



Cas9 ELISA Assay

Catalog# Cas9-1000

Introduction

Principle of the Assay

Microtitration wells coated with murine anti-Cas9 capture antibody are exposed to test specimens, which may contain Cas9 reactive determinants. The Cas9 antigen in the specimen is specifically captured onto the immobilized antibody during specimen incubation. The captured antigen is then reacted with a biotinylated mouse monoclonal Cas9 detection antibody. Subsequently, Streptavidin-HRP conjugate is then added. Following a wash cycle, specifically bound enzyme conjugate is detected by reaction with the Substrate Solution, tetramethylbenzidine (TMB). The assay is measured spectrophotometrically to indicate the level of Cas9 reactive determinants present in a sample. The assay can be used to monitor S. pyogenes Cas9 in cell culture lysate and is ideal for screening experimental samples utilizing the CRISPR/Cas9 gene editing system.

Kit Presentation

Materials Supplied

The reagents supplied in this pack are for research use only.

1	Coated microwell strips - plastic microtitration wells coated with anti-Cas9 murine monoclonal antibody in foil pouch with desiccant	1 plate (96 wells)
2	Cas9 Positive Control - 2.5 µg/mL	0.1 mL
3	Lysis Buffer - (NaCl, NP-40 Alt., Na-Deoxycholate, SDS, Tris-HCl, pH 7.5)	30 mL
4	Detector - anti-Cas9 murine monoclonal antibody conjugated to biotin	12 mL
5	Conjugate - streptavidin conjugated to horseradish peroxidase enzyme containing 0.01% Bromonitroiodoxane as preservative	12 mL
6	Wash Buffer (20x concentrated) - Tris buffered saline containing 0.05% Tween-20, must be diluted before use	60 mL
7	Substrate Solution - Ready to use, Tetramethylbenzidine	12 mL
8	Stop Solution - 1 N H ₂ SO ₄	12 mL

Additional Requirements for Manual Processing

- > Disposable tip micropipettes to deliver volumes of 5 µL, 10 µL, 25 µL, 100 µL and 200 µL (multichannel pipette preferred for dispensing reagents into microtiter plates).
- > Distilled or deionized water.
- > 37 (±1)° C incubator
- > Clean, disposable plastic/glass test tubes, approximate capacities 5 mL and 10 mL.
- > Range of standard, clean volumetric laboratory glassware consisting of, at least, 15 mL and 100 mL beakers, 1 L graduated cylinder, 1 mL, 5 mL, and 10 mL glass pipettes.

- > Absorbent paper towels.
- > Automatic microtitration plate washer or laboratory wash bottle.
- > Microtitration plate reader with 450 nm filter.
- > Latex gloves, safety glasses and other appropriate protective garments.
- > Biohazard infectious waste containers.
- > Safety pipetting devices for 1 mL or larger pipettes.
- > Timer
- > 1X PBS

Automatic or Semi-automatic Processing

The Cas9 Assay may be used with a variety of automatic or semi-automatic processors/liquid handling systems. It is essential that any such system is qualified by demonstrating that the Cas9 Assay results obtained using the automatic processor are equivalent to those obtained for the same specimens using the manual test method. Subsequently, the automatic processor should be periodically requalified.

Storage and Stability

The Cas9 Positive Control is to be stored immediately at -20°C or colder. The Cas9 Positive Control is stable for a maximum of 3 freeze-thaw cycles. If more cycles are required, aliquot into smaller portions and freeze tubes at -20°C or colder until needed.

All other reagents should be stored at 2-8°C and should not be used beyond the expiration date on the label. Once opened, microtitration strips may be stored at 2-8°C until the expiration date on the label, provided that desiccated conditions are maintained. Unused strips should be returned to their original foil pouch along with the sachet of desiccant. Opened pouches should be securely resealed.

The 1X Wash Buffer should not be stored for longer than 3 weeks at 2-8°C. It is recommended that Wash Buffer be freshly diluted before each assay. Do not use if the 1X Wash Buffer becomes visibly cloudy or develops precipitate during the 3 weeks.

Indications of Deterioration

The Cas9 Assay may be considered to have deteriorated if:

1. The kit fails to meet the required criteria for a valid test (see Interpretation of Results Section).
2. Reagents becoming visibly cloudy or develop precipitate. Note: When cold, concentrated Wash buffer normally develops crystalline precipitate, which can be redissolved with heating at 37°C.
3. The Substrate Solution turns dark blue. This is likely to be caused by chemical contamination of the Substrate Solution.

