy due to variations in viral titer. We will continue to measure library knockdown performance to rank constructs by level of knockdown efficacy. This information will be useful for determining the effects of gene dosage on phenotype for essential as well as nonessential genes.

A major concern with the use of RNAi in mammalian cells is off-target effects. To mitigate this problem, we designed shRNAs to contain at least three mismatches to all known cDNAs in the human or mouse genome. However, this does not eliminate the possibility of off-target effects with shorter stretches of identity (Zamore and Haley, 2005). To overcome this inherent property of shRNAs, we required that hit genes in our screen have at least two distinct shRNAs that induce a similar phenotype. Because distinct shRNAs are expected to have nonoverlapping spectra of off-target effects, this criterion should filter out most off-target effects. We also investigated nonspecific effects of viral infection using a small set of library shRNA vectors and found no evidence for interferon induction.

The two issues above, differential effectiveness of shRNAs and the possibility of off-target effects, underscore the importance of using multiple shRNAs to minimize false negatives and false positives in screens. For this reason, the TRC1 library was designed to include five shRNAs against each gene. We tested the effect of using fewer shRNAs per gene by randomly removing one construct from our data set. Using only four shRNAs, the number of hits that would be detected in our screen would fall from 102 to 75. Indeed, it would be desirable to use even more than five shRNAs per gene—especially in order to obtain "allelic" series with varying effects and to enable testing of essential genes by inducing moderate levels of knockdown.

We tested the utility of the TRC1 library in loss-of-function screening by infecting colon cancer cells with arrayed viral stocks to identify genes that alter mitotic progression. A screen surveying kinases in *Drosophila* S2 cells identified 80 genes that cause cell-cycle dysfunction upon downregulation (Bettencourt-Dias et al., 2004). Our screen tested human homologs for 59 of these 80 *Drosophila* genes, of which 21 were found to have altered mitotic phenotypes in our screen, suggesting that the function of many of these genes are evolutionarily conserved. We found that three of the genes identified as mitotic regulators in our screen of HT29 cells also regulate mitotic progression in human fibroblasts. Other genes identified as mitotic regulators in HT29 cells did not have similar effects in fibroblasts, suggesting that targeting these genes may confer specificity for cancer cells. Indeed, suppression of some of the genes identified in this screen also leads to cell death, suggesting that they are potential therapeutic targets. Further experiments are necessary to determine the roles of each of these genes in regulating mitotic progression. Although we have begun to investigate the role of some of these genes in other cell lines, it is clear that a definitive investigation of genes that regulate mitotic progression in normal and many types of cancer cells will require performing this screen in dozens of cell types. We believe that the library and methodologies described herein provide the means to undertake such a study.

In summary, we have produced a genome-scale lentiviral shRNA library to target human and mouse genes in a wide range of cell types, developed a pipeline to effectively convert this library into its plasmid and high-titer viral forms in an automated fashion, and used a subset of the library in its viral form to infect target cells in an arrayed format for phenotypic screens. Future advances in RNAi biology are expected to improve our ability to design and use RNAi libraries for genetic screening in mammals. In addition, methodologies to use RNAi in animals to study gene function are being developed by several groups and promise to provide a critical tool for the follow-up of genes identified in a cell-based RNAi screen (Dickins et al., 2005; Sandy et al., 2005). The use of genome-wide RNAi libraries for gene discovery should facilitate rapid identification of the major regulators of many biological processes, thereby annotating the genome and revealing the first global views of mammalian genetic circuits. The lentiviral library described here will facilitate comprehensive screening efforts and will be especially useful in enabling arrayed screens that focus on primary cells from mouse or human.

EXPERIMENTAL PROCEDURES

Library Production

Details of the library-production methods are provided in the Supplemental Data.

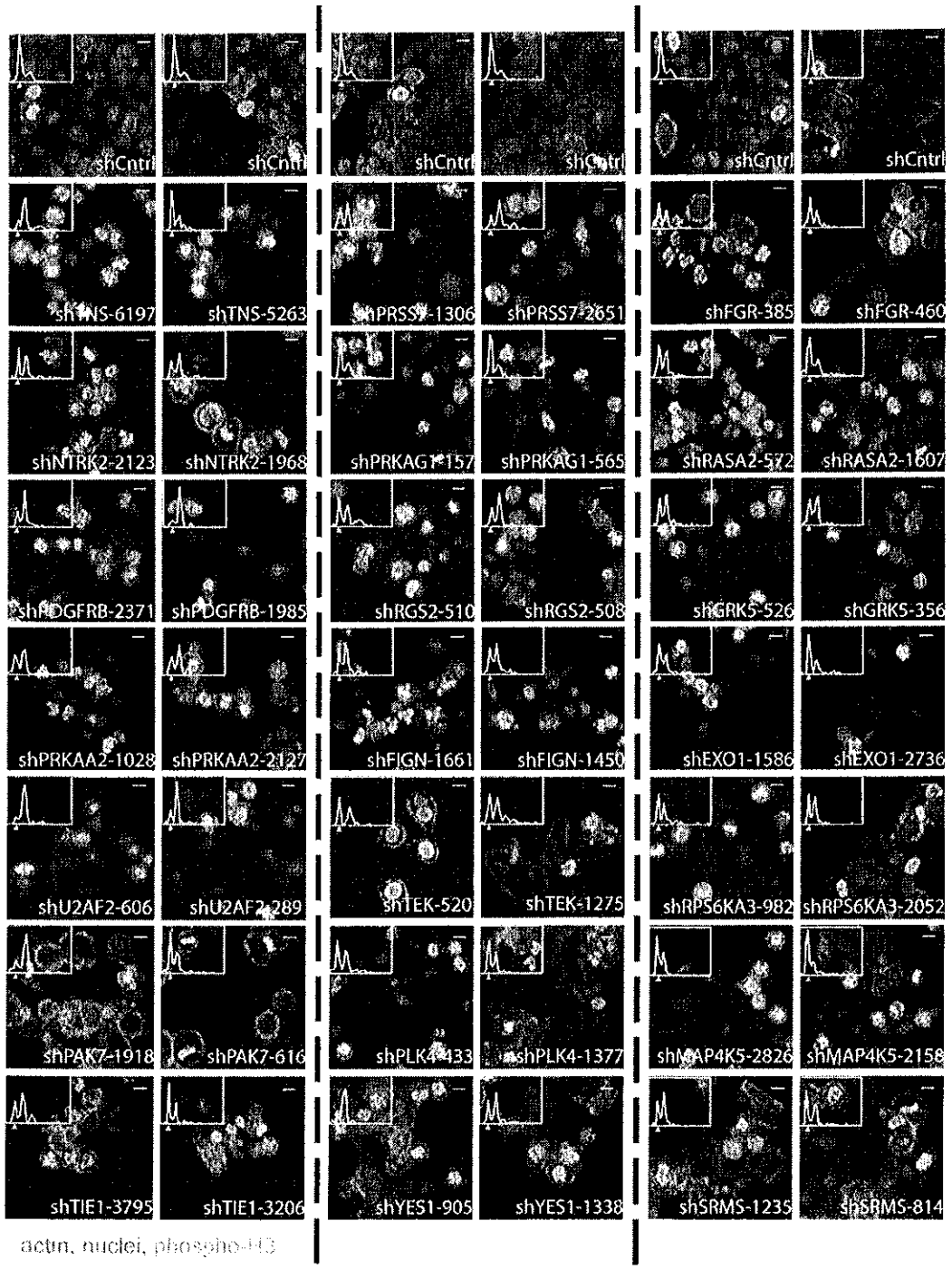

Figure 4. Images of HT29 Cells Infected with shRNAs for 21 Genes that Induce High Mitotic Indices

Pairs of images are shown for knockdowns by two distinct shRNA viruses for each hit gene. Scale bars = 10 μm. Corresponding DNA content histograms are superimposed as yellow traces in the left corner of each image. The percentage of total events is shown on the vertical axis and the integrated nuclear intensity on the horizontal axis. The control histogram is the average of ten images taken from control infections. The small yellow triangles underneath each histogram indicate the G1 peak in HT29 cells.

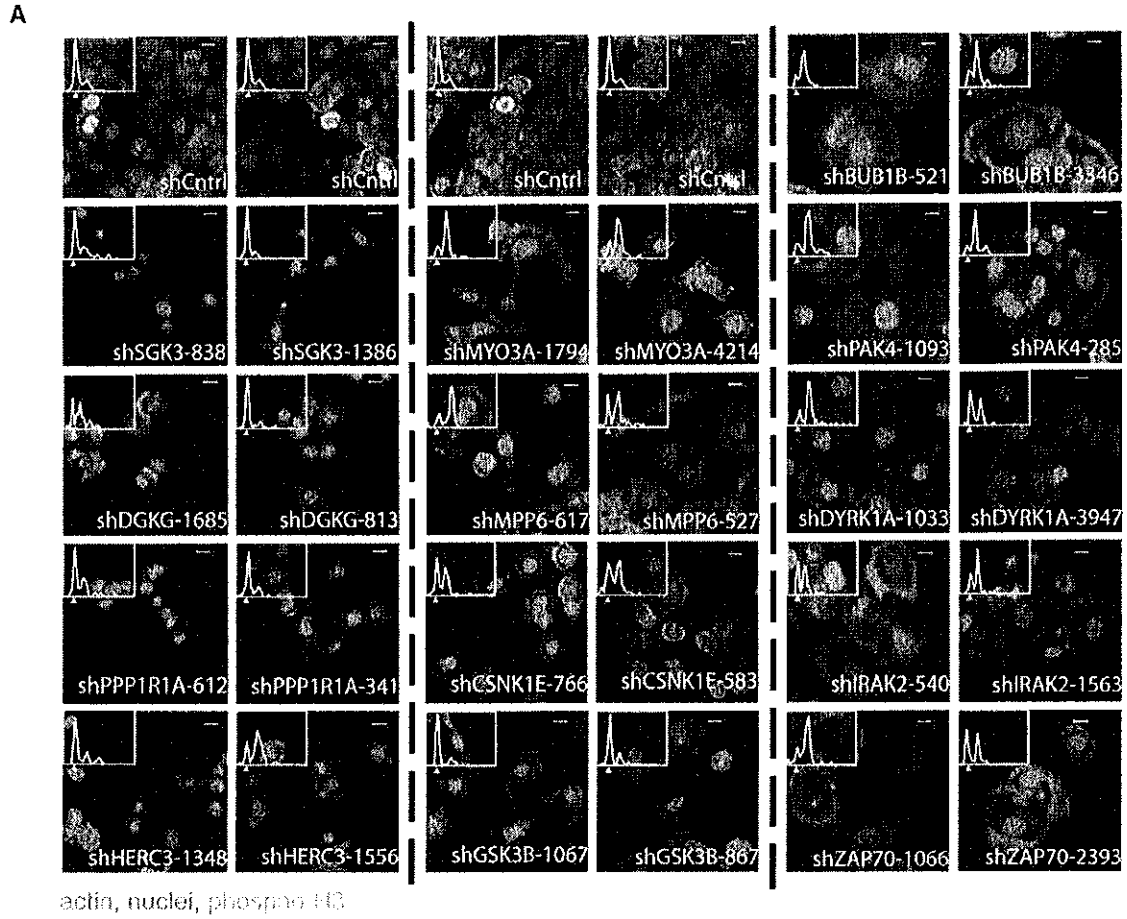

Figure 5. Images of HT29 Cells Infected with shRNAs for 13 Genes that Induce Low Mitotic Indices, and Association of High-Mitotic-Index-Inducing Genes with Mitotic Phase

(A) Pairs of images are displayed for knockdown by two distinct shRNAs for each low-MI-inducing gene. DNA histograms are displayed for each image as described for Figure 4.

(B) Genes identified by high MI for which a specific phase of mitosis is overrepresented were visually scored and are indicated below the corresponding phases (blue = nuclei, green = pH3, red = actin). Asterisks indicate cases where condensed staining may also be representative of apoptosis. Scale bars = 10 μm.

