CELLECTA

USER MANUAL

CRISPRuTest™ Functional Cas9 Activity Assay Kit

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Cellecta, Inc.

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Table of Contents

1. CRISPRuTest™ Functional Cas9 Activity Assay Kit 3

2. Kit Components 4

3. Additional Required Materials 5

4. Protocol Overview 7

4.1. Assay Procedure 7

4.2. Cas9 Activity Calculation 10

5. Technical Support 12

6. Safety Guidelines 13

7. Contact Us 14

1. CRISPRuTest™ Functional Cas9 Activity Assay Kit

The CRISPRuTest™ Functional Cas9 Activity Assay Kit can be used to measure the Cas9 nuclease activity in most mammalian cell systems. The choice of kit (CRISPRuTest™ or CRISPRuTest™ (EF1L)), depends on the cell line to be tested. In iPS cells, where the CMV promoter used in the CRISPRuTest™ Kit is quickly silenced, the CRISPRuTest™ Kit (EF1L) is strongly recommended.

The CRISPR system uses a target single-guide RNA molecule (sgRNA) to target and cut a specific sequence on the genomic DNA with the Cas9 nuclease. The CRISPRuTest Kit provides a FACS-based assay to assess the Cas9 nuclease activity in cells expressing *Streptococcus pyogenes* Cas9(spCas9) or Staphylococcus aureus_Cas9 (saCas9). The kit contains pseudoviral packaged lentiviral constructs with a green fluorescent protein (GFP) gene whose fluorescence is disrupted in cells with active Cas9. The kit provides enough reagents to measure this activity in 5-15 cell lines.

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

The CRISPRuTest™ Cas9 Activity Assay Kit comes in three formats:

- CRISPRuTest™ **Cas9** has the CMV promoter:

Component	Volume
CRISPRuTest™ CT-Active (CT-A) Viral Mix	500 µl
CRISPRuTest™ CT-Background (CT-B) Viral Mix	500 µl
Transduction Reagent (1000X)	50 µl

- CRISPRuTest™ **Cas9** (EF1L) has the EF1L promoter:

Component	Volume
CRISPRuTest™(EF1L) CT-Active (CT-A EF1L) Viral Mix	500 µl
CRISPRuTest™(EF1L) CT-Background (CT-B EF1L) Viral Mix	500 µl
Transduction Reagent (1000X)	50 µl

- CRISPRuSaTest™ **saCas9** has the CMV promoter:

Component	Volume
CRISPRuSaTest™ SaU-Active (SaU-A) Viral Mix	500 µl
CRISPRuSaTest™ SaU-Background (SaU-B) Viral Mix	500 µl
Transduction Reagent (1000X)	50 µl

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

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3. Additional Required Materials

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

> **Excitation:** 488nm and 561nm
> **Emission:** 530/20nm and 590/20nm

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

Cas9-Only Plasmids

Cat.#	Description	Quantity
SVC9B-PS	CRISPR Cas9 pRCCB-CMV-Cas9-2A-Blast (plasmid)	25 µg
SVC9-PS	CRISPR Cas9 pRCCH-CMV-Cas9-2A-Hygro (plasmid)	25 µg
SVC9G-PS	CRISPR Cas9 pRCCG-CMV-Cas9-2A-TagGFP2 (plasmid)	25 µg
SVC9R-PS	CRISPR Cas9 pRCCR-CMV-Cas9-2A-TagRFP (plasmid)	25 µg
SVC9P-PS	CRISPR Cas9 pRCCP-CMV-Cas9-2A-Puro (plasmid)	25 µg
SVC9B-VS	CRISPR Cas9 pRCCB-CMV-Cas9-2A-Blast (virus)	1×10^6 TU
SVC9-VS	CRISPR Cas9 pRCCH-CMV-Cas9-2A-Hygro (virus)	1×10^6 TU
SVC9G-VS	CRISPR Cas9 pRCCG-CMV-Cas9-2A-TagGFP2 (virus)	1×10^6 TU
SVC9R-VS	CRISPR Cas9 pRCCR-CMV-Cas9-2A-TagRFP (virus)	1×10^6 TU
SVC9P-VS	CRISPR Cas9 pRCCP-CMV-Cas9-2A-Puro (virus)	1×10^6 TU

Additional Cas9-Only plasmids are available. Please visit our CRISPR Cas9 Expression Construct web page.

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.

Cat.#	Description	Quantity
ZMDAMB231-C9	Cas9+ MDA-MB-231 Cells with Blast selection	1×10^6 cells

Transduction Control Virus

Non-Targeting CRISPR Control pRSG16-U6-sg-UbiC-TagRFP-2A-Puro (virus) may be used to assess transduction efficiency of the target cells.