Warnings and Precautions

Safety

1. The reagents supplied in this kit are for **research use only**.
2. Caution: All blood products should be treated as potentially infectious. Essential precautions can be summarized as follows:
 - >Do not pipette by mouth.
 - >Wear disposable gloves during all specimen and assay manipulations.
 - >Avoid use of sharp or pointed liquid handling devices, which may puncture skin.
 - >Do not smoke, eat, or drink in the laboratory work area.
 - >Avoid splashing of liquid specimens, reagents and formation of aerosols.
 - >Wash hands thoroughly on completion of a manipulation.
- >The Centers for Disease Control & Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.
3. The Cas9 kits contain reagent systems, which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers within or between kits.
4. The Substrate Solution and Stop Solution in this kit contain ingredients that can irritate the skin and cause eye damage. Handle them with care and wear suitable protective clothing and eye/face protection. In the case of contact with skin or eyes, immediately flush the affected area with plenty of water. For eye irritation, consider seeking medical attention.

Procedural

- This kit should be used in strict accordance with the instructions in the Package Insert.
- Do not use Cas9 Assay kits after the expiration date printed on the outer carton label.
- Do not cross contaminate reagents. Always use fresh pipette tips when drawing from stock reagent bottles.
- Always use clean, preferably disposable, glassware for all reagent preparation.
- Allow foil bags to warm to room temperature before opening. This avoids condensation on the inner surface of the bag, which may contribute to a deterioration of coated strips intended for future use.
- Reagents should be dispensed with the tip of the micropipettes touching the side of the well at a point about mid-section. Follow manufacturer's recommendations for automatic processors.
- Always keep the upper surface of the microtitration strips free from excess fluid droplets. Reagents and buffer over-spill should be blotted dry on completion of the manipulation.
- Do not allow the wells to completely dry during an assay.
- Automatic or semi-automatic EIA processors or liquid handling systems should be qualified specifically for use with Cas9 Assay by demonstration of equivalence to the manual processing methods.
- Consistent with good laboratory practice, it is recommended that all pipetting devices (manual or automatic), timers and thermometers are regularly calibrated according to the manufacturer's instructions.

Method of Use

Specimen Collection and Storage

Cas9 Assay is intended for use with cell culture lysate. The specimen should be tested as soon as possible. However, if the specimen needs storage, the specimens should be stored frozen at -60°C or below. Do not use self-defrosting freezers. Specimens that have been frozen and thawed should be thoroughly mixed before testing.

Cell Culture Lysate

Wash cell pellet containing 2.0×10^6 cells in 1X PBS and centrifuge at 500 x g for 5 minutes at 4°C to remove residual cell culture media. Lyse cell pellet in 250 µL of Lysis Buffer. Centrifuge sample again at 13,000 x g for 10 minutes at 4 °C to remove cellular debris. The supernatant is the experimental sample used for the Cas9 Assay. This will provide enough volume to run samples in duplicate wells (100 µL per well).

Rinse Cycle

Efficient rinsing to remove uncomplexed components is a fundamental requirement of enzyme immunoassay procedures. The Cas9 assay utilises one standard five-rinse cycle. Automatic plate washers may be used provided they meet the following criteria:

- All wells are completely aspirated.
- All wells are filled to the rim (350 µL) during the rinse cycle.
- Wash buffer is dispensed at a good flow rate.
- The microtitration plate washer must be well maintained to prevent contamination from previous use. Manufacturer's cleaning procedures must be followed diligently.

For the rinse cycle, the machine should be set to five consecutive washes. On completion of the cycle, invert the microtitration plate and tap firmly on absorbent paper towels. Check for any residual Wash Buffer in the wells and blot dry with a paper towel.

Alternatively, the following manual system may be employed:

- Aspirate well contents using a vacuum line fitted with a trap.
- Fill all wells to the brim with Wash Buffer dispensed from a squeeze-type laboratory wash bottle.
- Aspirate all wells.
- Repeat steps 2 and 3, four times.
- Invert the microtitration plate and tap firmly on absorbent paper towels.

Preparation for the Assay

1. Kit Positive Control 2.5 µg/mL

Prepare working strength Cas9 Positive Control by diluting 20 µL of Positive Control into 480 µL (1:25 dilution) of Lysis Buffer. This will give a final concentration of 100 ng/ml and will act as the top standard shown in Table 1.