RT DNA and Virus Production

Transfection-quality DNA was prepped using 96-well PureLink kits (Invitrogen) with average yields of 4 μg DNA/well, quantified using a PicoGreen assay (Molecular Probes), and normalized robotically in each plate. Lentiviruses were made in 96-well format by transfecting packaging cells (293T) with a three-plasmid system (Naldini et al., 1996;

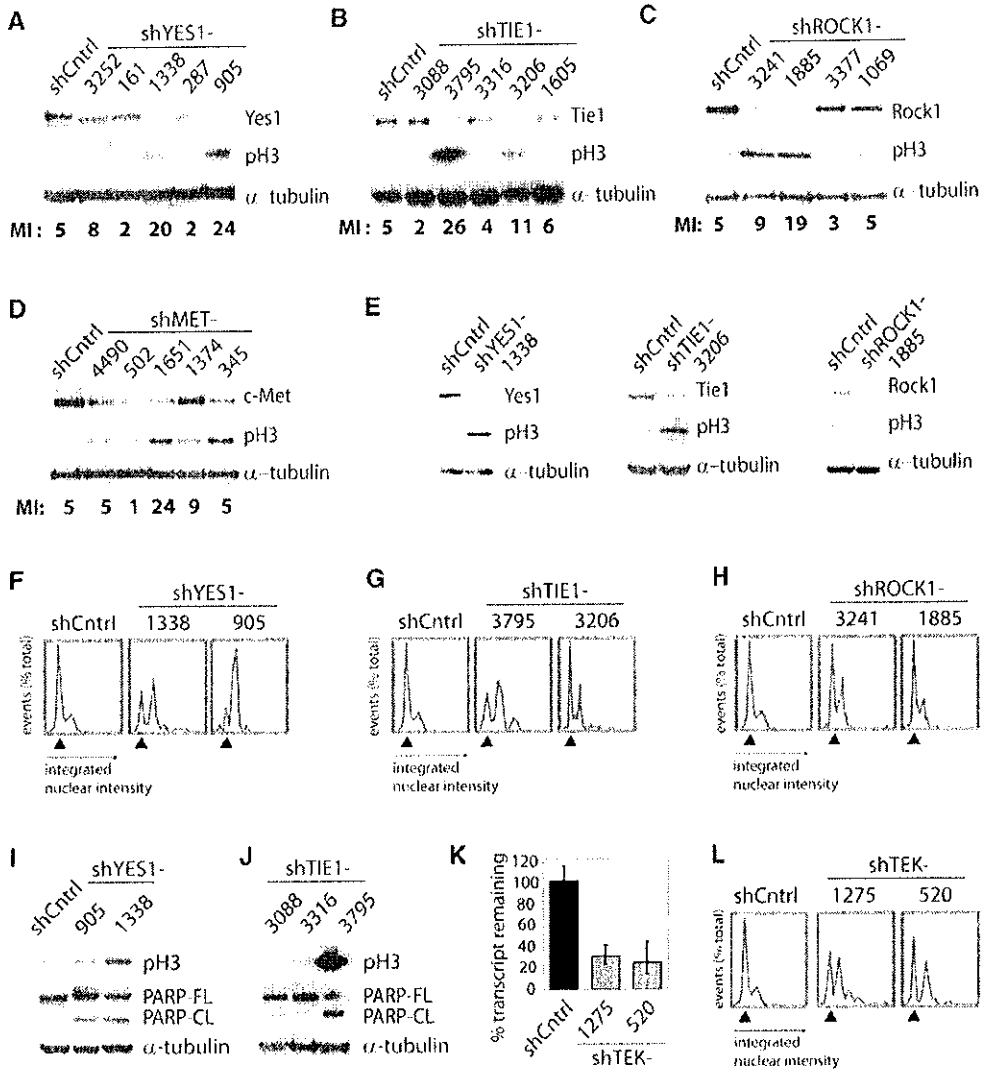

Figure 6. Correlation between Phenotype and Knockdown for Multiple Library shRNAs

(A–D) Immunoblot analyses of pH3, α-tubulin, and target protein levels following shRNA knockdown in HT29 cells targeting (A) *YES1* (shYES1-3252, shYES1-161, shYES1-1338, shYES1-287, shYES1-905), (B) *TIE1* (shTIE1-3088, shTIE1-3795, shTIE1-3316, shTIE1-3206, shTIE1-1605), (C) *ROCK1* (shROCK1-3241, shROCK1-1885, shROCK1-3377, shROCK1-1069), or (D) *MET* (shMET-4490, shMET-502, shMET-1651, shMET-1374, shMET-345). Control infections using a hairpin sequence targeting GFP knockdown are shown on the left of each blot (shCntrl). MIs from the primary screen data are indicated below each lane.

(E) Immunoblot analysis for indicated proteins and phosphorylation sites of BJ-TERT fibroblasts infected with shRNA viruses targeting *YES1*, *TIE1*, and *ROCK1*.

(F–H) DNA content histograms from primary screening data in HT29 cells for knockdowns of (F) *YES1* (shYES1-1338, shYES-905), (G) *TIE1* (shTIE1-3795, shTIE1-3206), and (H) *ROCK1* (shROCK1-3241, shROCK1-1885). The black triangles indicate the G1 peak, and DNA histograms from control infections are shown on the left of each panel for comparison.

(I and J) Immunoblot analyses of pH3, PARP (full length, FL, or cleaved, CL, indicating apoptosis), and α-tubulin protein levels following shRNA knockdown targeting *YES1* (shYES1-1338, shYES1-905), *TIE1* (shTIE1-3795, shTIE1-3088, shTIE1-3316), or a control shRNA targeting *GFP* (shCntrl) as indicated.

(K) Quantitative RT-PCR analysis of *TEK* transcript levels following lentiviral mediated RNAi with two different shRNAs that induced high MIs (shTEK-1275, shTEK-520). Error bars indicate the standard error for three qPCR measurements.

(L) DNA content histograms following knockdown with a control shRNA (shCntrl), shTEK-1275, and shTEK-520, from primary screen data. The black triangles indicate the G1 peak.

Zufferey et al., 1997; see also Supplemental Data and http://www.broad.mit.edu/genome_bio/trc/rnai.html).

HT Lentiviral Infections and Mitotic-Index Assay

Infection conditions were optimized in 384-well plates for growth conditions, plate types, viral dose, and assay times prior to HT screening. HT29 cells were seeded at a density of 300–350 cells/well in a 384-well assay plate (Costar 3712), incubated for 24 hr, infected using 3 μl of unconcentrated shRNA lentiviral supernatant from the 96-well viral production, and incubated for 4 days. All lentiviral infections were tested in duplicate, one replicate using 2 μg/ml puromycin during the final 3 days of incubation and the other replicate with no selection. Cells were ~50%–70% confluent at the time of fixation and fluorescent staining for HT image acquisition. Images were analyzed using Cellomics software to extract MI. Data for each lentiviral sample were rejected unless valid images were obtained for both selection conditions, the ratio of cell counts under +/− puromycin conditions exceeded 0.25, and the cell count was > 100 for the imaged area. MIs for + and −puromycin conditions were averaged. DNA content histograms were extracted from the same primary screening images using CellProfiler Software (http://jura.wi.mit.edu/cellprofiler/). For follow-up experiments, infections of HT29 and BJ-TERT cells were performed using a similar protocol as for the primary screen, scaled up to 6 cm dishes. Standard immunoblot analyses were performed for the hit proteins and for pH3. Details of infection and assay conditions and data analysis are provided in Supplemental Data.

Quantitative RT-PCR

mRNA was harvested in 96-well plates using GenePlate Hybridization (RNAture). RT reactions were performed with a SuperScript II RT Kit (Invitrogen). Quantitative PCR reactions were performed using Assays-on-Demand FAM-MGB primer/probe sets and TaqMan Universal PCR Master Mix (Applied Biosystems). Quantification of GAPDH levels in the same cDNA samples measured in separate qPCR reactions served as an endogenous control. All qPCR reactions were run in triplicate, and the average C_t (cycles to threshold) was used for the comparative C_t method (ABI User Bulletin #2). Control infections using an shRNA targeting lamin or an shRNA not targeting any human gene were used to define 100% expression.

Library Availability

The RNAi Consortium (TRC) human and mouse lentiviral shRNA libraries are available from Sigma-Aldrich Company (http://www.sigmaaldrich.com) and Open Biosystems (http://www.openbiosystems.com). Updated contents of the library can be found at http://www.broad.mit.edu/genome_bio/trc/rnai.html.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, four tables, and seven figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/6/1283/DC1/.

ACKNOWLEDGMENTS

This work is a project of the RNAi Consortium (TRC). The TRC was initiated by N.H., W.C.H., E.S.L., D.E.R., D.M.S., S.A.S., and B.R.S. We are grateful to the members of TRC—Academia Sinica, Bristol-Myers Squibb, Eli Lilly, Novartis, and Sigma-Aldrich—for their financial support and scientific advice. We are indebted to Bristol-Myers Squibb, Broad Institute, Dana-Farber Cancer Institute, and Whitehead Institute for Biomedical Research for their support of early phases of this effort. We thank S. Ali, N. Berkowitz, S. Bailey, J. Bridges, L. Brody, S. Bulmer, A. Burds Connor, J. Davies, T.R. Jones, M. Lamprecht, M. Lynes, H. Mizuno, J. Morawiak, C. Nguyen, S. Saif, D. Sarbassov, and S. Yadav for technical assistance; and we are grateful to C. Nusbaum for helpful discussions. We thank J. Evans, A. Davis, the Whitehead-MIT Bioimaging Center, and N. Durso at Cellomics for providing imaging resources and technical advice and Applied Biosystems for providing reagents. This work was also supported by a Dana-Farber/Harvard Cancer Center Core Grant Opportunity Award 2003-31C-NOPA (W.C.H.), NIH P50 CA112962 (W.C.H.), the Tisch Family Fund for Research in Solid Tumors (W.C.H.), an NSERC postdoctoral fellowship (J.M.), NIH CA103866 (D.M.S.), Keck Foundation (D.M.S.), Edith C. Blum Foundation, Stewart Trust (D.M.S.), NIH R01CA97061 (B.R.S), and a Career Award at the Scientific Interface (B.R.S).

Received: September 25, 2005
Revised: November 29, 2005
Accepted: January 4, 2006
Published: March 23, 2006

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MSDS FOR REPLICATION-DEFECTIVE LENTIVIRAL VECTORS (Biosafety Level 2)

Cultures of replication defective lentiviral vectors are non-infectious and are not hazardous materials as defined by OSHA 1919.1200. However, these materials are produced in cells where there is the possibility of recombination to form wild type virus. As such, they should be handled as potentially infectious material.

Description:

Lentiviral vectors consist of recombinant transgene sequences (e.g., marker or human genes), and viral packaging and regulatory sequences which are then flanked by lentiviral long terminal repeats (LTRs). The removal of the viral structural genes renders the vector replication defective and dependent upon a helper vector(s) or packaging cell line. Lentiviruses are enveloped viruses and upon leaving the producer cell line, the viral capsid becomes enclosed in a lipid by layer derived from the host cell. The vectors' LTRs are self-inactivating (SIN), thus restricting mRNA production from integrating vectors to the internal promoter, severely reducing full-length vector transcripts. By default, the lentiviral vectors are pseudotyped with the VSV-G Indiana envelope serotype; however the envelope protein can be customized as desired.

Lentiviral cultures are provided as either low concentration (>$1x10^6$ infectious units/ml) virus in tissue culture media, or as high concentration, purified (>$1x10^9$ infectious units/ml) virus in phosphate buffered saline. Trace components present in the purified virus include, but are not limited to, inorganic salts, vitamins and other nutrients, and human cellular proteins, carbohydrates, amino acids, and fats. The material is normally shipped and stored frozen. Further vector application and handling is described in the following publication:

Kafri, Tal. (2004). Gene delivery by lentivirus vectors an overview. Methods Mol Biol. 2004; 246:367-90. Review.

SECTION I

Hazardous Ingredients

None

SECTION II

Physical Data

Liquid or frozen particle suspensions

University of North Carolina
Gene Therapy Center
Joint Vector Laboratories

7017 Neurosciences Hospital
Chapel Hill, NC 27599-7592
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SECTION III
Health Hazards

Replication-defective lentiviral vectors are not known to cause any diseases in humans or animals. However, lentiviruses can integrate into the host cell genome and thus pose some risk of insertional mutagenesis.

SECTION IV
Fire and Explosion

None

SECTION V
Reactivity

Not chemically reactive. Will enter permissive mammalian cells and interact or react with cellular components.

SECTION VI
Method of Disposal

Spill: Contain spill and decontaminate the area using a disinfectant such as chlorine bleach (10% f.c.), Wescodyne, or detergent-based disinfectant.

Waste Disposal: Dispose of viral stocks by autoclaving at 121°C for 30-45 minutes
Dispose of infected liquid cultures by decontamination with chlorine bleach (10% f.c.) for 10 minutes and then dispose of in sink.
Dispose of infected animal carcasses or tissues by incineration

Follow all Federal, State, and Local regulations.

SECTION VII
Special Protective Information

Handle as biohazardous material under Biosafety Level 2 containment

University of North Carolina
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SECTION VIII

Special Precautions or Comments

The Gene Therapy Center recommends that all Lentiviral vectors and cultures be handled by qualified microbiologists using appropriate safety procedures and precautions. Upon accidental exposure to Lentiviral vectors, seroconversion towards HIV-1 viral proteins could result and health provider should be contacted. Detailed discussions of laboratory safety procedures are provided in **Laboratory Safety: Principles and Practice** (Fleming et al., ASM Press, Washington D.C., 1995), and in the U.S. Government Publication, **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999). This and other publications are available at the Centers for Disease Control Office of Health and Safety's website at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Information on the classification of human etiologic agents on the basis of hazard can be found as Appendix B in the NIH **Guidelines for Research Involving Recombinant DAN Molecules** at http://www.grants.nih.gov/grants/guide/notice-files/NOT-OD-02-005.html

The above information is accurate to the best of our knowledge. All materials and mixtures may present unknown hazards and should be used with caution. The user should exercise independent judgment as to the hazards based on all sources of information available. The Gene Therapy Center shall not be held liable for any damage resulting from the handling or use of the above product.

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THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: September 25, 2009
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 or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous 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 biohazards 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).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard 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

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Location of experimental work to be carried out: Building(s) RRI, Med Science Room(s) 4244, 4250, ACVS

*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 12.0, Approvals).

