ELISA kit for the Sensitive Detection of Ubiquitin C-terminal Hydrolase 1 (UCHL1)
Version 1.1
Lot 062315

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Abstract

This kit is an enzyme linked immunosorbent assay (ELISA) for the detection of ubiquitin C-terminal Hydrolase 1 (UCHL1), one of the most abundant proteins of the nervous system where it is expressed exclusively in neurons. UCHL1 may be released in large amounts into cerebrospinal fluid (CSF), blood and other body fluids following neuronal injury or degeneration. The level of UCHL1 detected can give information about the degree and location of an injury, the progression of a damage or disease state, the process of recovery and potentially the response of an individual to therapies. This kit has been used to detect informative levels of UCHL1 released from damaged or degenerating neurons in several human disease states and in a variety of animal models of human disease. The kit has been shown to work on blood and CSF samples from human, pig, rat, mouse, cat and dog. Since UCHL1 is a highly conserved protein, the kit is expected to work in many other mammalian species also.

1 Introduction

Ubiquitin C-terminal hydrolase 1 (UCHL1) was independently discovered by several different research groups and so has several other names, such as ubiquitin carboxyl esterase L1, ubiquitin thiolesterase, neuron-specific protein Pgp9.5 and Park5. It was originally identified as a major neuron specific cytoplasmic protein from 2-dimensional gel analysis of brain tissues and immunostaining, and was given the name "protein gene product 9.5" or Pgp9.5 (1). The protein was found to be extremely abundant and very heavily concentrated in brain, where it was estimated to represent 200-500 µg/g wet weight. Immunocytochemistry showed that UCHL1 was neuron-specific and it has been claimed to represent 1-2% of total brain protein (2). It was later found that a ubiquitin C-terminal hydrolase enzyme activity was associated with the Pgp9.5 protein, resulting in the renaming of Pgp9.5 to ubiquitin C-terminal hydrolase 1.

UCHL1 is the first characterized member of a family of ubiquitin C-terminal hydrolases with 4 members in the human. These enzymes cleave ubiquitin from other molecules, an activity important to generate mono-ubiquitin from ubiquitin genes which encode either polyubiquitin chains or ubiquitin fused to other proteins. Ubiquitin functions as a protein tag which covalently attaches to other proteins and targets them for proteosomal degradation. UCHL1 activity may also remove ubiquitin from partially degraded proteins, allowing the ubiquitin monomer to be recycled. Regulation of the ubiquitin pathway is very important and many disease states are associated with defects in this pathway.

Genetic knockout of the UCHL1 gene in mice results in motor neuron degeneration (3). The pattern of degeneration was similar to that seen in the spontaneous gracile axonal dystrophy (*gad*) mice which were found to have a mutation in the UCHL1 gene which destroyed the UCHL1 enzymatic activity. Both findings suggest that UCHL1 is an essential enzyme.

UCHL1 was also discovered as a gene mutated in some rare familial forms of Parkinson's disease, and so was named Park5. Park5 proved to encode an I93M point mutations in the UCHL1 gene, which reduces the ubiquitin hydrolase activity. Interestingly a common allelic variant of UCHL1, the S18Y polymorphism is actually protective against Parkinson's disease.

Pure recombinant human UCHL1 was used to produce the antibodies included in this kit. We developed a panel of mouse monoclonal, rabbit polyclonal and chicken polyclonal antibodies as outlined (4). We chose mouse monoclonal clone MCA-BH7 as the optimal capture antibody, and an affinity purified rabbit polyclonal antibody RPCA-UCHL1-AP was selected for UCHL1 detection. Detection of the rabbit antibody is performed using an appropriate anti-rabbit horseradish peroxidase (HRP) conjugate.

The original assay was developed and used to show that UCHL1 was released in large amounts into the CSF of aneurysmal subarachnoid hemorrhage (ASAH) patients in a manner closely matching that of pNF-H, which we had studied in the same patients previously (5).

2 Test Principle

The kit is an ELISA capture assay, in which a 96-well-format plate is coated with an affinity purified mouse monoclonal antibody raised against the purified recombinant human UCHL1. The plate has been blocked with a protein solution to remove any further non-specific binding, and is supplied with 50 µL of Tris buffered saline (TBS) plus 5 mM sodium azide (NaN_3) preservative. Standards and samples are incubated in microplate wells for 3 hours at room temperature or overnight at 4°C. Binding is detected with a rabbit polyclonal antibody to UCHL1 for another 1 hour. The amount of specifically bound rabbit polyclonal antibody is then detected using a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP). Finally, the wells are incubated with 3, 3`, 5, 5`-Tetramethyl Benzidine (TMB), a reagent which produces a blue color when exposed to HRP in the presence of hydrogen peroxide. The reaction is stopped by the addition of sulfuric acid (H_2SO_4), and the absorbance of the resulting yellow product is measured at 450 nm. Absorbance is directly proportional to the concentration of UCHL1 in the samples and standards. A standard curve is generated by plotting absorbance values against concentrations of the standard, and concentrations of unknown samples are calculated using the standard curve.

