CELLECTA

USER MANUAL

CRISPRtest™ Essential-Gene Cas9 Activity Assay Kit

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Cellecta, Inc.

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1. CRISPRtest™ Essential-Gene Cas9 Activity Assay Kit (Human or Mouse Cells)

Cellecta's CRISPRtest™ Essential-Gene Cas9 Activity Kits are easy-to-use, highly sensitive assays to measure functional Cas9 activity in human cell lines. It is intended as a companion product for high-throughput, CRISPR-based genetic screening or generation of isogenic knockout cell lines.

There are different versions of the CRISPRtest Essential-Gene Cas9 Assay for human and mouse cells, and versions with either a blue or green fluorescence readout.

- CRISPRtest™ Functional Cas9 Essential-Gene Knockout Assay for **Human** Cells, **GFP** / **RFP** Fluorescence (cat.# CRTEST)
- CRISPRtest_Blue™ Functional Cas9 Essential-Gene Knockout Assay for **Human** Cells, **BFP** / **RFP** Fluorescence (cat.# CRTESTB)
- CRISPRtest™ Mouse Functional Cas9 Essential-Gene Knockout Assay for **Murine** Cells, **GFP** / **RFP** Fluorescence (cat.# CRTESTM)
- CRISPRtest_Blue™ Mouse Functional Cas9 Essential-Gene Knockout Assay for **Murine** Cells, **BFP** / **RFP** Fluorescence (cat.# CRTESTMB)
- CRISPRtest_GBlue™ Mouse Functional Cas9 Essential-Gene Knockout Assay for **Murine** Cells, **GFP** / **BFP** Fluorescence (cat.# CRTESTMGB)
- CRISPRtest_GBlue™ Functional Cas9 Essential-Gene Knockout Assay for **Human** Cells, **GFP** / **BFP** Fluorescence (cat.# CRTESTGB)

This basic protocol is used with all the above assays. However, please confirm which version of the assay you are working and ensure that it is compatible with your target cells and that the fluorescence detection settings are correctly configured. Please read the entire user manual before proceeding with your experiment.

References and Product Citations for all Cellecta products can be found on the Cellecta website: https://cellecta.com/pages/citations-publications.

Please read the entire user manual before proceeding with your experiment. Also, please note that, when working with pseudoviral particles, you should follow the recommended guidelines for working with Biosafety Level 2 (BSL-2) materials.

Click the ? Download as PDF link located at the bottom of the left menu to download the PDF version of this user manual.

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2. Kit Components

Component	Volume
CRISPRtest™ or CRISPRtest_Blue Virus	500 µl
Transduction Reagent (1000X)	50 µl

The CRISPRtest™ Kit should be stored at -80°C until ready for use.

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3. Additional Required Materials

Use of the CRISPRtest assay requires access to a flow cytometer with the following excitation and emission requirements:

Flow Cytometry Settings for TagRFP	
Excitation:	561nm (yellow laser) [530nm laser is still acceptable]
Emission:	590/20nm band-pass filter, or similar
Flow Cytometry Settings for TagGFP2	
Excitation:	488nm (blue laser)
Emission:	530/20nm band-pass filter, or similar
Flow Cytometry Settings for TagBFP	
Excitation:	405nm (violet laser)
Emission:	470/20nm band-pass filter, or similar

Cellecta also offers the following products that may be useful when running this assay:

- Cas9 and dCas9-Variant Cells and Constructs
- Positive Control Cell Line
  - Cas9+ MDA-MB-231 Cells may be run in parallel with your target cells as a positive control for the assay.
- Transduction Control Virus
  - Non-Targeting CRISPR Control virus (e.g. sgNT in pRSG16-U6-sg-UbiC-TagRFP-2A-Puro) may be used to assess transduction efficiency of the target cells.

Non-targeting controls in other Cellecta vectors are available. Please visit our CRISPR Non-Targeting sgRNA Control Construct in Standard Lentiviral Vector web page.