Cat.#	Description	Quantity
SGCTL-NT-pRSG16-V	Non-Targeting CRISPR control pRSG16-U6-sg-UbiC-TagRFP-Puro Vector (virus)	1×10^7 TU

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

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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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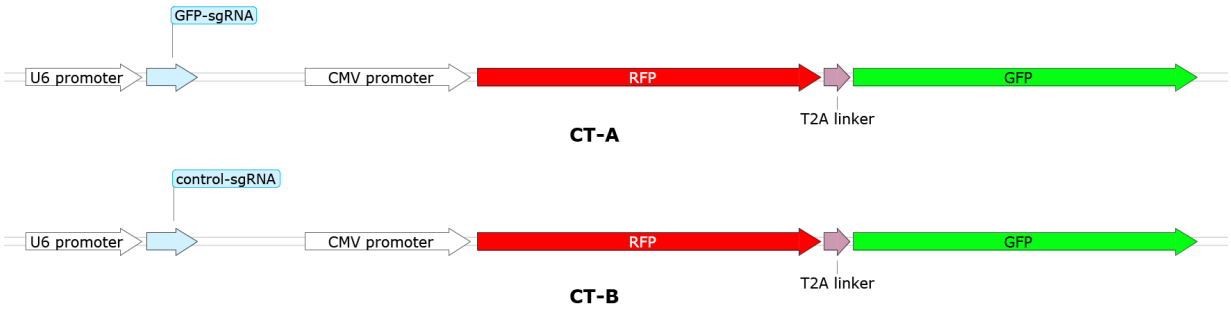
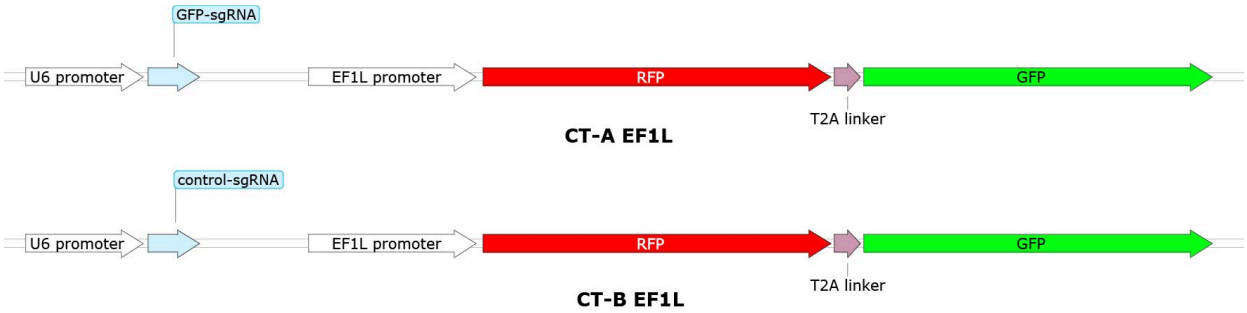
4. Protocol Overview

Both the CT-Active (CT-A/CT-A EF1L) and CT-Background (CT-B/CT-B EF1L) reagents contain a lentivirus expressing RFP and GFP from one transcript, separated by a T2A peptide linker sequence (see Figure below). Transduction of either of these reagents into cells will initially produce cells expressing both GFP and RFP fluorescence.



CRISPRuTest Vector Diagrams CT-A and CT-B



CRISPRuTest EF1L Vector Diagram CT-A EF1L and CT-B EF1L

The difference between the two viral reagents is that the CT-A expresses a GFP-targeting sgRNA, whereas the CT-B viral reagent expresses a non-targeting sgRNA. Therefore, within a few days after transduction, CT-A transduced cells will lose GFP and only have RFP fluorescence, whereas CT-B transduced cells will continue to exhibit both RFP and GFP fluorescence. The decline in GFP fluorescence in the CT-A cell population, then, can be used to assess Cas9/saCas9 activity and forms the basis of the CRISPRuTest assay.

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4.1. Assay Procedure

Two populations of putative Cas9-positive cells are transduced with the CRISPRuTest™ CT-A and CT-B viral mix reagents, respectively. After 4 days, each population is analyzed by flow by cytometry. The disappearance of GFP in the CT-A population as compared with the CT-B population provides a quantitative measure of the Cas9 activity in the target cells.

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 (see Additional Required Materials). 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.

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

Day 0

1. Quickly thaw the CRISPRuTest™ CT-A and CT-B lentiviral particles 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 Cas9+ cells in growth medium supplemented with 1X Transduction Reagent, at a density of ca. 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. Aliquot 1 ml of cell suspension (100,000 cells) into each of 2 wells of a 12-well plate.

