CHOP

MANUAL

ISG15-CHOP2-Reporter DeISGylation Assay Kit
Catalog Number PR1105

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BACKGROUND

Ubiquitin and Ubiquitin Like Proteins

In cells, proteins are tagged for degradation by ubiquitin and sent to the proteasome. In contrast, covalent modification of cellular proteins by the ubiquitin-like modifier SUMO (small ubiquitin-like modifier) regulates various cellular processes, such as nuclear transport and signal transduction. The ubiquitin family of proteins fall into two classes: the first class, ubiquitin-like proteins (UBLs) function as modifiers in a manner analogous to that of ubiquitin. Examples of UBLs are SUMO, Nedd8 (also called Rub1), ISG15, Apg8, Apg12, and Fat10. Proteins of the second class include parkin, RAD23 and DSK2, are designated ubiquitin-domain proteins (UDPs). These proteins contain domains that are related to ubiquitin but are otherwise unrelated.

Conjugation Machinery

The conjugation of Ub/UBLs to target proteins requires an orchestrated addition of Ub/UBLs to lysine residues in the target protein by E1 (activating enzyme), E2 (conjugating enzyme), and E3 (ligase) in an ATP dependent manner. The enzymes form an isopeptide bond between the carboxy-terminus of the UBL and the ε-amino group of the lysine residue of target proteins.

DeISGylating Enzymes

ISGylation is a reversible process in which deconjugation is performed in cells by deISGylating enzymes, otherwise known as isopeptidases. Isopeptidases are cysteine proteases that can be divided into multiple familes. The roles of isopeptidases include recycling of fused ubiquitin/UBL and processing pro-ubiquitin/UBL by cleavage to the mature form. Removal of ubiquitin or UBL moieties can affect cellular physiology in a number of ways, and several isopeptidases have been linked to pathologies such as cancer and cardiovascular disease.

ABOUT THE ASSAY

The ISG15-CHOP2-Reporter DeISGylation Assay consists of ISG15 fused to a reporter enzyme, as well as a separate reagent substrate for the reporter enzyme. Upon conjugation to ISG15, the reporter is rendered catalytically inactive. Following cleavage of the ISG15-reporter system by the isopeptidase, the free (and no active) reporter subsequently acts upon its substrate. Thus, in the coupled assay, the signal generated by cleavage of the reporter enzyme's substrate is a quantitative measure of isopeptidase activity.

BENEFITS

1. Rapid and robust readout for deISGylating activity within 60 minutes; signal to background ratio >10.
2. Reporter substrates are non-radioactive.
3. Amenable to high throughput screeing (HTS) and miniaturization.
4. Assay tests deconjugating activity between ISG15 and a physiologically relevant protein.

SUGGESTED USES

1. Demonstration of novel isopeptidase activity.
2. High throughput screening (HTS) for antagonists or agonists of isopeptidase activity.

COMPONENTS

1. **ISG15-CHOP2-Reporter (Reporter System)**

   Size: 1 x 375 µl (4 µM)

   Buffer: 20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol

   Storage: -80° C, avoid cycles of freezing and thawing

2. **PLpro (Control Isopeptidase)**

   Size: 2 x 10 µl (2 µM)

   Buffer: 20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 200µg/ml BSA, 2 mM β-mercaptoethanol

   Storage: -80° C, avoid cycles of freezing and thawing

3. **Reporter Substrate 2**

   Size: 1 x 30 µl (25 µM)

   Buffer: DMSO

   Storage: -80° C

   Misc.: light sensitive

   excitation/emission wavelength maxima are 485/531 nm

4. **Control Fluorophore 2 (Please refer to Fluorophore Control section, Pg 6 for information concerning the use of this reagent for plate reader validation)**

   Size: 1 x 20µl (1mM)

   Buffer: DMSO

   Storage: -80° C

ADDITIONAL ITEMS REQUIRED

1. **Assay Buffer (For Use In Step 1 of Protocol)**

   20 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 2 mM β-mercaptoethanol, 0.05% CHAPS

   Prepare fresh assay buffer on a daily basis.

2. **Fluorescence Plate Reader**

Appropriate filters required for excitation and emission wavelengths

Excitation: 485 nm

Emission: 531 nm

3. **Black 96 Well Plate, or Desired Template**
4. **15 ml Falcon Tube**
5. **1.5 ml snap cap tubes**

SOLUTIONS

PLpro (Control Isopeptidase) (For Use In Step 2 of Protocol)

1. Add 990 µl of assay buffer to one of the tubes labeled PLpro.
2. Vortex the tube.

You are now ready to add 50 µl aliquots into the wells of a black 96 well plate.