Other than the specific reagents and instruments listed above, the protocols assume the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

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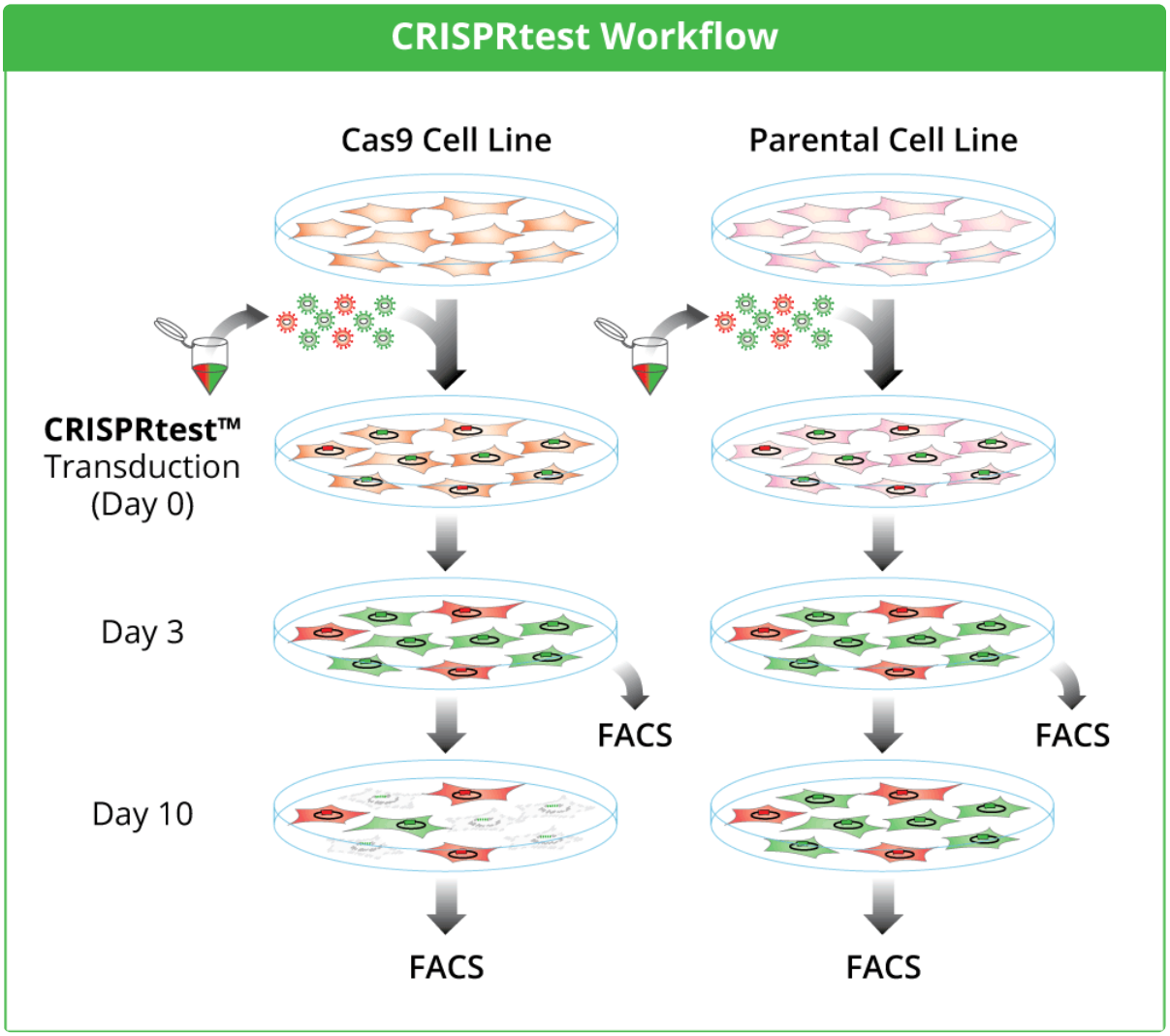
4. Protocol Overview

Cellecta's CRISPRtest™ Essential-Gene Cas9 Activity Kit allows you to assess the level of Cas9 activity in your human cell line using the CRISPRtest virus which contains two pre-mixed lentiviral-packaged vectors:

- One vector containing a green (GFP) or blue fluorescent protein (BFP) marker (depending on which version of the kit you are using) and an sgRNA sequence targeting an essential gene in human or mouse cells (also depending on the version of the assay you are using).
- A second vector containing a red fluorescent protein (RFP) marker and a non-targeting sgRNA.