4. Add 20 µl of CT-A virus into one well and 20 µl of CT-B virus into the other well, and then mix and return cells to incubator.

> **Note:** For most cell lines, 20 µl of CT viral reagents will suffice to obtain at least 50% RFP+ cells (the recommended minimum percentage of transduced cells for optimal assay sensitivity). For hard-to-transduce cell lines, more virus might be needed. If in doubt, it is recommended to set up two sets of transductions with 20 µl of CT-A and CT-B as described above, and 50 µl of each in the second transductions. For the final calculation, use the samples that have 50%-80% RFP+ cells on Day 4. A control lentiviral vector may be used before running the assay to test the transduction efficiency of your cells (see Additional Required Materials).

Days 1-6

Exchange medium with fresh growth medium and grow cells under standard conditions for 3 days. Passage cells as needed. Cells should not become confluent.

Day 7

1. Analyze cells by flow cytometry, using settings below:

> **Channel 1:** excitation 488nM, emission 530/20nm (GFP)
> **Channel 2:** excitation 561nM, emission 590/20nm (RFP)

2. For both CT-B and CT-A samples, gate in RFP+ cells as shown below:

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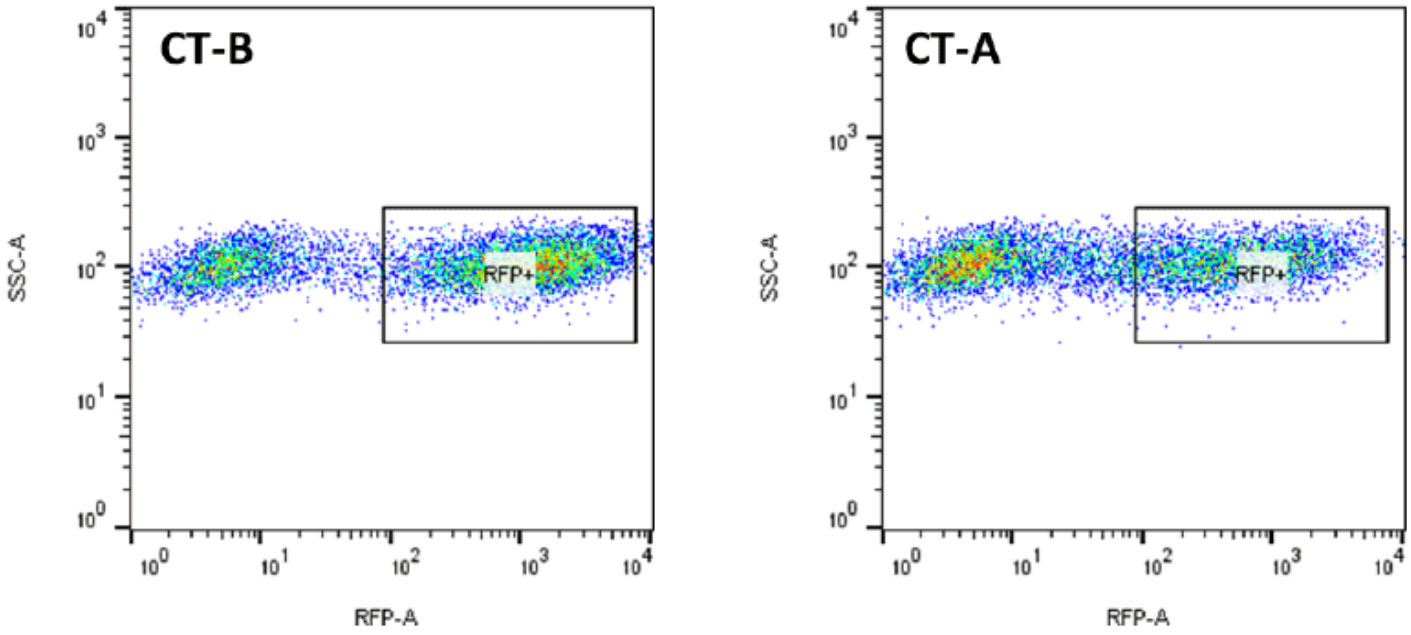
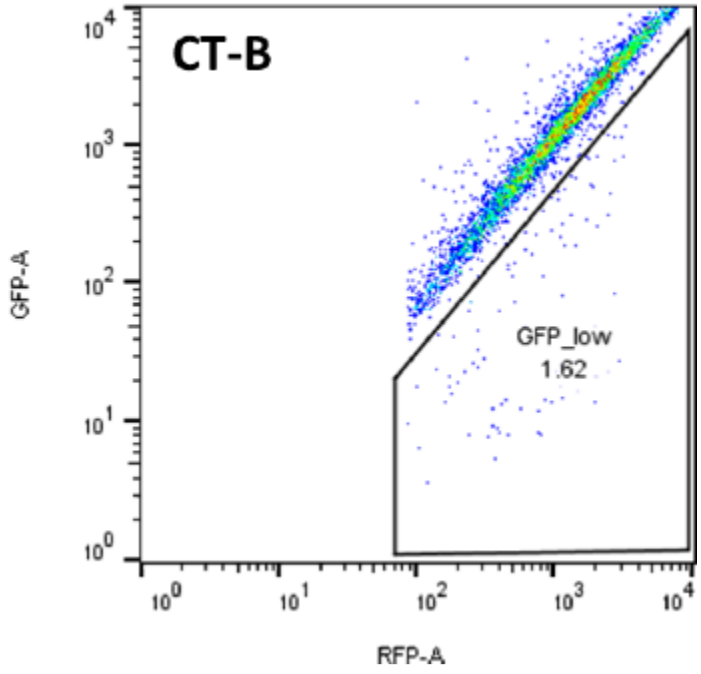