3. Each 50 µl aliquot contains 20nM PLpro.

Reporter Substrate Solution (For Use In Step 4 of Protocol)

1. Perform this step in a timely manner as to minimize exposure of the reporter substrate to light.
2. Dilute Reporter Substrate 2 in a 1:10 ratio with DMSO: Add 10 µl reporter substrate 2 to 90 µl of DMSO.
3. Combine 3.2 µl of diluted reporter substrate 2, and 196.8 µl of assay buffer in a 1.5 ml snap cap tube.
4. Vortex the tube.
5. You are now ready to add 50 µl aliquots into the wells of a black 96 well plate.
6. Each 50µl aliquot contains 40nM of reporter substrate 2.

ISG15-CHOP2-Reporter (Reporter System) and Reporter Substrate Solution (For Use In Step 5 of Protocol)

1. Perform this step in a timely manner as to minimize exposure of the reporter substrate to light.
2. Combine 150 µl of ISG15-CHOP2-Reporter, 9.6 µl stock reporter substrate 2, and 5.86 ml of assay buffer in a 15 ml Falcon Tube.
3. Vortex the tube.
4. You are now ready to add 50 µl aliquots into the wells of a black 96 well plate
5. Each 50 µl aliquot contains 100nM of ISG15 -CHOP2-Reporter and 40 nM of reporter substrate 2.

Test Isopeptidase (For Use In Step 3 of Protocol)

We suggest an initial dilution series of your test isopeptidase to optimize its concentration/activity. As a positive control, the kit includes the catalytic core domain of the enzyme PLpro, to be used at a concentration of 20nM in the final reaction. Make the dilutions of your test isopeptidase in assay buffer such that a final volume of 50 µl is added to each well. A suggested protocol is listed below.

1. Label seven 1.5 ml snap cap tubes T1 through T7.
2. Place 200 µl of assay buffer in tubes T2 through T7.
3. Dilute the test isopeptidase to a concentration of 800nM in 400µl of Assay Buffer in Tube T1.
4. Vortex Tube T1 and perform a 2-fold dilution by transferring 200 µl of solution from Tube T1 into Tube T2.
5. Perform another 2 fold dilution by taking 200 µl from Tube T2 and placing it into Tube T3.
6. Repeat for Tubes T4 through T7.

tube	Concentration of test isopeptidase in 50 µl (nM)	Concentration of test isopeptidase in 100 µl (nM)
T1	800	400
T2	400	200
T3	200	100
T4	100	50
T5	50	25
T6	25	12.5
T7	12.5	6.25

PROTOCOL

Optimization of Your Test Isopeptidase, Suggested Protocol

1. Add 50 µl of Assay Buffer in triplicate to columns 1 and 2.
2. Add 50 µl of PLpro (control isopeptidase) in triplicate to column 3.
3. Add 50 µl of Tubes T1-T7 of your test isopeptidase in triplicate to columns 4 through 10.
4. Add 50 µl of reporter substrate solution in triplicate to column 1.
5. Add 50 µl of reporter and reporter substrate solution in triplicate to columns 2 through 10.
6. Below gives a representation of the 96 well plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50 µl Assay Buffer + 50 µl Reporter Substrate	50 µl Assay Buffer + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl PLpro + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T1 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T2 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T3 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T4 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T5 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T6 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T7 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate		
B	50 µl Assay Buffer + 50 µl Reporter Substrate	50 µl Assay Buffer + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl PLpro + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T1 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T2 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T3 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T4 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T5 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T6 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T7 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate		
C	50 µl Assay Buffer + 50 µl Reporter Substrate	50 µl Assay Buffer + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl PLpro + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T1 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T2 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T3 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T4 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T5 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T6 + 50 µl ISG15-CHOP2-Reporter & Reporter Substrate	50 µl Tube T7 + 50 µl ISG15-CHOP2120 -Reporter &		

7. There are two options for fluorescence detection. First, the plate can be incubated at room temperature for 30 minutes, protected from light, and a single time point reading can be measured using the appropriate excitation and emission filters, 485 and 531 nm, respectively. Alternatively, an in-plate kinetic reading can be performed by measuring the change in fluorescence over time.

8. Determine the mean plate blank value (column 1 in the representative example) and subtract from each data point to give a true representation of the change in fluorescence intensity. It is recommended that each experimental condition is measured in triplicate.