Transduction of the CRISPRtest virus into cells will result in an initial ratio of GFP (or BFP) cells to RFP cells. After cell growth in the presence of active Cas9, the ratio of GFP (or BFP) cells to RFP cells will decrease proportionally to Cas9 activity.



CRISPRtest Essential-Gene Cas9 Activity Kit Workflow

Cas9 cell line and Parental cells are transduced with the CRISPRtest Virus. At Day 3 and Day 10 after transduction, a portion of the cells are analyzed by flow cytometry to determine GFP:RFP ratios. These ratios are then used to calculate the percentage knockout which is an assessment of Cas9 activity

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4.1. Assay Procedure

For this assay, you should transduce the CRISPRtest Virus into the Cas9-expressing cells and the parental cells which were used to generated the Cas9 cell line.

The assay was optimized using MDA-MB-231 cells. These Cas9-expressing MDA-MB-231 with blasticidin selection marker cells are available from Cellecta for use in the assay as a positive control. Some optimization may be needed based on the growth characteristics of your target cells. If a negative control is also desired, include a cell line not expressing Cas9 (ideally, the parental cells for the Cas9-positive target cells) in a parallel run of the assay.

Start the experiment with actively growing cells. Maintain cells in log phase throughout the entire experiment.

Day 0 – Transduction

1. Quickly thaw the CRISPRtest Virus in a water bath at 37°C. Transfer the thawed particles to a laminar flow hood, gently mix by rotation, inversion, or gentle vortexing, and keep on ice. Unused reagent can be aliquoted, refrozen at -80°C, and used again for subsequent experiments.

2. Suspend parental cells and Cas9 cells in growth media supplemented with 1X CRISPRtest Transduction Reagent (dilute supplied Transduction Reagent 1000-fold in growth media), at a density of 100,000 cells/ml.

> **Note:** This cell density was calculated for MDA-MB-231 cells. Depending on cell size and growth, you may need to use a different concentration and correspondingly-sized plate. As a rule of thumb, cells should be transduced at a density such that they would become confluent in ~48 hours. For the assay, you should plate at least 100,000 cells.

3. For each cell line, aliquot 1 ml of cell suspension per well in 4 wells of a 12-well plate.

4. Add 0 µl, 3 µl, 10 µl, and 30 µl of CRISPRtest (or CRISPRtest_Blue) Virus in the 4 different wells, mix, and incubate for 24 hours at 37°C, 5% CO2.

Day 1 – Change Medium

24 hours post-transduction, change cell growth medium with fresh growth medium. Continue incubation in the CO2 incubator for an additional 48 hours.

Day 3 – CRISPRtest Dilution Selection

1. Collect an aliquot of cells from each well and analyze by flow cytometry (FACS analysis) using the following settings:

Flow Cytometry Settings for TagRFP	
Excitation:	561nm (yellow laser) [530nm laser is still acceptable]
Emission:	590/20nm band-pass filter, or similar
Flow Cytometry Settings for TagGFP2	
Excitation:	488nm (blue laser)
Emission:	530/20nm band-pass filter, or similar

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Flow Cytometry Settings for TagBFP	
Excitation:	405nm (violet laser)
Emission:	470/20nm band-pass filter, or similar

2. For each sample, calculate the percentage of GFP- RFP+ cells (i.e., %RFP) and the percentage of GFP+ RFP- cells (i.e., %GFP) or, if using CRISPRtest_blue, calculate BFP- RFP+ cells (i.e., %RFP) and BFP+ RFP- cells (i.e., %BFP), accordingly.

3. For each cell line, choose the CRISPRtest Virus dilution (from Step 3) where the RFP is between 10%-20 (it should be the same dilution for both cell lines), then discard the other dilutions.