FUNDING AGENCY/AGENCIES: CIHR & HSFO
GRANT TITLE(S): Smooth Muscle Cells and Vascular Disease

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:

Caroline O'Neil	Faran Vafaie
Zengxuan Nong	Paul Comartin
Theodore Small	Alanna Watson
Matthew Frontini	Oula Akawi

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

Research Summary

The purpose of this study is to ascertain molecular details of processes that contribute to the development of diseased arteries and the formation of new blood vessels. Smooth muscle cells, fibroblasts and endothelial cells are prominent constituents of the human atherosclerotic plaque and will be utilized to study their role in vascular disease. These cells are particularly abundant in lesions that rapidly develop after angioplasty-induced vascular injury. Functionally, these cells contribute to atherosclerosis by replicating within the growing lesion, and by synthesizing and secreting extracellular matrix. We will specifically examine molecules, such as Nampt, WTAP and FGF-9, which enable cells in the artery to organize and stabilize the artery wall. These target genes will be inserted into various plasmids and nucleofected into the different cells lines to elucidate their role. Nucleofection protocol will prevent the production of virus. In addition, the mouse and rat animal model can mimic the complex events that take place in individuals with diseased arteries. Use of an animal system enables the retrieval of suitable amounts of tissue, at defined stages of the disease, so that a thorough analysis can be performed. To determine the response of the candidate genes to vascular injury, the carotid artery of rats or the femoral artery of mice, will be injured. Adenovirus will be administered locally to the site of mechanical injury of the artery during surgery and gene expression will be evaluated during various stages of lesion development. The adenovirus will be produced in a level 2 facility at Robarts and the mice will be housed in a level 2 facility at ACVS. The messenger RNA will be assessed by Laser Capture Microdissection and Real Time RT-PCR. Protein elaboration will be assessed immunohistochemically. This proposal aims to elucidate the role of selected genes that are expressed in the vascular lesions which may be critical to lesion development or angiogenesis.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ⊗ YES O NO
(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? O 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:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
E.coli, DH5a competent cells	O Yes ⊗ No	O Yes ⊗ No	O Yes ⊗ No	2	Invitrogen	⊗ 1 O 2 O 3
AdMax: adenovirus type 5	O Yes ⊗ No	O Yes ⊗ No	O Yes ⊗ No	0.1	Microbix Biosystems Inc	O 1 ⊗ 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ⊗ YES O 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 O No	Primary smooth muscle cells derived from arteries	Not applicable
Rodent	⊗ Yes O No	Fibroblasts derived from mouse embryos	2006-064-08
Non-human primate	O Yes ⊗ No		
Other (specify)	O Yes ⊗ No		

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

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)*	Supplier / Source
Human	⊗ Yes O No	HEK 293, HAEC, Hela, HT-1080, Fibroblasts (transformed)	ATCC and Lonza
Rodent	⊗ Yes O No	Renca, 3T3-L1, 3T3-Swiss albino, C3H/10T 1/2	ATCC
Non-human primate	⊗ Yes O No	Cos-7	ATCC
Other (specify)	O 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 O 1 ⊗ 2 O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ⊗ YES O 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/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid	Dr. Michael Chu LHSC	O Yes O No ⊗ Unknown		O 1 ⊗ 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (unpreserved)	Dr. Michael Chu LHSC	O Yes O No ⊗ Unknown		O 1 ⊗ 2 O 3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

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 O NO If no, please proceed to Section 5.0

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

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
E.coli DH5alpha competent cells	pEGFP-N3, pIRES2-EGFP, pLNCX2, pQCXIN, pQCXIP	Clontech, addgene	Genes involved in vascular disease such as Nampt, WTAP & FGF-9	Genes play a role in the development of vascular lesions

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

4.3 Will genetic modification(s) involving viral vectors be made? ⊗ YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results
Admax (adenovirus type 5)	Adeno E1A, Admax vectors (pD311, pDC411, pDC511)	Microbix Biosystems	Genes involved in vascular disease such as Nampt, WTAP & FGF-9	Genes play a role in the development of vascular lesions

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

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

4.5 Will virus be replication defective? ⊗ YES O NO

4.6 Will virus be infectious to humans or animals? ⊗ YES O NO

4.7 Will this be expected to increase the containment level required? O YES ⊗ NO

5.0 Human Gene Therapy Trials

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

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

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

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

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? ⊗ YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used mouse, rat

6.3 AUS protocol # 2006-064-08

6.4 Will any of the agents listed in section 4.0 be used in live animals ⊗ YES, specify: adenovirus O NO

6.5 Will the agent(s) be shed by the animal: ⊗ YES O NO, please justify:

_____

_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

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

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES ⊗ NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s)_____
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin_____

8.4 How much of the toxin is handled at one time*? _____

8.5 How much of the toxin is stored*? _____

8.6 Will any biological toxins be used in live animals? O YES, Please provide detais:_____ O NO

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

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES ⊗ NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

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

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:
_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O 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 plant? _____

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

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

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

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 ⊗ NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

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

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 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 biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 ⊗ 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

⊗ YES, permit # if on-campus BIO-RRI-0028
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 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.wph.uwo.ca/

SIGNATURE J. S. Pilon Date: Feb 18, 2010

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, or 3 measures, that are unique to this agent.

14.3 Please outline what will be done if there is an exposure to the biohazards listed, such as a needlestick injury:

Health and safety protocol: report to first-aid person and follow-up with university occupational health and safety

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: [signature] Date: 3 March 2010

Safety Officer for Institution where experiments will take place: SIGNATURE: [signature] Date: Feb. 24, 2010

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: [signature] Date: March 2, 2010

Approval Number: Bio-RR-0028 Expiry Date (3 years from Approval): March 2, 2013

Special Conditions of Approval:

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

Subject: Re: Pickering Protocol
From: Jennifer Stanley <jstanle2@uwo.ca>
Date: Tue, 02 Mar 2010 13:08:30 -0500
To: Greg Dekaban <dekaban@robarts.ca>

Greg
No changes have been made - thanks.
Jen

On 3/2/2010 12:35 PM, Greg Dekaban wrote:

> Yes I am as long as nothing has changed from when Ron Noseworthy and I looked at it together.
> Greg
>
> -----Original Message-----
> From: Jennifer Stanley [mailto:jstanle2@uwo.ca]
> Sent: March 1, 2010 2:51 PM
> To: Greg Dekaban; rsn@uwo.ca
> Subject: Pickering Protocol
>
> Hi Greg
> At the meeting on Friday, Jerry wanted me to confirm with you that you are comfortable with Level 2 for this project.
> Thanks
> Jennifer

invitrogen

Material Safety Data Sheet

Revision Date: 09-Nov-2006

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

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

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

Chemical Name	CAS-No	Weight %
Glycerol	56-81-5	5-10

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
Liquid

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

HMIS

Health	0
Flammability	0
Reactivity	0

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

Chemical Name	OSHA PEL (TWA)	OSHA PEL (Ceiling)	ACGIH OEL (TWA)	ACGIH OEL (STEL)
Glycerol	15 mg/m³ total dust 5 mg/m³ respirable fraction	-	10 mg/m³	-

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

Chemical Name	LD50 (oral,rat/mouse)	LD50 (dermal,rat/rabbit)	LC50 (inhalation,rat/mouse)
Glycerol	12600 mg/kg (Rat)	10 g/kg (Rabbit)	570 mg/m³ (Rat)

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

Chemical Name	TSCA	PICCS	ENCS	DSL	NDSL	AICS
Glycerol	Listed	Listed	Listed	Listed	-	Listed

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

Chemical Name	Massachusetts - RTK	New Jersey - RTK	Pennsylvania - RTK	Illinois - RTK	Rhode Island - RTK
Glycerol	Listed	-	Listed	-	Listed

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.

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 be present unknown hazards and should be used with caution. Since Invitrogen Corporation 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, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY PARTCULAR PURPOSE.

End of Safety Data Sheet

Adenovirus types 1, 2, 3, 4, 5 and 7 - Material Safety Data Sheets (MSDS)

MATERIAL SAFETY DATA SHEET - INFECTIOUS SUBSTANCES

SECTION I - INFECTIOUS AGENT

NAME: ***Adenovirus types 1, 2, 3, 4, 5 and 7***

SYNONYM OR CROSS REFERENCE: ARD, acute respiratory disease, pharyngoconjunctival fever

CHARACTERISTICS: *Adenoviridae*; non-enveloped, icosahedral virions, 70-90 nm diameter, doubled-stranded, linear DNA genome.

SECTION II - HEALTH HAZARD

PATHOGENICITY: Varies in clinical manifestation and severity; symptoms include fever, rhinitis, pharyngitis, tonsillitis, cough and conjunctivitis; common cause of nonstreptococcal exudative pharyngitis among children under 3 years; more severe diseases include laryngitis, croup, bronchiolitis, or severe pneumonia; a syndrome of pharyngitis and conjunctivitis (pharyngoconjunctival fever) is associated with adenovirus infection

EPIDEMIOLOGY: Worldwide; seasonal in temperate regions, with highest incidences in the fall, winter and early spring; in tropical areas, infections are common in the wet and colder weather; annual incidence is particularly high in children; adenovirus types 4 and 7 are common among military recruits (ARD)

HOST RANGE: Humans

INFECTIOUS DOSE: >150 plaque forming units when given intranasally

MODE OF TRANSMISSION: Directly by oral contact and droplet spread; indirectly by handkerchiefs, eating utensils and other articles freshly soiled with respiratory discharge of an infected person; outbreaks have been related to swimming pools; possible spread through the fecal-oral route

INCUBATION PERIOD: From 1-10 days

COMMUNICABILITY: Shortly prior to and for the duration of the active disease

SECTION III - DISSEMINATION

RESERVOIR: Humans

ZOONOSIS: None

VECTORS: None

SECTION IV - VIABILITY

DRUG SUSCEPTIBILITY: No specific antiviral available; cidofovir has shown promise in the treatment of adenoviral ocular infections.

SUSCEPTIBILITY TO DISINFECTANTS: Susceptible to 1% sodium hypochlorite, 2%

glutaraldehyde, 0.25% sodium dodecyl sulfate

PHYSICAL INACTIVATION: Sensitive to heat>56°C; unusually stable to chemical or physical agents and adverse pH conditions

SURVIVAL OUTSIDE HOST: Resistance to chemical and physical agents allows for prolonged survival outside of the body. Adenovirus type 3 survived up to 10 days on paper under ambient conditions; adenovirus type 2 survived from 3-8 weeks on environmental surfaces at room temperature

SECTION V - MEDICAL

SURVEILLANCE: Monitor for symptoms; confirm by serological analysis

FIRST AID/TREATMENT: Mainly supportive therapy

IMMUNIZATION: Vaccine available for adenovirus types 4 and 7 (used for military recruits)

PROPHYLAXIS: None available

SECTION VI - LABORATORY HAZARDS

LABORATORY-ACQUIRED INFECTIONS: Ten cases documented up to 1988

SOURCES/SPECIMENS: Respiratory secretions

PRIMARY HAZARDS: Ingestion; droplet exposure of the mucous membrane

SPECIAL HAZARDS: Contact with feces from infected animals

SECTION VII - RECOMMENDED PRECAUTIONS

CONTAINMENT REQUIREMENTS: Biosafety level 2 practices and containment facilities for all activities involving the virus and potentially infectious body fluids or tissues

PROTECTIVE CLOTHING: Laboratory coat; gloves when skin contact with infectious materials is unavoidable

OTHER PRECAUTIONS: None

SECTION VIII - HANDLING INFORMATION

SPILLS: Allow aerosols to settle; wearing protective clothing gently cover the spill with absorbent paper towel and apply 1% sodium hypochlorite starting at the perimeter and working towards the centre; allow sufficient contact time (30 min) before clean up

DISPOSAL: Decontaminate all wastes before disposal; steam sterilization, incineration, chemical disinfection

STORAGE: In sealed containers that are appropriately labelled

SECTION IX - MISCELLANEOUS INFORMATION

Date prepared: November 1999

Prepared by: Office of Laboratory Security, PHAC

Although the information, opinions and recommendations contained in this Material Safety Data Sheet are compiled from sources believed to be reliable, we accept no responsibility for the accuracy, sufficiency, or reliability or for any loss or injury resulting from the use of the information. Newly discovered hazards are frequent and this information may not be completely up to date.

Date Modified: 2001-01-23

MSDS FOR ANIMAL CELL CULTURES (Biosafety Level 1 or 2)

ATCC cultures are not hazardous as defined by OSHA 1910.1200. However, as live cells they are potential biohazards.

ATCC Emergency Telephone: (703) 365-2710 (24 hours)
Chemtrec: (800) 424-9300
To be used only in the event of an emergency involving a spill, leak, fire, exposure or accident.

Description

Either frozen or growing cells shipped in liquid cell culture medium (a mixture of components that may include, but is not limited to: inorganic salts, vitamins, amino acids, carbohydrates and other nutrients dissolved in water).

SECTION I
Hazardous Ingredients

Frozen cultures may contain 5 to 10% Dimethyl sulfoxide (DMSO)

SECTION II
Physical data

Pink or red aqueous liquid

SECTION III
Health hazards

For Biosafety Level 1 Cell Lines

This cell line is not known to harbor an agent known to cause disease in healthy adult humans. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Handle as a potentially biohazardous material under at least Biosafety Level 1 containment.

For Biosafety Level 2 Cell Lines

This cell line is known to contain an agent that requires handling at Biosafety Level 2 containment [U.S. Government Publication **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999)]. These agents have been associated with human disease. This cell line has **NOT** been screened for Hepatitis B, human immunodeficiency viruses or other adventitious agents. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

SECTION IV
Fire and explosion

Not applicable

SECTION V
Reactivity data

Stable. Hazardous polymerization will not occur.

SECTION VI
Method of disposal

Spill: Contain the spill and decontaminate using suitable disinfectants such as chlorine bleach or 70% ethyl or isopropyl alcohol.
Waste disposal: Dispose of cultures and exposed materials by autoclaving at 121°C for 20 minutes. Follow all Federal, State and local regulations.

SECTION VII
Special protection information

For Biosafety Level 1 Cell Lines

Handle as a potentially biohazardous material under at least Biosafety Level 1 containment. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

For Biosafety Level 2 Cell Lines

Handle as a potentially biohazardous material under at least Biosafety Level 2 containment. Cell lines derived from primate lymphoid tissue may fall under the regulations of 29 CFR 1910.1030 Bloodborne Pathogens.