FLUOROPHORE CONTROL

Validation of fluorometric plate reader settings with fluorophore control (if desired)

1. Combine 2µl of Control Fluorophore 2 with 4mL of Assay Buffer. (Final concentration of 500nM for fluorophore solution)

2. Prepare a set of two-fold serial dilutions by combining 500µl of diluted fluorophore with 500µl of assay buffer. (This will result in concentrations of 250, 125, 62.5, 31.2, 15.6, 7.81, and 0nM).

3. Place 100µl of each dilution into the wells of 96-well plate in triplicate.

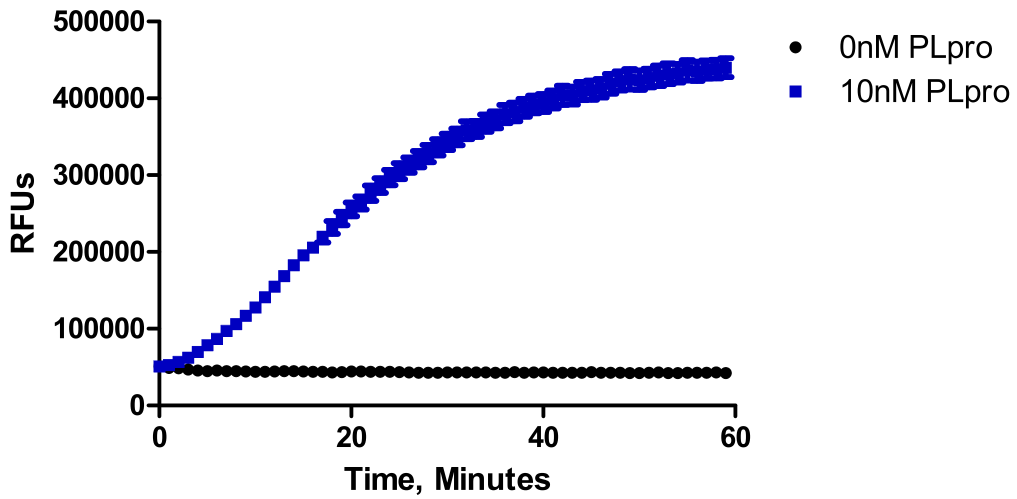
4. Read plate on fluorescent plate reader with excitation and emission filters compatible with measurements of 485 nm and 531 nm, respectively.

QUALITY CONTROL

Quality Control

In a 96 well plate, 50nM ISG15-CHOP2-Reporter and 20nM reporter substrate were incubated in the presence and absence of 10nM PLpro. Liberation of the fluorescent reporter substrate was monitored on a fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 531 nm. Net RFU was determined by subtracting the blank (reporter substrate alone) from each data point. Results are shown in the figure below.

Representative experiment. Data = mean ± standard deviation



REFERENCES

Nicholson, B, Leach, C.A., et al (**2008**). “Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities.” *Prot. Sci.*, in press

Arnold, J.J., Bernal, A., et al. (**2005**). “Small ubiquitin-like modifying protein isopeptidase assay based on poliovirus RNA polymerase activity.” *Anal Biochem*, Nov 17.

Hall, J., Mattern, M., and Butt, T. (**2005**). “Mining the ubiquitin pathway.” *The Scientist* 19 (23): 42-43.

Lindner, H. A., Fotouhi-Ardakani, N. et al. (**2005**). “The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme.” *J Virol* 79(24): 15199-15208

Ciechanover, A. (**2003**) "The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting." *Biochem Soc Trans* 31(2): 474-81.

Hochstrasser, M. (**2002**). "Molecular biology. New proteases in a ubiquitin stew." *Science* 298(5593): 549-52.

Ciechanover, A. (**2001**). "Ubiquitin-mediated degradation of cellular proteins: why destruction is essential for construction, and how it got from the test tube to the patient's bed." *Isr Med Assoc J* 3(5): 319-27.

Wilkinson, K. D. (**2000**). "Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome." *Semin Cell Dev Biol* 11(3): 141-8.

Chung, C. H. and S. H. Baek (**1999**). "Deubiquitinating enzymes: their diversity and emerging roles." *Biochem Biophys Res Commun* 266(3): 633-40.

Dang, L. C., F. D. Melandri, et al. (**1998**). "Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes." *Biochemistry* 37(7): 1868-79.

Hershko, A. and A. Ciechanover (**1998**). "The ubiquitin system." *Annu Rev Biochem* 67: 425-79.

Wilkinson, K. D. (**1997**). "Regulation of ubiquitin-dependent processes by deubiquitinating enzymes." *Faseb J* 11(14): 1245-56.

Hochstrasser, M. (**1996**). "Ubiquitin-dependent protein degradation." *Annu Rev Genet* 30: 405-39.

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