> **Note** CRISPRtest Virus dilutions should give similar %RFP in both parental and Cas9 cell lines. At Day 3, the GFP:RFP (or BFP:RFP) ratio should be ~1 in both cell lines.

Calculations	Parental Cells Day 3	Cas9 Cells Day 3
%RFP	13%	13%
%GFP (or BFP)	18%	17%
GFP (BFP) : RFP Ratio	= 18 / 13 = 1.38	= 17 / 10 = 1.3

4. Grow the selected CRISPRtest dilutions for an additional 7 days. Cells must be in log phase for the whole duration of the experiment so passage the cells as needed.

Day 10 – Read

1. Analyze cells by flow cytometry, using the same settings as on Day 3.

2. Calculate the fluorescent ratio in both cell lines and assess Cas9 activity

3. Use the following formula to determine Cas9 activity:

> **%KO = 1 – [(GFP:RFP)Cas9 / (GFP:RFP)Parental]** (for GFP/RFP kits)

> **%KO = 1 – [(BFP:RFP)Cas9 / (BFP:RFP)Parental]** (for BFP/RFP kits)

The calculated KO value is the percentage of cells in the Cas9 cell line where the PCNA gene was knocked out (i.e., where CRISPR knockout was effective for both alleles). The closer this value is to 100, the more active Cas9 is in the cells.

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4.2. CRISPRtest Data (GFP/RFP)

The following is an example of data generated from a CRISPRtest experiment in MDA-MB-231 cells.

Flow Cytometry Scatter

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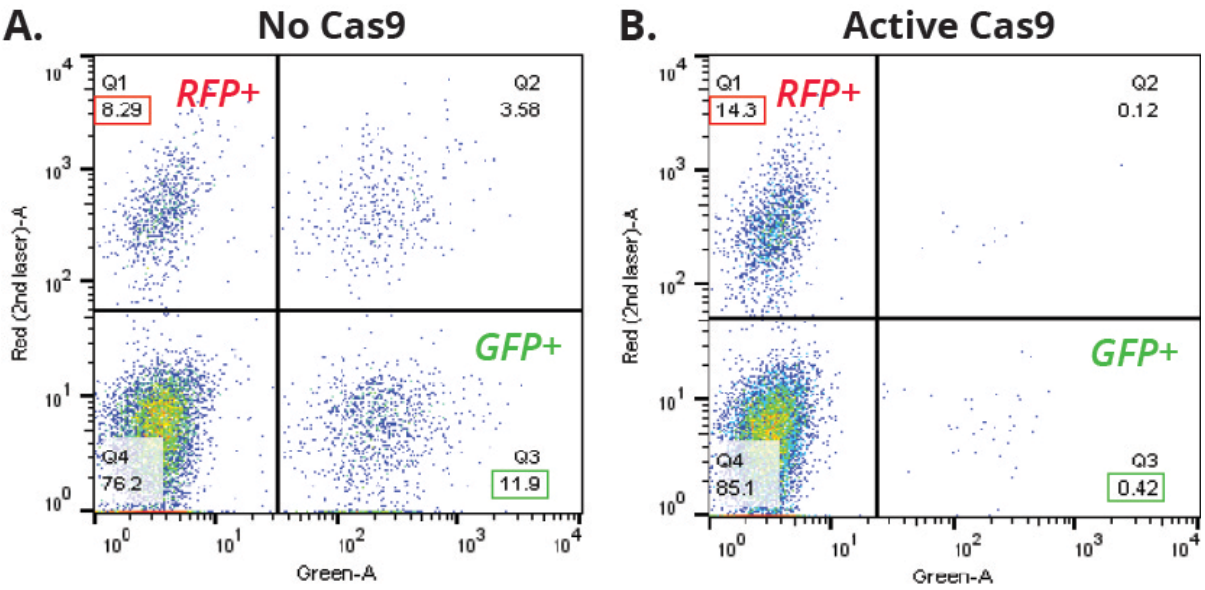

Figure. CRISPRtest experimental data, showing flow cytometry scatter plot analysis of GFP and RFP expressing cells at Day 10 in (A) Parental and (B) Cas9 expressing MDA-MB-231 cells. The percentages of RFP-positive cells calculated in the GFP-RFP+ quadrant Q1 and GFP-positive cells in the GFP+ RFP- quadrant Q3 are used to calculate the percent knockout in the table below.