SECTION VIII
Special precautions or comments

ATCC recommends that appropriate safety procedures be used when handling all cell lines, especially those derived from human or other primate material. Detailed discussions of laboratory safety procedures are provided in **Laboratory Safety: Principles and Practice** (Fleming, et al., 1995) the ATCC manual on quality control (Hay, et al., 1992), the Journal of Tissue Culture Methods (Caputo, 1988), and in the U.S. Government Publication, **Biosafety in Microbiological and Biomedical Laboratories** (CDC, 1999). This publication is available in its entirety in the Center for Disease Control Office of Health and Safety's web site at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

THE ABOVE INFORMATION IS CORRECT TO THE BEST OF OUR KNOWLEDGE. ALL MATERIALS AND MIXTURES MAY PRESENT UNKNOWN HAZARDS AND SHOULD BE USED WITH CAUTION. THE USER SHOULD MAKE INDEPENDENT DECISIONS REGARDING THE COMPLETENESS OF THE INFORMATION BASED ON ALL SOURCES AVAILABLE. ATCC SHALL NOT BE HELD LIABLE FOR ANY DAMAGE RESULTING FROM HANDLING OR CONTACT WITH THE ABOVE PRODUCT.

February 2002

Cell Biology

ATCC® Number:	**CRL-1573™** Order this Item	Price:	**$256.00**
Designations:	293 [HEK-293]		
Depositors:	FL Graham		
Biosafety Level:	2 [CELLS CONTAIN ADENOVIRUS]		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	adherent		
Organism:	*Homo sapiens* (human)		
Morphology:	epithelial 		
Source:	**Organ:** embryonic kidney **Cell Type:** transformed with adenovirus 5 DNA		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Restrictions:	These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties.		
Applications:	efficacy testing [92587] transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents) viruscide testing [92579]		
Receptors:	vitronectin, expressed		
Tumorigenic:	Yes		
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 12,14 D16S539: 9,13 D5S818: 8,9 D7S820: 11,12 THO1: 7,9.3 TPOX: 11 vWA: 16,19		

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Cytogenetic Analysis:	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.
Age:	fetus
Comments:	Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome [RF32764], it is now clear that only left end sequences are present. [39768] The line is excellent for titrating human adenoviruses. The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit. [23406] 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). [39768] **ATCC 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%.
Propagation:	**Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C 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.

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.An inoculum of 2 X 10 (3) to 6 X 10 (3) viable cells/cm2 is recommended.
6. Incubate cultures at 37°C.6. Subculture when cell concentration is between 6 and 7 X 10(4) cells/cm2.

Preservation:

Subcultivation Ratio: 1:10 to 1:20 weekly.
Medium Renewal: Every 2 to 3 days

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

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2003
derivative: ATCC CRL-10852
derivative: ATCC CRL-12006
derivative: ATCC CRL-12007
derivative: ATCC CRL-12013
derivative: ATCC CRL-12479
derivative: ATCC CRL-2029
derivative: ATCC CRL-2368
purified DNA:ATCC CRL-1573D

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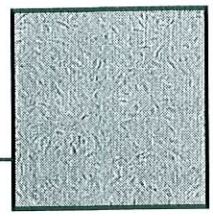
HAEC



Introduction

Clonetics® Aortic Endothelial Cell Systems contain Normal Human Aortic Endothelial Cells (HAEC) and optimized media for their growth. Each System can quickly generate HAEC cultures for experimental applications in cardiovascular pharmaceutical development and vascular pathology, including atherosclerosis. Clonetics® Aortic Endothelial Cell Systems are convenient and easy to use, allowing the researcher to focus on results. Cryopreserved HAEC are shipped in third passage. Proliferating HAEC are shipped in fourth passage.

Clonetics® Cells, Medium and Reagents are quality tested together and guaranteed to give optimum performance as a complete Cell System.

Cell System Components

- One Aortic Endothelial Cell Product (Cryopreserved or Proliferating)
- Clonetics® EGM®-2 BulletKit® (CC-3162) contains one 500 ml bottle of Endothelial Cell Basal Medium-2 and the following growth supplements: Hydrocortisone, 0.2 ml; hFGF-B, 2 ml; VEGF, 0.5 ml; R^3-IGF-1, 0.5 ml; Ascorbic Acid, 0.5 ml; Heparin, 0.5 ml; FBS, 10 ml; hEGF, 0.5 ml; GA-1000, 0.5 ml.
- One ReagentPack™ (CC-5034) Containing:

Trypsin/EDTA	100 ml
Trypsin Neutralizing Solution	100 ml
HEPES Buffered Saline Solution	100 ml

Characterization of Cells

Routine characterization of HAEC includes immunofluorescent staining. Cells stain positive for acetylated LDL and von Willebrand (Factor VIII) antigen. Cells stain negative for smooth muscle α-actin.

Performance

Recommended seeding density for subculture	2,500 - 5,000 cells/cm^2
Typical time from subculture to confluent monolayer	5 - 9 days
Additional population doublings guaranteed using Clonetics® System	15

Quality Control

All cells are performance assayed and test negative for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi. Cell viability, morphology and proliferative capacity is measured after recovery from cryopreservation. Clonetics® Media are formulated for optimal growth of specific types of normal human cells. Each lot of medium is tested for the support of cell viability and proliferative capacity. Certificates of Analysis (CA) for each cell strain are shipped with each order. CA for all other products are available upon request.

Ordering Information

Cryopreserved Cells

CC-2535	HAEC	≥ 500,000 cells

Proliferating Cells – Flasks and Multiwell Plates

CC-2635	T-25 Flask
CC-0222	T-75 Flask
CC-0132	96-well Plate

Other proliferating formats are available. Contact Technical Service or refer to the Lonza website for details.

CC-3162	EGM®-2 BulletKit®, EBM®-2 plus SingleQuots® of Growth Supplements	500 ml
CC-3156	EBM®-2, Endothelial Basal Medium-2	500 ml
CC-4176	EGM®-2 SingleQuots®, Formulates EBM®-2 to EGM®-2	
CC-5034	ReagentPack™	
	Trypsin Neutralizing Solution	100 ml
	Trypsin/EDTA Solution	100 ml
	HEPES Buffered Saline Solution	100 ml

Lonza

When placing an order or for technical service, please refer to the product numbers and descriptions listed above. For a complete listing of all Clonetics® Products, refer to the Lonza website or the current Lonza catalog. To obtain a catalog, additional information or technical service you may contact Lonza by web, e-mail, telephone, fax or mail.

Product Warranty

CULTURES HAVE A FINITE LIFESPAN IN VITRO. Lonza guarantees the performance of its cells only if Clonetics® Media and Reagents are used exclusively, and the recommend protocols are followed. The performance of cells is not guaranteed if any modifications are made to the complete Cell System. Cryopreserved HAEC are assured to be viable and functional when thawed and maintained properly.

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or in vitro procedures.

WARNING: CLONETICS® AND POIETICS® PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA approved method for the presence of HIV-I, Hepatitis B Virus and Hepatitis C Virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV, Hepatitis B Virus, and Hepatitis C Virus. Testing can not offer complete assurance that HIV-1, Hepatitis B Virus, and Hepatitis C Virus are absent. All human sourced products should be handled at the Biological Safety Level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH Manual, Biosafety in Microbiological and Biomedical Laboratories, 1999. If you require further information, please contact your site Safety Officer or Technical Services.

Product Description

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

ATCC® Number: CCL-2™ Order this Item
Price: $256.00

Designations: HeLa

Depositors: WF Scherer

Biosafety Level: 2 [CELLS CONTAIN PAPOVAVIRUS]

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Homo sapiens* (human)

Morphology: epithelial

Source:
Organ: cervix
Disease: adenocarcinoma
Cell Type: epithelial

Cellular Products:
keratin
Lysophosphatidylcholine (lyso-PC) induces AP-1 activity and c-jun N-terminal kinase activity (JNK1) by a protein kinase C-independent pathway [26623]

Permits/Forms:
In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Applications:
transfection host ([21491] Nucleofection technology from Lonza
Roche FuGENE® Transfection Reagents)
screening for Escherichia coli strains with invasive potential [21447] [21491]

Virus Susceptibility:
Human adenovirus 3
Encephalomyocarditis virus
Human poliovirus 1
Human poliovirus 2
Human poliovirus 3

Reverse Transcript: negative

DNA Profile (STR):
Amelogenin: X
CSF1PO: 9,10
D13S317: 12,13.3
D16S539: 9,10
D5S818: 11,12
D7S820: 8,12
THO1: 7
TPOX: 8,12
vWA: 16,18

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Cytogenetic Analysis: Modal number = 82; range = 70 to 164.
There is a small telocentric chromosome in 98% of the cells. 100% aneuploidy in 1385 cells examined. Four typical HeLa marker chromosomes have been reported in the literature.HeLa Marker Chromosomes: One copy of Ml, one copy of M2, four-five copies of M3, and two copies of M4 as revealed by G-banding patterns. M1 is a rearranged long arm and centromere of chromosome 1 and the long arm of chromosome 3. M2 is a combination of short arm of chromosome 3 and long arm of chromosome 5. M3 is an isochromosome of the short arm of chromosome 5. M4 consists of the long arm of chromosome 11 and an arm of chromosome 19.

Isoenzymes: G6PD, A

Age: 31 years adult

Gender: female

Ethnicity: Black

HeLa Markers: Y

Comments: The cells are positive for keratin by immunoperoxidase staining.
HeLa cells have been reported to contain human papilloma virus 18 (HPV-18) sequences.
P53 expression was reported to be low, and normal levels of pRB (retinoblastoma suppressor) were found.

Propagation: **ATCC 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%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C

Subculturing: **Protocol:**

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

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:6 is recommended
Medium Renewal: 2 to 3 times per week

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

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2003
recommended serum:ATCC 30-2020
derivative:ATCC CCL-2.1
derivative:ATCC CCL-2.2
derivative:ATCC CCL-2.3

References:

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Cell Biology

ATCC® Number: CCL-121™ Order this Item
Price: $272.00

Designations: HT-1080

Biosafety Level: 1

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Homo sapiens* (human)

Morphology: epithelial

Source:
Tissue: connective tissue
Disease: fibrosarcoma

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Isolation: **Isolation date:** July, 1972

Applications: transfection host (Nucleofection technology from Lonza
Roche FuGENE® Transfection Reagents)

Virus Susceptibility:
Human poliovirus 1
RD-114 Feline
Feline leukemia virus
Vesicular stomatitis virus

Tumorigenic: Yes

Reverse Transcript: negative

Oncogene: ras +

DNA Profile (STR):
Amelogenin: X,Y
CSF1PO: 12
D13S317: 12,14
D16S539: 9,12
D5S818: 11,13
D7S820: 9,10
THO1: 6
TPOX: 8
vWA: 14,19

Cytogenetic Analysis: modal number = 46; range = 44 to 48.
Pseudodiploidy was frequently noted. About 40% of the cells had rearranged karyotypes with an extra E-group chromosome and a group C chromosome, probably chromosome 11, was missing.

Isoenzymes: G6PD, B

Age: 35 years

Gender: male

Ethnicity: Caucasian

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Comments: The cells contain an activated N-ras oncogene.

Propagation: **ATCC 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%.
Temperature: 37.0°C

Subculturing: **Protocol:**

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is recommended
Medium Renewal: Every 2 to 3 days

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

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2003
recommended serum:ATCC 30-2020

References:
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Cell Biology

ATCC® Number: CRL-2947™ Order this Item

Price: $355.00

Designations: Renca

Depositors: R.Wiltrout

Biosafety Level: 1

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Mus musculus* (mouse)

Morphology: epithelial-like

Source:
Organ: kidney
Disease: renal adenocarcinoma
Cell Type: epithelial
Strain: Balb/cCr

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Isolation: Isolation date: 1969

Tumorigenic: YES

Age: 6 weeks

Gender: male

Comments: The Renca cell line was derived from a tumor that arose spontaneously as a renal cortical adenocarcinoma in Balb/cCr mice. The pattern of growth of this tumor accurately mimics that of human adult renal cell carcinoma, particularly with regard to spontaneous metastasis to lung and liver. [PubMed: 4703766, 4057425]The cells do not express transforming growth factor-beta type II receptor (TbetaR-II). [PubMed: 10414746]

Propagation:
ATCC 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:
- 10% fetal bovine serum (final conc.)
- non-essential amino acids (NEAA) (0.1mM extra)
- additional sodium pyruvate (1mM extra)
- additional L-glutamine (2mM extra)

Temperature: 37.0°C
Atmosphere: air, 95%; carbon dioxide (CO2), 5%

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Subculturing:

Protocol: Volumes used in this protocol are for 75 sq cm 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 Ca++/Mg++ free Dulbecco's phosphate-buffered saline (D-PBS) or 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 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 37C 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. An inoculum of 2 X 10(4) to 4 X 10(4) viable cells/sq. cm is recommended.
6. Incubate cultures at 37C. We recommend that you subculture when the culture reaches a cell concentration between 8 X 10(4) and 1.5 X 10(5) cells/sq. cm.
   Subcultivation ratio: A subcultivation ratio of 1:4 to 1:10 is recommended.
   Medium renewal: Every 2 to 3 days

Preservation: **Freeze medium:** RPMI-1640 Medium, 77.5%; FBS, 15% FBS; DMSO, 7.5%
Storage temperature liquid nitrogen vapor phase

Doubling Time: approximately 24 hours

Related Products:
Recommended medium (without the additional serum described under ATCC Medium): ATCC 30-2001
Recommended serum: ATCC 30-30-2020
0.25% (w/v) Trypsin - 0.53mM EDTA in Hank's BSS (w/o Ca++, Mg ++): ATCC 30-2101
Phosphate-buffered saline: ATCC 30-2200
Cell culture tested DMSO: ATCC 4-X
L-Glutamine solution, 200mM: ATCC 30-2214
Erythrosin B vital stain solution: ATCC 30-2404

References:
16172681: Murphy GP, Hrushesky WJ. A murine renal cell carcinoma. J. Natl. Cancer Inst. 50(4):1013-25, 1973. PubMed: 4703796
16172682: Salup RR, et al. Role of natural killer activity in development of spontaneous metastases in murine renal cancer. J. Urol. 134(6):1236-41, 1985. PubMed: 4052425
16172683: Engel J, et al. Transforming growth factor-beta type II receptor confers tumor suppressor activity in murine renal carcinoma (renca) cells . Urology. 54(1):164-70, 1999. PubMed: 10414746

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Cell Biology

ATCC® Number: CL-173™ Order this Item
Price: $256.00

Designations: 3T3-L1

Depositors: Massachusetts Institute of Technology

Biosafety Level: 1

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Mus musculus* (mouse)

Morphology: fibroblast

Source: **Organ:** embryo
Cell Type: fibroblast

Cellular Products: triglycerides [3491]

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Applications: transfection host (Nucleofection technology from Lonza
Roche FuGENE® Transfection Reagents)

Receptors: insulin, expressed

Reverse Transcript: negative

Age: embryo

Comments: L1 is a continuous substrain of 3T3 (Swiss albino) developed through clonal isolation. The cells undergo a pre-adipose to adipose like conversion as they progress from a rapidly dividing to a confluent and contact inhibited state. A high serum content in the medium enhances fat accumulation [PubMed ID: 4426090].
Tested and found negative for ectromelia virus (mousepox).
This line is also designated as ATCC CCL-92.1.ATCC CL-173 was deposited in 1974 without passage number information from the depositor. At the time of submission, ATCC prepared approximately 30 vials of seed stock at about 4 passages beyond the original depositor material (passage number: unknown +4).