2. Wash Buffer

Prepare working-strength Wash buffer by diluting 1 part concentrate with 19 parts of distilled or de-ionized water. If a kit is likely to be utilized over a period in excess of 4 weeks, then it is recommended that only enough stock concentrate be diluted sufficient for immediate needs. Each row of 8 wells may be adequately washed with 100 mL of working strength buffer.

Quantitative Assay Procedure

To test quantitatively, a standard curve should be prepared using Lysis Buffer as the diluent as shown in the table below. Each standard plus a Lysis Buffer (negative) control should be run in duplicate.

Table 1

Cas9 Quantitative Standard Curve Generation			
Tubes	Addition to Tube	Lysis Buffer (µL)	Cas 9 (ng/mL)
1	20 µL of 2.5 µg/mL Cas9	480	100
2	250 µL of Tube 1	250	50
3	250 µL of Tube 2	250	25
4	250 µL of Tube 3	250	12.5
5	250 µL of Tube 4	250	6.25
6	250 µL of Tube 5	250	3.125
7	250 µL of Tube 6	250	1.56
8	0	250	0

Assay Protocol

- Allow all reagents to reach room temperature (18-25°C).
 - The standards, test specimens and cell Lysis Buffer (for use as a negative control) should be tested in duplicate in every assay.
 - Select sufficient microtitration well strips to accommodate all test specimens, controls and reagent blank. Fit the strips into the holding frame. Label wells according to specimen identity using the letter/number cross-reference system molded into the plastic frame.
 - Dispense 100 µL of each standard and specimen into appropriate wells.
- Note: All standards and samples should be tested in duplicate.**
- Incubate at 37(±1)°C for 60 (±5) minutes.
 - Aspirate the contents of the wells and wash the microtitration plate as described in the Rinse Cycle section.
 - Pipette 100 µL of detector antibody into each well and incubate at 37(±1)°C for 60 (±5) minutes.
 - Aspirate the contents of the wells and wash the microtitration plate as described in the Rinse Cycle section.
 - Pipette 100 µL of Streptavidin HRP conjugate into each well and incubate at room temperature (18-25°C) for 30 (±5) minutes.
 - Aspirate the conjugate from the wells and wash the microtitration plate as described in the Rinse Cycle section.
 - Without delay, dispense 100 µL Substrate Solution into each well. A multichannel pipette should be used for best results. Leave at room temperature (18-25°C) protected from direct sunlight for 30 (±5) minutes.
 - Stop the reaction by adding 100 µL of Stop Solution to each well including the reagent blank. The blue solution should change to a uniform yellow color. Ensure that the undersides of the wells are dry and that there are no air bubbles in the well contents.
 - Immediately after adding the Stop Solution, read the absorbance values at 450 nm using a microtitration plate reader **blanked on the negative control well**.

Interpretation of Results

Quantitative Analysis

Manual Method: The calibration curve can be constructed manually on linear graph paper by plotting the mean absorbance for each standard on the y-axis versus the concentration of the standard (value printed on vial) on the x-axis. Connect the points to produce a point to point curve. Do not force the line to be linear. The concentration of the specimens can be found directly from the standard curve.

Table 2. Example Data at 450nm.

Standards	450 nm absorbance
Standard 1 (0 ng/mL)	0.0
"	
Standard 2 (1.56 ng/mL)	0.059
"	
Standard 3 (3.13 ng/mL)	0.122
"	
Standard 4 (6.25 ng/mL)	0.203
"	
Standard 5 (12.5 ng/mL)	0.421
"	
Standard 6 (25 ng/mL)	0.803
"	
Standard 7 (50 ng/mL)	1.421
"	
Standard 8 (100 ng/mL)	2.597

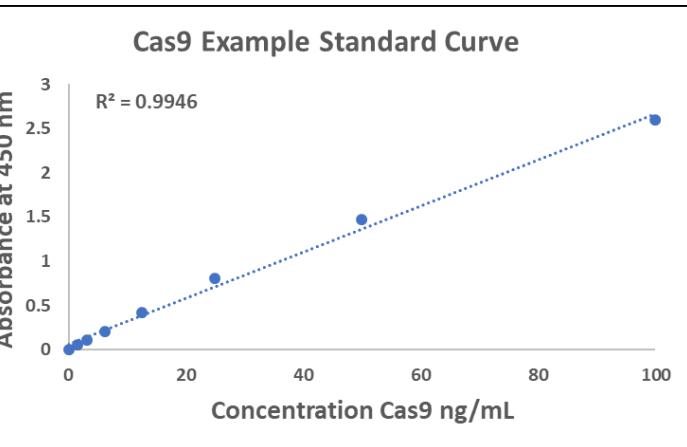


Figure 1: Cas9 Standard Curve. Note: This standard curve is only an example and should not be used to generate any results.