Calculations

Calculations	Parental Cells Day 10	Cas9 Cells Day 10
%RFP	8.29%	14.3%
%GFP	11.9%	0.42%
GFP:RFP	= 11.9 / 8.29 = 1.44	= 0.42 / 14.3 = 0.03

Calculate **%KO** using [(GFP:RFP)Cas9 / (GFP:RFP)Parental] as follows:

> **1 – (0.03 / 1.44) = 98%**

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4.3. CRISPRtest_Blue Data (BFP/RFP)

The following is an example of data generated from a CRISPRtest_Blue experiment in MDA-MB-231 cells.

Flow Cytometry Scatter

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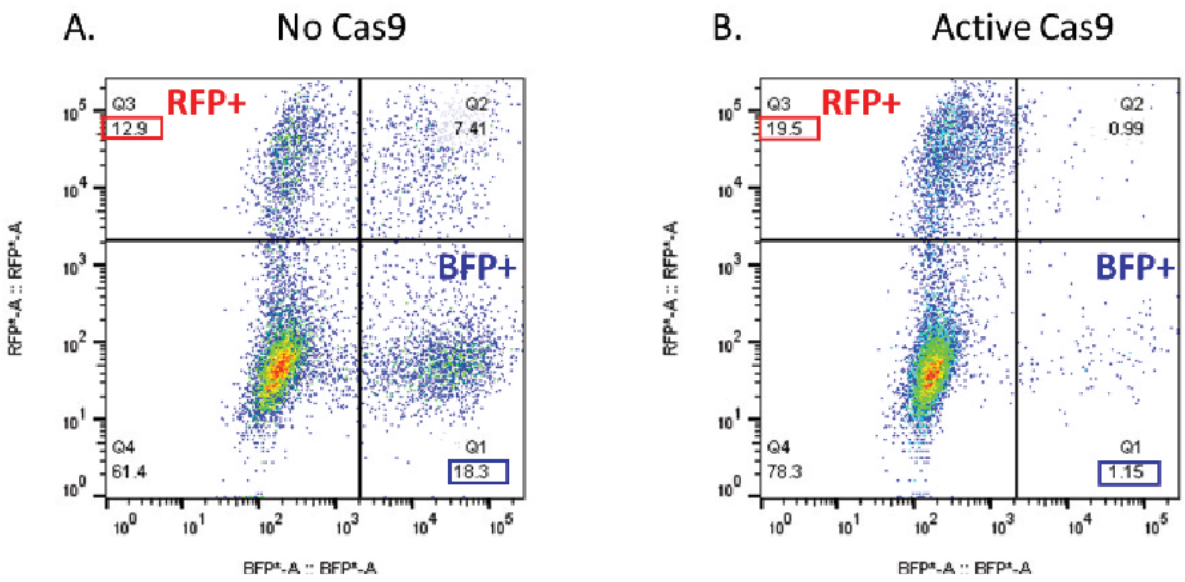

Figure. CRISPRtest_Blue experimental data, showing flow cytometry scatter plot analysis of BFP and RFP expressing cells at Day 10 in (A) Parental and (B) Cas9-
expressing 293T cells. The percentages of RFP-positive cells calculated in the BFP- RFP+ quadrant Q3 and BFP-positive cells in the BFP+ RFP- quadrant Q1 are used to calculate the percent knockout in the table below.

Calculations

Calculations	Parental Cells Day 10	Cas9 Cells Day 10
%RFP	12.9%	19.5%
%BFP	18.3%	1.15%
BFP:RFP	= 18.3 / 12.9 = 1.44	= 1.15 / 19.5 = 0.06

Calculate **%KO** using [(BFP:RFP)Cas9 / (BFP:RFP)Parental] as follows:

> **1 – (0.05 / 1.42) = 96%**

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4.4. CRISPRtest_GBlue Data (GFP/BFP)

The following is an example of data generated from a CRISPRtest_GB experiment in Neuroblastoma Cas9 cells.