Propagation: **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: bovine calf serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
Growth Conditions: The serum used is important in culturing this line. Calf serum is recommended and not fetal bovine serum.

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Subculturing:

Protocol: Never allow culture to become completely confluent.

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37C 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.
   The recommended inoculum is 2 to 3 X 10(3) cells/sq. cm. Subculture before cultures become 70 to 80% confluent or when cells reach 5 to 6 X10(4) viable cells/sq. cm.
6. Incubate cultures at 37C.

Preservation:

Interval: Every three days
Medium Renewal: 2 to 3 times per week
Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Doubling Time: 14 hrs

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002
formerly distributed as:ATCC CCL-92.1
0.25% (w/v) Trypsin - 0.53 mM EDTA in Hank' BSS (w/o Ca++, Mg++):ATCC 30-2101
Cell culture tested DMSO:ATCC 4-X
Recommended serum: ATCC 30-2030

References:

886: Green H, Meuth M. An established pre-adipose cell line and its differentiation in culture. Cell 3: 127-133, 1974. PubMed: 4426090
3491: Green H. Triglyceride-accumulating clonal cell line. US Patent 4,003,789 dated Jan 18 1977
32373: Goodrum FD, et al. Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. J. Virol. 70: 6323-6335, 1996. PubMed: 8709260
32455: Scherer PE, et al. Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. Proc. Natl. Acad. Sci. USA 93: 131-135, 1996. PubMed: 8552590
32787: Kallen CB, Lazar MA. Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. Proc. Natl. Acad. Sci. USA 93: 5793-5796, 1996. PubMed: 8650171

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Cell Biology

ATCC® Number: CCL-92™ Order this Item
Price: $256.00

Designations: 3T3-Swiss albino

Depositors: H Green

Biosafety Level: 1

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Mus musculus* (mouse)

Morphology: fibroblast

PHOTO

Source: **Organ:** embryo
Cell Type: fibroblast

Cellular Products: Lysophosphatidylcholine (lyso-PC) induces AP-1 activity and c-jun N-terminal kinase activity (JNK1) by a protein kinase C-independent pathway [25623]

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Isolation: **Isolation date:** 1962

Virus Susceptibility: polyomavirus; SV40

Reverse Transcript: negative

Cytogenetic Analysis: This is a hypertriploid mouse cell line. The modal chromosome number was 68 occurring in 30% of cells. The rate of cells with higher ploidies was 2.4%.

Age: embryo

Comments: The 3T3 cell line was established by G. Todaro and H. Green in 1962 from disaggregated Swiss mouse embryos. [5732]
The cells are contact inhibited.
A confluent monolayer yields 40000 cells/sq cm.
Tested and found negative for ectromelia virus (mousepox).
The cells should be grown in plastic flasks, they do not grow well on some types of glass surfaces.
A saturation density of approximately 50000 cells per sq cm can be reached.

Propagation: **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: bovine calf serum to a final concentration of 10%.
Temperature: 37.0°C

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Subculturing:	**Protocol:** Never allow culture to become completely confluent. Remove medium, and rinse with 0.25% trypsin, 0.53 mM EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks. For plates (50mm) use an inoculum of 3 X 10 exp5 cells per plate and subculture every 3 days. For 75 sq cm flasks use 4 X 10 exp5 cells per flask and subculture every 3 days. **Medium Renewal:** Twice per week
Preservation:	**Freeze medium:** Complete growth medium 95%; DMSO, 5% **Storage temperature:** liquid nitrogen vapor temperature
Doubling Time:	18 hrs
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002 recommended serum:ATCC 30-2020 irradiated to be used as feeder cells:ATCC 46-X
References:	5732: Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17: 299-313, 1963. PubMed: 13985244 21632: Bennicelli JL, et al. Mechanism for transcriptional gain of function resulting from chromosomal translocation in alveolar rhabdomyosarcoma. Proc. Natl. Acad. Sci. USA 93: 5455-5459, 1996. PubMed: 8643596 26261: Vogt M, Dulbecco R. Studies on cells rendered neoplastic by polyoma virus: the problem of the presence of virus-related materials. Virology 16: 41-51, 1962. PubMed: 13926482 26262: Todaro GJ, et al. Antigenic and cultural properties of cells doubly transformed by polyoma virus and SV40. Virology 27: 179-185, 1965. PubMed: 4284055 26263: Todaro GJ, et al. Transformation of properties of an established cell line by SV40 and polyoma virus. Proc. Natl. Acad. Sci. USA 51: 66-73, 1964. PubMed: 14104605 26623: Fang X, et al. Lysophosphatidylcholine stimulates activator protein 1 and the c-Jun N-terminal kinase activity. J. Biol. Chem. 272: 13683-13689, 1997. PubMed: 9153219 32307: Chen ST, et al. Generation of packaging cell lines for pseudotyped retroviral vectors of the G protein of vesicular stomatitiis virus by using a modified tetracycline inducible system. Proc. Natl. Acad. Sci. USA 93: 10057-10062, 1996. PubMed: 8816759 32500: Campbell M, et al. The simian foamy virus type 1 transcriptional transactivator (Tas) binds and activates an enhancer element in the gag gene. J. Virol. 70: 6847-6855, 1996. PubMed: 8794326 33069: Hsu DK, et al. Identification of a murine TEF-1-related gene expressed after mitogenic stimulation of quiescent fibroblasts and during myogenic differentiation. J. Biol. Chem. 271: 13786-13795, 1996. PubMed: 8662936

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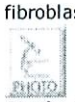
Cell Biology

ATCC® Number: CCL-226™ Order this Item
Designations: C3H/10T1/2, Clone 8
Depositors: C Heidelberger
Biosafety Level: 1
Shipped: frozen
Medium & Serum: See Propagation
Growth Properties: adherent
Organism: *Mus musculus* (mouse)
Morphology: fibroblast



Source: **Strain:** C3H
Organ: embryo
Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications: transfection host (Roche FuGENE® Transfection Reagents)
Tumorigenic: No
Reverse Transcript: negative
Antigen Expression: H-2k
Cytogenetic Analysis: Mouse karyotype with a modal number of 80 chromosomes.
Age: embryo

Price: $269.00

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Comments:
C3H/10T1/2, Clone 8 was isolated by C. Reznikoff, D. Brankow and C. Heidelberger in 1972 from a line of C3H mouse embryo cells. [23019]
The cells are very sensitive to post confluence inhibition of cell division, do not produce tumors in syngeneic mice, have no background of spontaneous transformation, nor do they contain overt endogenous transforming murine leukemia or sarcoma viruses. [22697]
The cells are contact sensitive.
There is no detectable background spontaneous transformation.
They are highly susceptible to transformation by chemical agents. [1208]
Tested and found negative for ectromelia virus (mousepox).
NOTE: THE INOCULATION DENSITY, FEEDING AND HARVESTING SCHEDULES MUST BE FOLLOWED RIGIDLY IF THE LINE IS TO RETAIN ITS ESSENTIAL CHARACTERISTICS.
THE BATCH OF SERUM USED FOR GROWTH AND FOR TRANSFORMATION ASSAYS MAY AFFECT BOTH THE MORPHOLOGY OF THIS LINE AND THE RESULTS OBTAINED.
Monolayers established and maintained for the standard transformation assay should be free of all foci after 6 weeks. [1208]
The donor recommends that the line be used between the 5th and 15th passages only.

Propagation:
ATCC complete growth medium: The base medium for this cell line is Eagle's Basal medium with 2 mM L-glutamine , 1.5 g/L sodium bicarbonate and Earle's BSS. To make the complete growth medium, add the following components to the base medium: heat-inactivated fetal bovine serum to a final concentration of 10%.
Temperature: 37.0°C

Subculturing:
Protocol: Remove medium, and rinse with 0.25% trypsin, 0.53 mM EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks. SUBCULTURE MUST BE DONE BEFORE THE CULTURE REACHES CONFLUENCE.
Subcultivation Ratio: Seed new flasks at 2000 viable cells/sq cm.
Medium Renewal: Once between subcultures if necessary

Preservation:
Freeze medium: Complete growth medium 95%; DMSO, 5%
Storage temperature: liquid notrogen vapor temperature

References:
1208: Reznikoff CA, et al. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. Cancer Res. 33: 3239-3249, 1973. PubMed: 4796600
1209: Terzaghi M, Little JB. Repair of potentially lethal radiation damage in mammalian cells is associated with enhancement of malignant transformation. Nature 253: 548-549, 1975. PubMed: 1167940
1210: Mondal S, Heidelberger C. Transformation of C3H/10T1/2 CL8 mouse embryo fibroblasts by ultraviolet irradiation and a phorbol ester. Nature 260: 710-711, 1976. PubMed: 1264202
22440: Smith GJ, et al. Clonal analysis of the expression of multiple transformation phenotypes and tumorigenicity by morphologically transformed 10T1/2 cells. Cancer Res. 53: 500-508, 1993. PubMed: 8425183
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23019: Reznikoff CA, et al. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Res. 33: 3231-3238, 1973. PubMed: 4357355
33039: Jain MK, et al. Molecular cloning and characterization of SmLIM, a developmentally regulated LIM protein preferentially expressed in aortic smooth muscle cells. J. Biol. Chem. 271: 10194-10199, 1996. PubMed: 8626552

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Cell Biology

ATCC® Number:	**CRL-1651™** Order this Item
Designations:	COS-7
Depositors:	Y Gluzman
Biosafety Level:	2 [Cells Contain SV-40 viral DNA sequences]
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Cercopithecus aethiops*
Morphology:	fibroblast
	PHOTO
Source:	**Organ:** kidney **Cell Type:** SV40 transformed
Cellular Products:	T antigen
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Virus Susceptibility:	SV40 (lytic growth); SV40 tsA209 at 40C; SV40 mutants with deletions in the early region
Comments:	This is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. This line contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40C, and supports the replication of pure populations of SV40 mutants with deletions in the early region.The 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.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C

Price: **$269.00**

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Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is recommended
Medium Renewal: 2 to 3 times per week

Preservation:

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

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002
recommended serum:ATCC 30-2020
parental cell line:ATCC CCL-70
0.25% (w/v) Trypsin - 0.53 mM EDTA in Hank' BSS (w/o Ca++, Mg++):ATCC 30-2101
Cell culture tested DMSO:ATCC 4-X

References:

1822: Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23: 175-182, 1981. PubMed: 6260373
32447: Fernandez LM, Puett D. Lys583 in the third extracellular loop of the lutropin/choriogonadotropin receptor is critical for signaling. J. Biol. Chem. 271: 925-930, 1996. PubMed: 8557706
32459: 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: 8570611
32500: Campbell M, et al. The simian foamy virus type 1 transcriptional transactivator (Tas) binds and activates an enhancer element in the gag gene. J. Virol. 70: 6847-6855, 1996. PubMed: 8794326
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32547: Jang SI, et al. Activator protein 1 activity is involved in the regulation of the cell type-specific expression from the proximal promoter of the human profilaggrin gene. J. Biol. Chem. 271: 24105-24114, 1996. PubMed: 8798649
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32720: Chen Y, et al. Demonstration of binding of dengue virus envelope protein to target cells. J. Virol. 70: 8765-8772, 1996. PubMed: 8971005
32728: Russell DW, Miller AD. Foamy virus vectors. J. Virol. 70: 217-222, 1996. PubMed: 8523528
32861: Wright DA, et al. Association of human fas (CD95) with a ubiquitin-conjugating enzyme (UBC-FAP). J. Biol. Chem. 49: 31037-31043, 1996. PubMed: 8940092
32893: Zhang J, et al. Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. J. Biol. Chem. 271: 18302-18305, 1996. PubMed: 8702465
33011: Ozcelebi F, et al. Phosphorylation of cholecystokinin receptors expressed on chinese hamster ovary cells. J. Biol. Chem. 271: 3750-3755, 1996. PubMed: 8631990
33013: Gibson S, et al. Functional LCK is required for optimal CD28-mediated activation of the TEC family tyrosine kinase EMT/ITK. J. Biol. Chem. 271: 7079-7083, 1996. PubMed: 8636141
33016: Shaul PW, et al. Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. J. Biol. Chem. 271: 6518-6522, 1996. PubMed: 8626455
33032: Ladner RD, et al. Identification of a consensus cyclin-dependent kinase phosphorylation site unique to the nuclear form of human deoxyuridine triphosphate nucleotidohydrolase. J. Biol. Chem. 271: 7752-7757, 1996. PubMed: 8631317
33038: Wu X, et al. Demonstration of a physical interaction between microsomal triglyceride transfer protein and apolipoprotein B during the assembly of ApoB-containing lipoproteins. J. Biol. Chem. 271: 10277-10281, 1996. PubMed: 8626595
33052: Hawes BE, et al. Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. J. Biol. Chem. 271: 12133-12136, 1996. PubMed: 8647803
33137: Arai H, Charo IF. Differential regulation of G-protein-mediated signaling by chemokine receptors. J. Biol. Chem. 271: 21814-21819, 1996. PubMed: 8702980
33163: Hsieh CM, et al. APEG-1, a novel gene preferentially expressed in aortic smooth muscle cells, is down-regulated by vascular injury. J. Biol. Chem. 271: 17354-17359, 1996. PubMed: 8663449
33175: Holtmann MH, et al. Multiple extracellular loop domains contribute critical determinants for agonist binding and activation of the secretin receptor. J. Biol. Chem. 271: 14944-14949, 1996. PubMed: 8663161