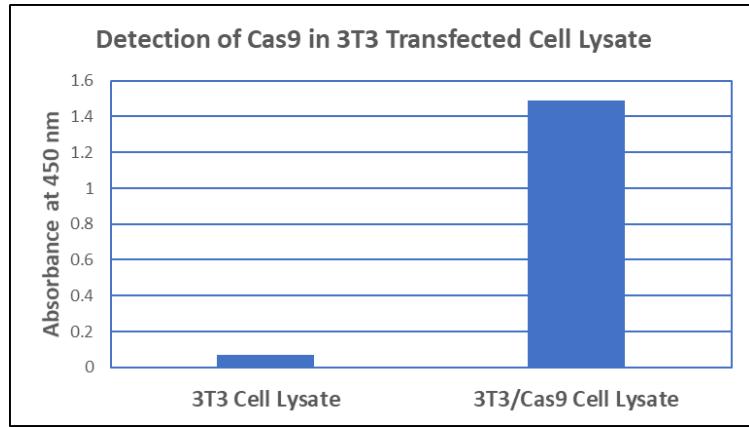


Figure 2: Detection of Cas9 in cell lysate from mock or Cas9 transfected 3T3 cells.

Computer-Assisted Method: Computer assisted data reduction may be used to create the standard curve. Software providing a point to point curve fitting routine provides acceptable results.

Assay validation

The Cas9 assay should be considered valid if:

The negative control should be ≤ 0.10

The 50 ng/ml control should be ≥ 0.60

Procedure for Samples with Cas9 Assay values greater than the highest standard.

Many cell lysate samples will have Cas9 values greater than the highest standard. In order to obtain accurate results for samples with Cas9 assay values greater than the highest standard it is necessary to dilute and re-test the sample. Diluting the serum specimen 10-fold is recommended. For example: Make a 10-fold dilution by adding 0.1 mL of the initial specimen to 0.90 mL of 1X PBS. Mix thoroughly and repeat the assay according to the Assay Procedure. Multiply the results by 10 to determine the correct Cas9 assay values in the sample.

Limitations of Use

1. Assay values determined using assays from different manufacturers or different methods may not be used interchangeably.
2. The assay cannot be used to quantitate samples with Cas9 assay values greater than the highest standard without further serial dilution of the samples. See the Interpretation of Results section for directions on testing such samples.
3. The performance characteristics have not been established for any matrices other than cell culture lysate.

Performance Characteristics

Analytical Sensitivity: To determine the sensitivity of the assay, the 0 standard was assayed 20 times. The minimal detectable level was calculated by adding two standard deviations to the mean absorbance for the 0 standard. The minimal detectable level is 1.1 ng/mL.

Linearity: Four strongly reactive samples were serially two-fold diluted and run on the assay. The values obtained were compared to the expected values by standard linear regression. The r^2 values obtained ranged from 0.993 to 0.999.

Precision: Four samples with different levels of activity were assayed ten times each on three different assays. The results are summarized in the following table.

Precision Data

		Sample 1	Sample 2	Sample 3	Sample 4
Assay 1 (n = 10)	Mean (ng/mL)	72.96	45.33	20.99	9.49
	SD	4.34	2.96	2.39	1.09
	CV	5.94%	6.54%	11.37%	11.48%
Assay 2 (n = 10)	Mean (ng/mL)	76.94	45.78	22.09	9.98
	SD	3.39	4.36	2.48	1.24
	CV	4.41%	9.53%	11.23%	12.47%
Assay 3 (n = 10)	Mean (ng/mL)	79.13	48.13	25.11	11.55
	SD	3.32	4.58	1.79	1.02
	CV	4.20%	9.51%	7.14%	8.87%
Inter-Assay (n = 30)	Mean (ng/mL)	76.34	46.42	22.26	10.34
	SD	4.43	4.09	2.71	1.41
	CV	5.80%	8.81%	12.19%	13.59%

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