#_Plot gated cells of CT-B sample GFP vs. RFP as shown below, adjusting Channel 1 and 2 intensity so that GFP and RFP signal falls into a diagonal. Then, gate all cells below diagonal (“GFP_low” cells):



3. Do the same for CT-A sample, applying the same “GFP_low” gate:

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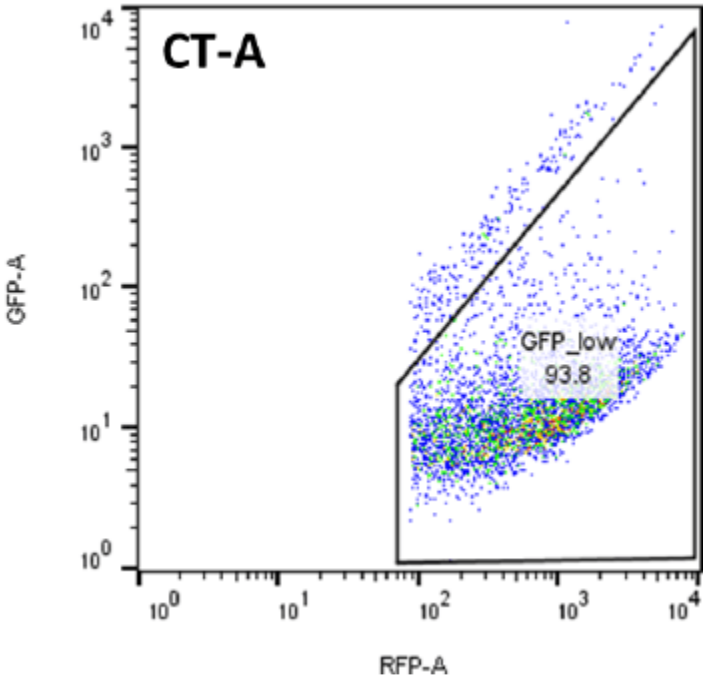

While some Cas9 lines can show a significant loss of GFP activity as soon as Day 7, depending on the cell line readings may have to be taken at multiple time points until the loss of GFP activity plateaus. Some cell lines may take 10, 14, or even 21 days to reach maximum activity.

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4.2. Cas9 Activity Calculation

The difference between the percentage of “GFP_low” cells in the CT-A population and percentage of “GFP_low” cells in the CT-B population indicates the percentage of cells which underwent Cas9-mediated GFP knockout in the CT-A sample. The percentage of “GFP_low” cells in the CT-B population should be close to 0% (i.e., no sgRNA targeting GFP), where as in the CT-A population it should be near 100% (sgRNA targeting GFP).

The flow cytometry data from the the previous section using the positive control cell line MDA-MB-231-Cas9-Blast (see Additional Required Materials to obtain this cell line) can be used to calculate the percentage of GFP knockout, which is a direct measurement of the Cas9 activity, as follows:

Target Cell Population	“GFP low”
CT-A Cells	0.938
CT-B Cells	0.0162

Percentage GFP Knockout in Target Cas9 cells = 93.8% – 1.62% = 92.2%

> **Note:** If the GFP Knockout in the target cells is less than 80%, you should continue to incubate the cells for a few more days. For some cell lines, it may take 10, 14, or even 21 days to reach maximum activity.

> **Note:** For a Cas9-negative cell line, the percentage of “GFP_low” cells in both CT-A and CT-B should be similarly close to

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> 0%.

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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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