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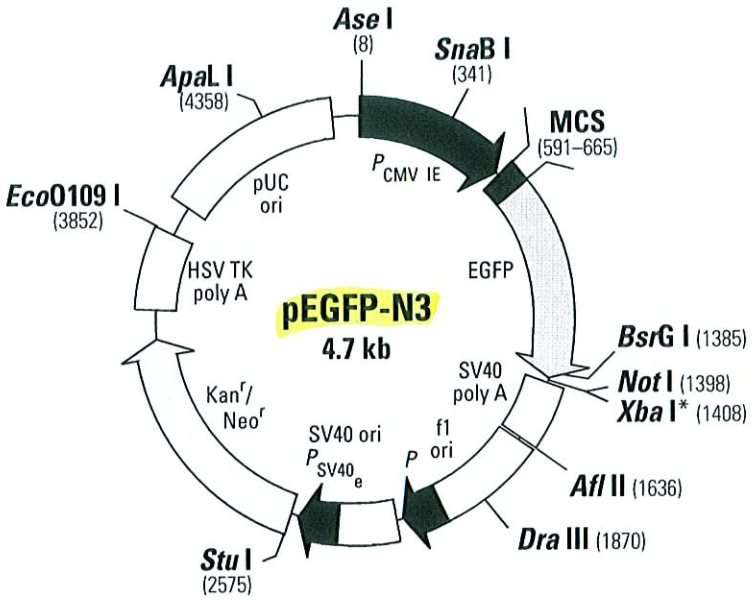
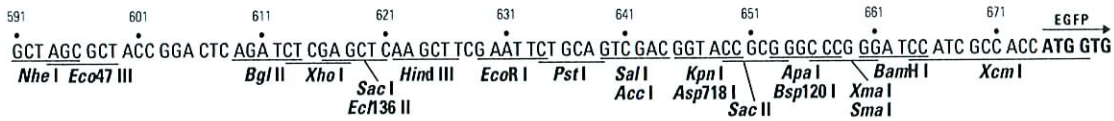
pEGFP-N3 Vector Information

PT3054-5

GenBank Accession #: U57609

Catalog #6080-1





Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N3 (Unique restriction sites are in bold). 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*⁻ host and make fresh DNA.

Description:

pEGFP-N3 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-N3 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-N3 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 (Neor), 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-N3 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

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(PR29967; published 03 October 2002)

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-N3 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-N3 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: 591–665
- Enhanced green fluorescent protein gene
  Kozak consensus translation initiation site: 668–678
  Start codon (ATG): 675–677; Stop codon: 1392–1394
  Insertion of Val at position 2: 678–680
  GFPmut1 chromophore mutations (Phe-64 to Leu; Ser-65 to Thr): 867–872
  His-231 to Leu mutation (A→T): 1369
- SV40 early mRNA polyadenylation signal
  Polyadenylation signals: 1548–1553 & 1577–1582; mRNA 3' ends: 1586 & 1598
- f1 single-strand DNA origin: 1645–2100 (Packages the noncoding strand of EGFP)
- Bacterial promoter for expression of Kan^r gene:
  –35 region: 2162–2167; –10 region: 2185–2190
  Transcription start point: 2197
- SV40 origin of replication: 2441–2576
- SV40 early promoter
  Enhancer (72-bp tandem repeats): 2274–2345 & 2346–2417
  21-bp repeats: 2421–2441, 2442–2462 & 2464–2484
  Early promoter element: 2497–2503
  Major transcription start points: 2493, 2531, 2537 & 2542
- Kanamycin/neomycin resistance gene
  Neomycin phosphotransferase coding sequences: start codon (ATG): 2625–2627; stop codon: 3417–3419
  G→A mutation to remove *Pst* I site: 2807
  C→A (Arg to Ser) mutation to remove *Bss*H II site: 3153
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  Polyadenylation signals: 3655–3660 & 3668–3673
- pUC plasmid replication origin: 4004–4647

Primer Locations:
- EGFP-N Sequencing Primer (#6479-1): 741–720
- EGFP-C Sequencing Primer (#6478-1): 1328–1349

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

References:
1. 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. Glover, D. M. (IRL Press, Oxford, UK) pp. 143–190.

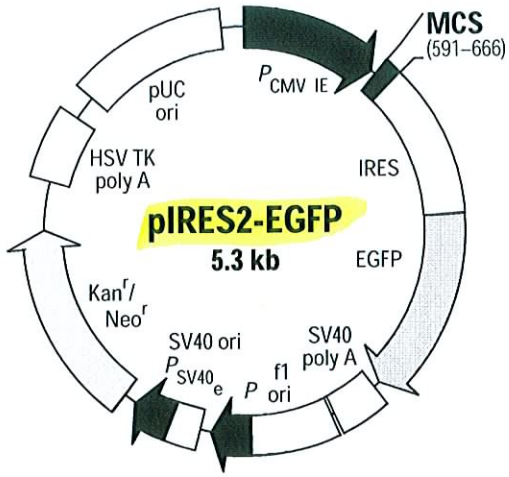
Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by BD Biosciences Clontech. This vector has not been completely sequenced.

Vector Information

pIRES2-EGFP Vector Information

PT3267-5

Catalog #6029-1



591 601 611 621 631 641 651 661

GCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGCCCGGGATCC...IRES

***Nhe* I** ***Eco*47 III** ***Bgl* II** ***Xho* I** *Hind* III ***Sac* I** ***Ecl*136 II** ***Eco*R I** ***Pst* I** ***Sal* I** ***Acc* I** *Kpn* I *Asp*718 I *Apa* I *Bsp*120 I ***Sac* II** ***Xma* I** ***Sma* I** ***Bam*H I**

Restriction Map and Multiple Cloning Site (MCS) of pIRES2-EGFP Vector. Unique restriction sites are in bold. Note that the *Eco*47 III site has not been confirmed in the final construct.

Description:

pIRES2-EGFP contains the internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. This permits both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA. pIRES2-EGFP is designed for the efficient selection (by flow cytometry or other methods) of transiently transfected mammalian cells expressing EGFP and the protein of interest. This vector can also be used to express EGFP alone or to obtain stably transfected cell lines without time-consuming drug and clonal selection.

EGFP is a red-shifted variant of wild-type GFP (3–5) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) EGFP encodes the GFPmut1 variant (6) 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 (7). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (8) to further increase the translation efficiency in eukaryotic cells. The MCS in pIRES2-EGFP is between the immediate early promoter of cytomegalovirus ($P_{CMV\ IE}$) and the IRES sequence. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the bicistronic 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 pIRES2-EGFP backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production. pIRES2-EGFP replaces (but is not derived from) the pIRES-EGFP Vector previously sold by BD Biosciences Clontech. pIRES2-EGFP is functionally similarly to pIRES-EGFP; however, pIRES2-EGFP gives brighter EGFP fluorescence than the older vector. Note that the *Xba* I site at position

(PR29951; published 03 October 2002)

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1987 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*⁻ host and make fresh DNA.

Use:
Genes inserted into the MCS should include the initiating ATG codon. pIRES2-EGFP and its derivatives can be introduced into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (9).

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: 591–665
- IRES sequence: 666–1250
- Enhanced green fluorescent protein (EGFP) gene
  Kozak consensus translation initiation site: 1247–1257
  Start codon (ATG): 1254–1256; Stop codon: 1971–1973
  Insertion of Val at position 2: 1257–1259
  GFPmut1 chromophore mutations (Phe-64 to Leu; Ser-65 to Thr): 1446–1451
  His-231 to Leu mutation (A→T): 1948
- SV40 early mRNA polyadenylation signal
  Polyadenylation signals: 2127–2132 & 2156–2161; mRNA 3' ends: 2165 & 2177
- f1 single-strand DNA origin: 2224–2679 (Packages the noncoding strand of EGFP.)
- Bacterial promoter for expression of Kan^r gene:
  –35 region: 2741–2746; –10 region: 2764–2769
  Transcription start point: 2776
- SV40 origin of replication: 3020–3155
- SV40 early promoter/enhancer
  72-bp tandem repeats: 2853–2996; 21-bp repeats (3): 3000–3063
  Early promoter element: 3076–3082
- Kanamycin/neomycin resistance gene: 3204–3998
  G→A mutation to remove *Pst* I site: 3386; C→A (Arg to Ser) mutation to remove *Bss*H II site: 3732
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signals: 4234–4252
- pUC plasmid replication origin: 4583–5226

Propagation in *E. coli*
- Suitable host strains: DH5α, HB101, and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as 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. Jackson, R. J., *et al.* (1990) *Trends Biochem. Sci.* **15**:477–483.
2. Jang, S. K., *et al.* (1990) *J. Virol.* **62**:2636–2643.
3. Cormack, B., *et al.* (1996) *Gene* **173**:33–38.
4. Yang, T. T., *et al.* (1996) *Nucleic Acids Res.* **24**:4592–4593.
5. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
6. Jackson, R. J., *et al.* (1990) *Trends Biochem.* **15**:477–483.
7. Jang, S. K., *et al.* (1988) *J. Virol.* **62**:2636–2643.
8. Huang, M. T. F. & Gorman, C. M. (1990) *Nucleic Acids Res.* **18**(4):937–947.
9. 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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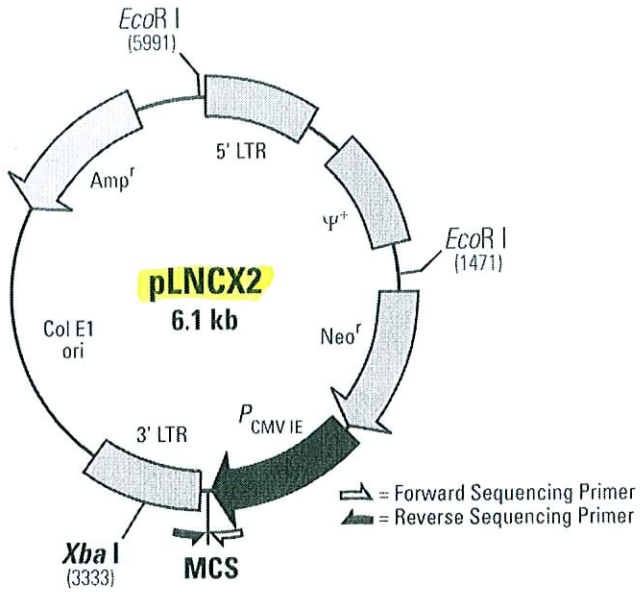
Vector Information

pLNCX2 Vector Information

PT3297-5

GenBank Accession #: Submission in progress.

Cat. No. 631503
631508
631511



2926 2936 2946 2956 2966 2976 2986 2996

AGATCTCGAGCTCAAGCTTGTTTGGCCGAGGCGGCCGCTTGTCGACAGGCCTTAATGGCCTAACATCGATA

Bgl II *Xho* I *Hind* III *Sfi* I *Not* I *Sal* I *Stu* I *Sfi* I *Cla* I

Restriction Map and Multiple Cloning Site (MCS) of pLNCX2. Unique restriction sites are in bold.

Description

pLNCX2 contains elements derived from Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV), and is designed for retroviral gene delivery and expression (1–3). Upon transfection into a packaging cell line, pLNCX2 can transiently express, or integrate and stably express, a transcript containing Ψ+ (the extended viral packaging signal) a selectable marker, and the gene of interest. The 5' viral LTR in this vector contains viral promoter/enhancer sequences that control expression of the neomycin resistance (Neo^r) gene for antibiotic selection in eukaryotic cells. A gene of interest can be cloned into the multiple cloning site immediately downstream of the human cytomegalovirus (CMV) immediate early promoter (P_{CMV}). pLNCX2 also includes the Col E1 origin of replication and *E. coli* Amp^r gene for propagation and antibiotic selection in bacteria.

Use

pLNCX2 can be transfected into a packaging cell line such as the RetroPack™ PT67 Cell Line (Cat. No. 631510). Once in the cell, RNA from the vector is packaged into infectious, replication-incompetent retroviral particles. pLNCX2 does not contain the structural genes (*gag*, *pol*, and *env)* necessary for particle formation and replication; these genes are stably integrated into PT67 (4–7). Subsequent introduction of pLNCX2, containing Ψ+, transcription and processing elements, and the gene of interest produces high-titer, replication-incompetent infectious virus. These retroviral particles can infect target cells and transmit the gene of interest (which is cloned between the viral LTR sequences), but cannot replicate within these cells since the cells lack the viral structural genes. The separate introduction and integration of the structural genes into the packaging cell line minimizes the chances of producing replication-competent virus due to recombination events during cell proliferation.