Flow Cytometry Scatter

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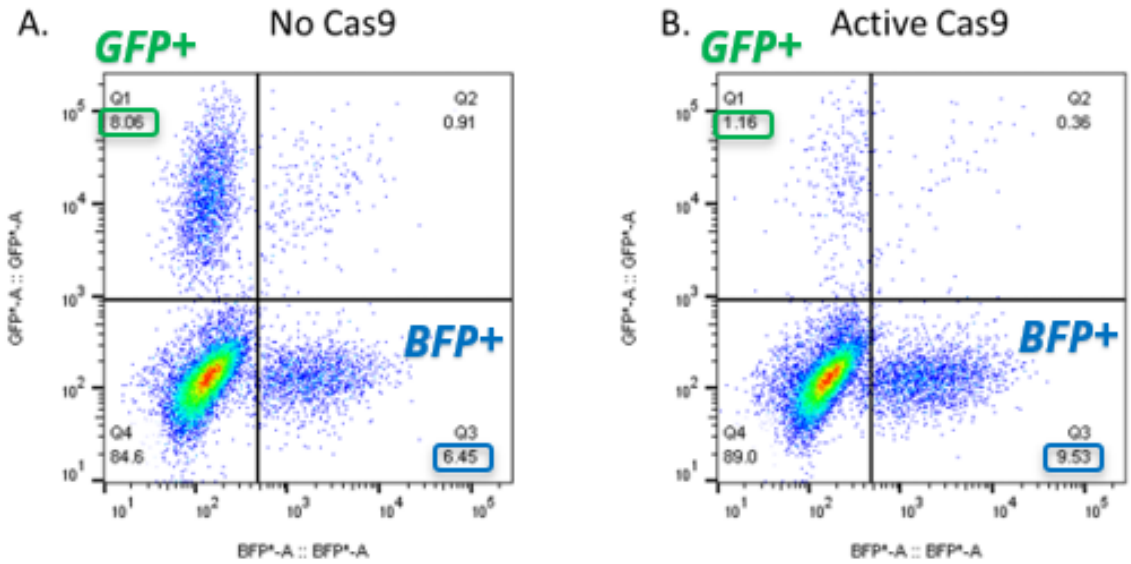

Figure. CRISPRtest_GB experimental data, showing flow cytometry scatter plot analysis of GFP and BFP expressing cells at Day 10 in (A) Parental and (B) Cas9-expressing Neuroblastoma cells. The percentages of BFP-positive cells calculated in the GFP(-) BFP(+) quadrant Q3 and GFP-positive cells in the GFP(+) BFP(-) quadrant Q1 are used to calculate the percent knockout in the table below.

Calculations

Calculations	Parental Cells Day 10	Cas9 Cells Day 10
%BFP	6.45 %	9.53%
%GFP	8.06%	1.16%
GFP:BFP	= 8.06 / 6.45 = 1.25	= 1.16 / 9.53 = 0.12

Calculate **%KO** using [(GFP:BFP)Cas9 / (GFP:BFP)Parental] as follows:

> **1 – (0.12 / 1.25) = 90%**

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5. Technical Support

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For the latest technical news and updates, visit Cellecta's blog at: https://cellecta.com/blogs/news

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6. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The tat-transactivator domain has been removed from the upstream 5'-LTR in the lentivector to disable transcription from the 5'-LTR.
- The three proteins (gag, pol, env) are necessary for replication and packaging of the lentiviral vector. When packaging lentiviral particles, the genes encoding these three packaging proteins are separately introduced and expressed in the packaging cells using separate ordinary plasmids that lack packaging signals and share no significant homology to the lentivector. As a result, none of the HIV-1 genes (gag, pol, env) will be present in the packaged lentiviral genome.
- None of the HIV-1 genes (gag, pol, env) will be present in the packaged lentiviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Lentiviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of replication-incompetent lentiviral particles falls within NIH Biosafety Level 2 criteria. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

> https://www.cdc.gov/labs/bmbl/

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with lentiviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

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7. Contact Us

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