(PR792366; published 10 September 2007)

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Location of Features

- 5' MoMuSV LTR: 1–589
- Ψ^+ (extended packaging signal): 659–1468
  Mutated *gag* (ATG–>TAG): 1049–1051
- Neomycin resistance gene (Neo^r):
  Start codon: 1512–1514; stop codon: 2304–2306
- Immediate early CMV promoter (P_{CMV}): 2374–2906
- Multiple Cloning Site (MCS): 2926–2996
- 3' MoMuLV LTR: 3035–3628
- Col E1 origin of replication:
  Site of replication initiation: 4164
- Ampicillin resistance gene (β-lactamase):
  Start codon: 5784–5782; stop codon: 4926–4924

Sequencing primer locations

- pLNCX Seq/PCR Primers:
  5' primer (2882-2906): 5'-AGCTGGTTTAGTGAACCGTCAGATC-3'
  3' primer (3057-3032): 5'-ACCTACAGGTGGGGTCTTTCATTCCC-3'

Propagation in *E. coli*

- Suitable host strains: DH5α, HB101, and other general purpose strains.
- Selectable marker: plasmid confers resistance to ampicillin (100 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: Col E1
- Copy number: low

References

1. Coffin, J. M. & Varmus, H. E., Eds. (1996) *Retroviruses* (Cold Spring Harbor Laboratory Press, NY).
2. Ausubel, F. M., *et al.* (1994) *Current Protocols in Molecular Biology* (Greene Publishing Associates, Inc. & John Wiley & Sons, Inc.).
3. Miller, A. D. & Rosman, G. J. (1989) *BioTechniques* **7**:980–990.
4. Mann, R., *et al.* (1983) *Cell* **33**:153–159.
5. Miller, A. D. & Buttimore, C. (1986) *Mol. Cell. Biol.* 6:2895–2902.
6. Morgenstern, J. P. & Land, H. (1990) *Nucleic Acids Res.* **18**:3587–3590.
7. Miller, A. D. & Chen, F. (1996) *J. Virol.* **70**:5564–5571.

Notes: The viral supernatants produced by this retroviral vector could, depending on your cloned insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant retrovirus. Appropriate NIH, regional, and institutional guidelines apply.

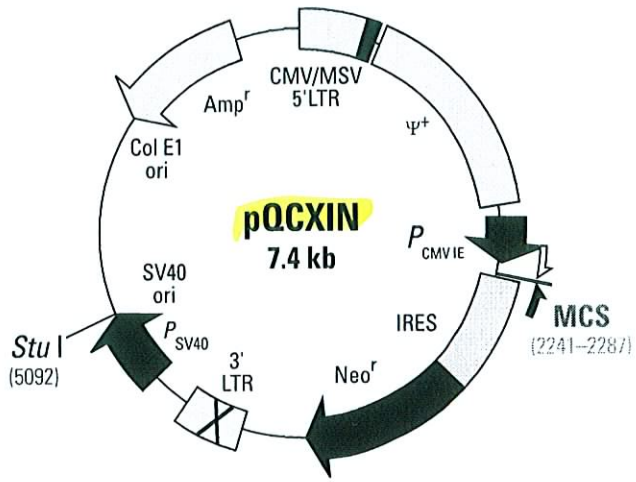
The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by Clontech. This vector has not been completely sequenced.

Vector Information

pQCXIN Retroviral Vector Information

PT3667-5

Cat. No. 631514



= Forward Sequencing Primer
= Reverse Sequencing Primer

2240 2250 2260 2270 2280

CGCGGCCGCACCGGTAGGCCTCGTACGCTTAATTAACGGATCCGGAATTCC

Not I *Age* I *Bsi* WI *Pac* I *Bam*H I *Eco*R I

Restriction Map and Multiple Cloning Site (MCS) of pQCXIN Vector. Unique restriction sites are in bold.

Description

pQCXIN Retroviral Vector is a bicistronic expression vector designed to express a target gene along with the neomycin selection marker (1). Upon transfection into a packaging cell line, this vector can transiently express, or integrate and stably express a viral genomic transcript containing the CMV immediate early promoter, gene of interest, IRES and the neomycin resistance gene (Neo^r). The gene of interest and the neomycin resistance gene are co-translated, via the internal ribosome entry site (IRES), from a bicistronic message in mammalian cells (2, 3).

This vector incorporates unique features including: optimization to remove promoter interference and self-inactivation. The hybrid 5' LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter. This construct drives high levels of transcription in HEK 293-based packaging cell lines due, in part, to the presence of adenoviral E1A (4, 5, 6, 7) in these cells. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. This may reduce the phenomenon known as promoter interference (8) and allow more efficient expression.

Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (Psi$^+$), and tRNA primer binding site. pQCXIN also contains a bacterial origin of replication and *E. coli* Amp^r gene for propagation and selection in bacteria.

(PR7Y2441; published January 2008)

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Use

pQCXIN is designed to deliver and express a gene along with the neomycin resistance marker from a bicistronic message. The design is optimized to produce high titers via the $P_{CMV\ IE}$ in the packaging cell line. The bicistronic transcript makes it unnecessary to screen the transformants since the neomycin resistance is expressed in concert with the DNA inserted into the multiple cloning site.

Once transfected into the packaging cell line (such as the RetroPack™ PT67 Cell Line (Cat. No.631510) AmphoPack293, EcoPack2-293, or Pantropic System), RNA from the vector is packaged into infectious, replication-incompetent retroviral particles since pQCXIN lacks structural genes (gag, pol, and env) necessary for particle formation and replication; however, these genes are stably integrated as part of the packaging cell genome. Once a high titer clone is selected, these retroviral particles can infect target cells and transmit the gene of interest but cannot replicate within these cells due to the absence of viral structural genes. The separate introductioin and integration of the structural genes into the packaging cell line minimizes the chances of producing replication-competent virus due to recomination events during cell proliferation.

Location of Features

- 5' LTR (CMV/MSV): 1–728
  Cytomegalovirus (CMV)/ mouse sarcoma virus (MSV) hybrid promoter:1–511
  R region: 584–654
  U5 region: 655–728
- Ψ^+ (extended packaging signal): 758–1567
- Cytomegalovirus (CMV) immediate early promoter ($P_{CMV\ IE}$): 1601–2132
- Multiple Cloning Site (MCS): 2238–2287
- Internal ribosome entry site (IRES): 2289–2862
- Neomycin resistance gene (Neo^r): 2876–3670
- 3' MoMuLV LTR (deletion in U3): 4087–4512
  Poly A signal: 4415–4420
  cleavage site: 4435–4436
- SV40 promoter: 4792–5059
- SV40 ori: 5013–5078
  Site of replication initiation
- Col E1 ori (Site of replication initiation): 5399
- Ampicillin resistance gene (β-lactamase): 7019–6159
  Start codon (ATG): 7019–7017 stop codon (TAA): 6161–6159

Sequencing Primer Locations

- pQC Seq/PCR Primers:
  5' primer (2141–2164): 5'-ACGCCATCCACGCTGTTTTGACCT-3'
  3' primer (2311–2334): 5'-AAGCGGCTTCGGCCAGTAACGTTA-3'

Propagation in *E. coli*

- Suitable host strains: DH5α, DH10B, and other general purpose strains.
- Selectable marker: plasmid confers resistance to ampicillin (100 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: ColE1
- Copy number: low

References

1. Julius, M. A., Yan, Q., Zheng, Z., & Kitajewski, J. (2000) *BioTechniques* **28**(4):702–707.
2. Adam, M. A., Ramesh, N., Miller, A. D. & Osborne, W. R. (1991) *J. Virol.* **65**:4985–4990.
3. Ghattas, I. R., Sanes, J. R. & Majors, J. E. (1991) *Mol. Cell Biol.* **11**:5848–5859.
4. Kinsella, T. M. & Nolan G. P. (1996) *Hum. Gene Ther.* **7**:1405–1413.
5. Ory, D. S., Neugeboren, B. A. & Mulligan, R. C. (1996) *Proc. Nat. Acad. Sci. USA* **93**:11400–11406.
6. Pear, W. S., Nolan, G. P., Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**(18):8392–8396.
7. Yang, S., Delgado, R., King, S. R., Woffendin, C., Barker, C. S., Yang, Z. Y., Xu, L., Nolan, G. P. & Nabel, G. J. (1999) *Hum. Gene Ther.* **10**:123–132.
8. Emerman, M. & Temin, H. M. (1984) *Cell* **39**:449–467.

Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with sequences obtained by Clontech Laboratories, Inc. This vector has been completely sequenced.

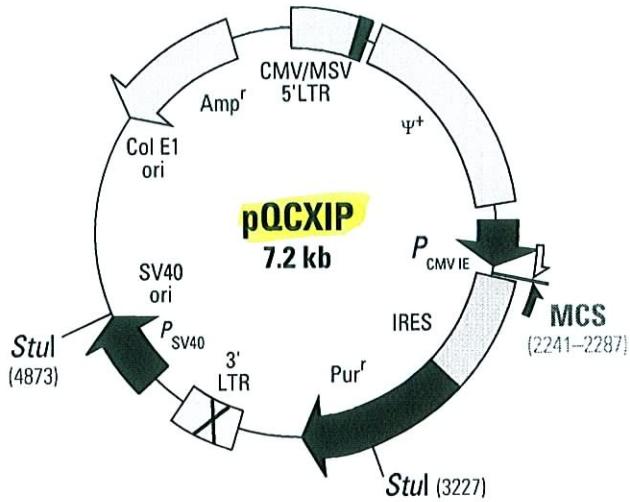
The viral supernatants produced by this retroviral vector could, depending on your cloned insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant retrovirus. Appropriate NIH, regional, and institutional guidelines apply.

Vector Information

pQCXIP Vector Information

PT3669-5

Cat. No. 631516



= Forward Sequencing Primer
= Reverse Sequencing Primer

2240 2250 2260 2270 2280

CGCGGCCGCACCGGTAGGCCTCGTACGCTTAATTAACGGATCCGGAATTCC

Not I **Age I** **Pac I** **BamH I** **EcoR I**

Restriction Map and Multiple Cloning Site (MCS) of pQCXIP Vector. Unique restriction sites are in bold.

Description

pQCXIP Retroviral Vector is a bicistronic expression vector designed to express a target gene along with the puromycin selection marker (1). Upon transfection into a packaging cell line, this vector can transiently express, or integrate and stably express a viral genomic transcript containing the CMV immediate early promoter, gene of interest, IRES and the puromycin resistance gene (Pur^r). The gene of interest and the puromycin resistance gene are co-transcribed, via the internal ribosome entry site (IRES), as a bicistronic message in mammalian cells (2, 3).

This vector incorporates unique features including: optimization to remove promoter interference and self-inactivation. The hybrid 5' LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter. This construct drives high levels of transcription in HEK 293-based packaging cell lines due, in part, to the presence of adenoviral E1A (4, 5, 6, 7) in these cells. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. This may reduce the phenomenon known as promoter interference (8) and allow more efficient expression.

Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (Psi$^+$), and tRNA primer binding site. pQCXIP also contains a bacterial origin of replications and *E. coli* Amp^r gene for propagation and selection in bacteria.

(PR7Y2443; published January 2008)

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Use

pQCXIP is designed to deliver and express a gene along with the puromycin resistance marker from a bicistronic message. The design is optimized to produce high titers via the $P_{CMV\ IE}$ in the packaging cell line. The bicistronic transcript makes it unnecessary to screen the transformants since the puromycin resistance is expressed in concert with the gene inserted into the multiple cloning site.

Once transfected into the packaging cell line (such as the RetroPack™ PT67 Cell Line (Cat. No.631510) AmphoPack293, EcoPack2-293, or Pantropic System), RNA from the vector is packaged into infectious, replication-incompetent retroviral particles since pQCXIP lacks structural genes (gag, pol, and env) necessary for particle formation and replication; however, these genes are stably integrated as part of the packaging cell genome. Once a high titer clone is selected, these retroviral particles can infect target cells and transmit the gene of interest but cannot replicate within these cells due to the absence of viral structural genes. The separate introduction and integration of the structural genes into the packaging cell line minimizes the chances of producing replication-competent virus due to recombination events during cell proliferation.

Location of Features

- 5' LTR (CMV/MSV): 1–728
  Cytomegalovirus (CMV)/ mouse sarcoma virus (MSV) hybrid promoter: 1–511
  R region: 584–654
  U5 region: 655–728
- Ψ^+ (extended packaging signal): 758–1567
- Immediate early CMV promoter ($P_{CMV\ IE}$): 1601–2132
- Multiple Cloning Site (MCS): 2239–2287
- Internal ribosome entry site (IRES): 2289-2862
- Puromycin resistance gene (Pur^r): 2898–3494
  Start codon (ATG): 2895–2897; stop codon (TGA): 3492–3494
- 3' MoMuLV LTR (deletion in U3): 3868–4293
  Poly A region: 4195–4216
- SV40 promoter: 4573–4840
- SV40 ori: 4794–4859
- Col E1 ori (Site of replication initiation): 5180
- Ampicillin resistance gene (β-lactamase): 6800–5940
  Start codon (ATG): 6800–6798 stop codon (TAA): 5940–5942

Sequencing Primer Locations

- pQC Seq/PCR Primers:
  5' primer (2141–2164): 5'-ACGCCATCCACGCTGTTTTGACCT-3'
  3' primer (2311–2334): 5'-AAGCGGCTTCGGCCAGTAACGTTA-3'

Propagation in *E. coli*

- Suitable host strains: DH5α, DH10B, and other general purpose strains.
- Selectable marker: plasmid confers resistance to ampicillin (100 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: Col E1
- Copy number: low

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

The viral supernatants produced by this retroviral vector could, depending on your cloned insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant retrovirus. Appropriate NIH, regional, and institutional guidelines apply.

Home : AdMax

Last Updated 1/07/09

AdMax

"We continue to have great success with the AdMax™ system for producing a wide range of vectors for our research. We can go from plasmid clone to high titre vector ready for in vitro or in vivo evaluation in just a few weeks."
Michael Parr Ph.D., Scientist, Gene Therapy/Delivery Group, Validation Biology, Biogen Inc.

"Our lab uses the AdMax™ system for construction of E1/E3-deleted Adenovirus vectors routinely, with excellent results. We have found this technology to be easy and reliable."
C.S.H. Young, Professor of Microbiology, Columbia University, New York, NY USA.

"This method works really great, it proved to be efficient and reliable. We think that so far this is the best available method for constructing the recombinant viruses."
Dr. Elena Burova, Associate Manager, Adenovirus Facility, Regeneron Pharmaceuticals, Inc.

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You will require the ACROBAT plug-in to view PDF files. If you do not have the plug-in, please download the latest version here.

Clone, cotransfect and GO!
Small shuttle plasmids, single cloning step, cotransfections without restriction, 95% reliability. The simplest, most efficient, most flexible system for construction of adenovirus expression vectors.

How fast?
How fast can you clone your gene into a small pUC based shuttle plasmid and prepare 100ug plasmid DNA? Add 7 to 10 days to that!

How efficient?
Approximately 100 fold more plaques rescued than with previous two plasmid methods.

How reliable?
If your expression cassette is less than 7-8 kb and your transgene product is nontoxic, 95% of recombinant viruses should contain and express the transgene. Use your favourite promoter or use the high efficiency MCMV IE promoter provided with our kits.

How simple?
Only two steps. No homologous recombination in difficult to handle bacterial systems; use your favourite bacterial strain. No transfer of candidate plasmids from one bacterial strain to another. No need for expensive, exotic restriction enzymes or for linearization of plasmid DNA prior to cotransfection of 293 cells. The system does not require lambda packaging or yeast technologies that are not standard procedures in the majority of labs.

How flexible?
Cassettes can be inserted in E1 or E3 or transgenes can be cloned into both regions. For example a transactivator can be inserted in E3 and a regulated expression cassette in E1. Vectors can be designed with an E3 deletion, a wild type E3 region or, if the transgene in E1 is small, a stuffer sequence can be inserted in E3 to prevent formation of RCA. You have a choice of two site specific recombinases: Cre or FLP, with similar high rescue efficiencies.

How expensive?
The initial cost of our kits is competitive with other systems, but unlike other kits ours allow for an infinite number of vector rescues. If you can grow plasmid DNA there is no need to purchase our kits more than once.

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System Overview

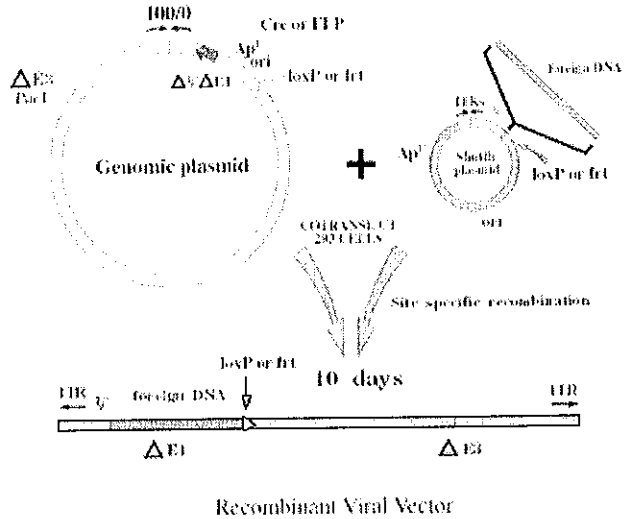

Figure 1 outlines the principles of the AdMax™ system with Cre-lox as an example. Recombination in cotransfected cells introduces the gene of interest into infectious Ad DNA while simultaneously excising the recombinase gene (Ng et al., 1999, 2000).

Neither the small shuttle plasmid nor the genomic plasmid need be digested with restriction enzymes prior to cotransfection. Any E1 complementing cell line such as 293 cells (Graham et al., 1977), 911 cells (Fallaux et al., 1996) or PERC6 cells (Fallaux et al., 1998) can be used for cotransfections.

Although rescue of viral vectors is highly efficient (over 100 fold greater than with the original two plasmid method of Bett et al., (1994)), and 95% of viruses generated by cotransfection should carry the transgene, it is good laboratory practice to build up working stocks of virus from plaque isolates before extensive experimentation.

Microbix provides low passage 293 cells that are especially cultured to maintain the strong adherence and plaque forming properties of the original 293 cells. For rapid production of vectors to be used in preliminary experiments, it may be possible to produce recombinant viruses by incubating cell cultures under liquid medium following cotransfections.

Transgenes are cloned into one of our small high copy number shuttle plasmids (Figures 2 and 4) which are then cotransfected with an Ad genomic plasmid (Figures 3 and 5) into 293 cells. High efficiency site specific recombination catalyzed by Cre or FLP recombinase results in "rescue" of the expression cassette into the left end of an E1 deleted (first generation) Ad vector.

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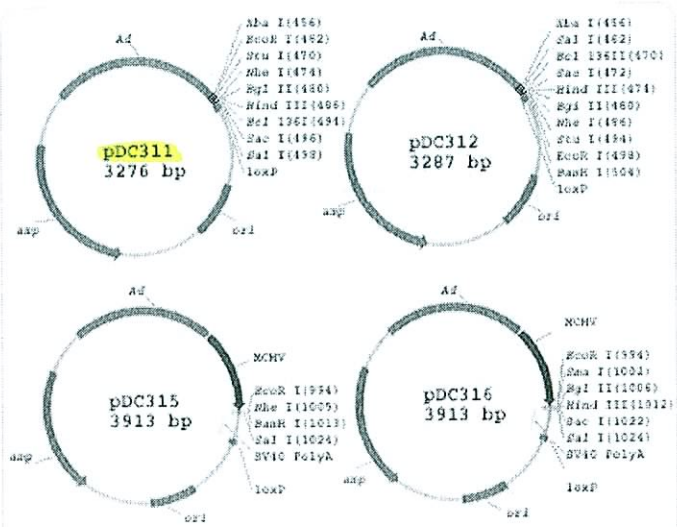

Figure 2. Shuttle plasmids for Cre-lox Ad vector construction

Shuttle plasmids **(Figure 2)** designed for insertion of the transgene are small, simple and pUC based for high yields. Promoterless plasmids with polycloning sites comprising recognition sites for 8 enzymes are only 3.2 kb in size. Plasmids containing an expression cassette utilizing the Murine Cytomegalovirus Immediate Early Gene promoter (MCMV Pr) are only 3.9 kb and have up to 6 restriction enzyme cloning sites. The genomic plasmids containing most of the Ad genome plus cassettes expressing recombinase and carrying the recombinase recognition site are approximately 34 kb in size. Two recombination systems are available, based on Cre-lox or FLP-frt.

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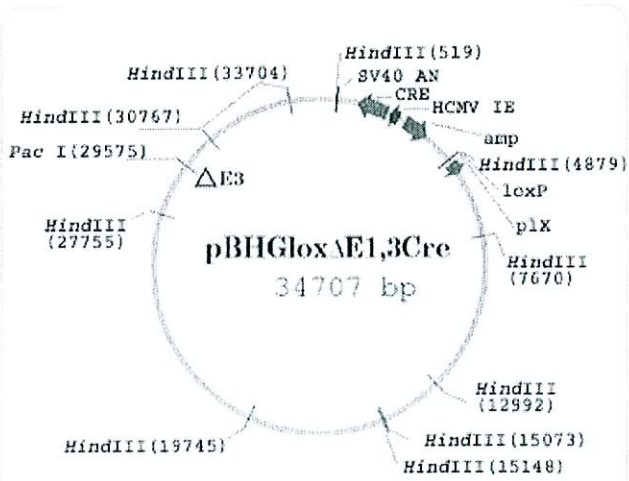

Figure 3. Ad genomic plasmid for construction of Ad vector by Cre-lox recombination

Figure 3 shows an example of one of the available Ad genomic plasmids containing a Cre expression cassette (which is excised during recombination with the shuttle plasmid). This plasmid can be purified and aliquoted and stored frozen for multiple vector rescue cotransfections. As little as 2 ug DNA/dish suffices to generate numerous plaques following cotransfection of 293 cells with a shuttle plasmid.

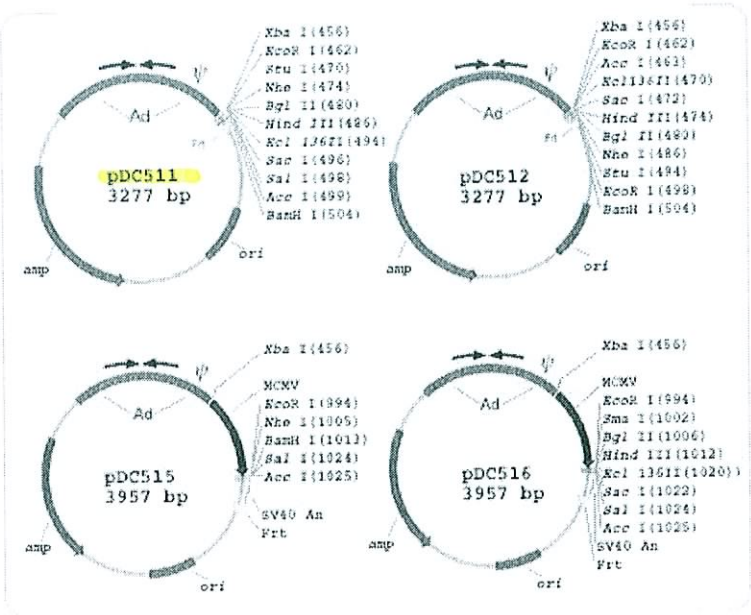

Figure 4 illustrates a set of shuttle plasmids analogous to those shown in Figure 2 but containing frt sites for recombination by the site specific recombinase, FLP, encoded by the yeast 2u plasmid (O'Gorman et al. Science 251, 1351, 1991).

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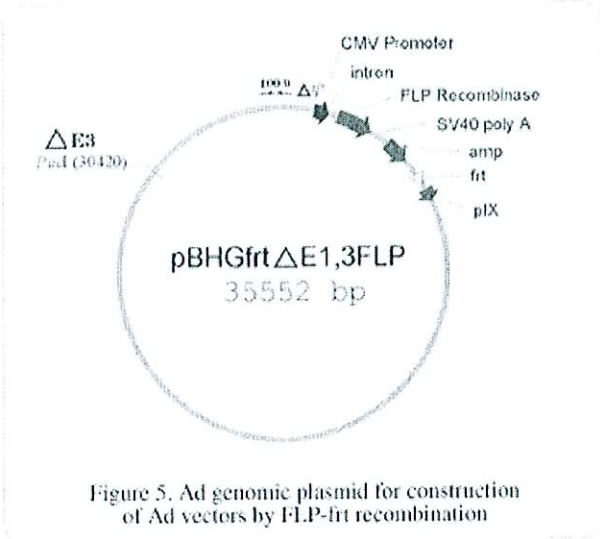

Figure 5. Ad genomic plasmid for construction of Ad vectors by FLP-frt recombination

The genomic plasmid encoding FLP and carrying an frt site for FLP mediated recombination with the shuttle plasmids of Figure 4 is illustrated in Figure 5. FLP functions as efficiently as Cre for production of adenovirus recombinants by site specific recombination between two cotransfected plasmids (Ng., et al., submitted). Plasmids can be propagated in any of the common bacterial strains such as DH5 alpha.

For recombinant DNA cloning any commonly used protocols will suffice but it is recommended that plasmid DNA to be used in cotransfections be prepared using the protocol provided with the kits.

Also we recommend that the simple cotransfection protocol provided with the kits be followed as closely as possible at least initially. Once the users have successfully rescued a number of transgenes and feel comfortable with the system, they are invited to try other plasmid DNA purification protocols and transfection methods.

For beginners we recommend that initial transfections be done using pFG140 (Graham, 1984), an infectious Ad genomic plasmid that serves as a positive control and which is provided free with all kits.

Because the only restriction enzymes required with the AdMax™ system are common enzymes used for cloning into the small

shuttle plasmids the AdMax™ system is simpler and more economical than methods requiring rare cutters (Chartier et al., 1996; He et al., 1998; Mizuguchi & Kay, 1998).

Moreover those rescue protocols typically use enzymes such as Pac I or SwaI to linearize plasmid DNA prior to transfection. If the transgene contains these sites then these methods are not practical. PacI sites, for example, are found surprisingly often in eukaryotic DNA. (There is one PacI site in the Murine Cytomegalovirus Immediate Early Gene promoter (one of the strongest viral promoters available (Addison et al., 1997)) and one also in the gene encoding luciferase, a popular reporter gene.)

The E3 deleted genomic plasmids contain a unique PacI cloning site in E3. It is possible to insert a reporter gene (Parks et al., 1996) or a gene for a transactivator in the E3 region to create a modified genomic plasmid that can then be combined with cassettes inserted in the E1 shuttle plasmid. Thus, for example, a series of vectors expressing genes under regulation by tet or by RU486 can be readily constructed using the AdMax™ system.

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Ordering Information

AdMax™ Kits Available

Catalogue#	**Microbix Product**
PD-01-64	Kit D (contains pDC311, pDC312, pDC315, pDC316, pBHGloxΔE1,3Cre, and pFG140)
PD-01-65	Kit E (contains pDC511, pDC512, pDC515, pDC516, pBHGfrtΔE1,3FLP, and pFG140)
PD-01-67	Kit F (contains pDC411, pDC412, pDC415, pDC416, pBHG10, pBHGE3 and pFG140)

AdMax™ Plasmids must be ordered in complete kits. Each plasmid is priced at 10 ug per vial.

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Individual AdMax™ Plasmids

Catalogue#	**Microbix Product**
PD-01-29	pDC411
PD-01-30	pDC412
PD-01-31	pDC415
PD-01-32	pDC416

AdMax™ is covered by US patents 7,132,290; 6,855,534; 6,756,226; and 6,379,943

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