3 Kit Contents and Quantity

96 well plate coated with MCA-BH7	1
Detection antibody-RPCA-UCHL1-AP, 0.15mg/ml	10 µL
Goat anti-rabbit HRP Conjugate: use at 1:10,000	10 µL
10x TBST	5 mL
Protein Blocker– Non-Fat Dry Milk	0.5 g
Recombinant Human UCHL1 Protein Standard at 0.5 mg/mL	10 µL
Development Buffer	12 mL

4 Required but Not Included in the Kit

1X TBST for washing
2N H_2SO_4 HRP stop solution
Microplate reader (450 nm)
Multichannel 8 or 12 tip pipette
Reservoirs for multichannel pipette
Automated plate washer
Plate shaker
15 or 50 mL polypropylene tubes for dilutions
Software for data analysis

5 Storage of Reagents

The 96 well plate can be split into 12 well strips if required and can be stored for up to 3 months at 4°C. The dilution buffer and UCHL1 standard should be stored frozen at -20°C.

6 Preparation of Reagents

A. Dilution Buffer - 50 mL:
Weigh out 0.5 g of non-fat milk powder and dissolve in 50 mL of 1x TBST. The buffer can be stored at 4°C for 2-3 days.

B. Detection Buffer
The solution can be stored at 4°C prior to mixing. It is better to use the reagent when it is warmed up to room temperature.

7 Standard Curve

Concentration of UCHL1 Standard (ST) is 0.5 mg/mL. The highest standard point (5 ng/mL) is obtained by serial dilution of stock solution with Dilution Buffer (SDB see **6A**). For example, dilute the standard from 0.5 mg/mL 1:48 (5 µL ST+245 µL of DB) to give 10 µg/mL. Vortex and dilute that 1:50 (5 µL ST+245 µL of DB) to get 200 ng/mL. Vortex and then dilute that 1:40 (25 µL ST+975 µL of DB) to give 5 ng/mL.

Designate two columns of wells (16 wells) for the standard curve. Add 50 µL/well of the DB (see **6a**) to wells "B" through "H" in two columns. Add 100 µL diluted UCHL1 Standard (5 ng/mL) to the "A" wells of the first two columns.

Perform serial 1:2 dilutions (50 µL/well) down the plate in the columns designated for the standard curve. Do not add any standard solution to the last "H" wells, these are **BLANK**. Discard 50 µL of the solution from well "G".

Column	Sample Dilution Buffer (SDB)	Standard	concentration (ng/mL)
A	0	100	5
B	50	50 (A)	2.5
C	50	50 (B)	1.25
D	50	50 (C)	0.625
E	50	50 (D)	0.3125
F	50	50 (E)	0.156
G	50	50 (F)	0.078
H	50	0	0

8 Sample Preparation

Serum, Plasma, CSF and Urine
The kit measures UCHL1 in mammalian CSF, serum, plasma and urine. Mix thawed samples thoroughly just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. We recommend the dilution of samples 1:1 with DB followed by vortexing at the beginning of analysis. Serum and plasma samples should be stored at -20°C, or preferably at -70°C for long-term storage. CSF and urine samples should be stored at -70°C.
Do not store diluted samples.

9 Procedure

1. Unpack the ELISA plate and aspirate the solution from the wells.
2. Obtain a standard curve (see **7** above). Apply a total sample amount of 50 µL/well. For samples: add Dilution Buffer (see **6a**) into the wells, then an appropriate amount of tested sample, preferably in duplicates to make a final volume of 50 µL/well.
3. Incubate the plate at room temperature for 3 hours with gentle shaking, or at 4°C overnight.
4. Wash the plate 3 times with TBST (0.3 mL per well) using an automated plate washer. Turn the plate 180° and wash for 3 times again to ensure even washing.
5. Dilute rabbit polyclonal anti-UCHL1 (RPCA UCHL1-AP) antibody 1:600 in Antibody Dilution Buffer (10µL/6mL DB), final concentration 0.25 µg/mL. Add 50 µL to each well.

6. Incubate the plate at room temperature for 1 hour with gentle shaking.
7. Wash the plate 3 times with TBST using an automatic plate washer. Turn the plate 180° and wash 3 times again.
8. Add 1 μL of goat anti-rabbit-HRP to 10 mL of Dilution Buffer . Vortex and apply 50 μL to each well.
9. Incubate the plate at room temperature for 1 hour with gentle shaking.
10. Wash the plate 3 times with TBST using an automatic plate washer. Turn the plate 180° and wash 3 times again.
11. Add 100 μL of the Detection Buffer (**6B**) into each well.
12. Incubate the plate with gentle shaking for 10-15 minutes at room temperature. The incubation time may be extended (up to 30 minutes). Look for the blue color in the standards and samples.
13. At an appropriate time (10-15 minutes) stop the reaction by adding 50 μL of 2N H_2SO_4. The blue color will change to yellow upon acidification. Take care to avoid creating bubbles which will create a strong interfering absorbance signal.
14. Measure the absorbance of each well using a microplate reader set to 450 nm absorbance within 5 minutes following step 13.

10 Interpretation of results

The level of UCHL1 detected in a particular situation should provide an unbiased quantification of this protein in the relevant situation. Analysis of the levels in serum, plasma, CSF and urine should provide information on the degree of ongoing neuronal injury or degeneration which has occurred. We typically use the top two rows of the plate for a standard curve leaving the last wells without standard (See Example Worksheet, Page 8). It is important to leave the last well blank, containing no protein standards but with SDB and reacted with all antibody and development reagents which will indicate the non-specific background.

The data below shows, in the second column, OD450 values corrected for blank readings. The standard curve is constructed by plotting the mean absorbance (Y) of standards against the known concentration (X) of Standards. Results are reported as concentration of UCHL1 (pg/mL) in samples. The graph of this data (Fig. 1, top right) is a typical one showing an asymptotic curve resulting from high concentration of the standard.

Thus, we chose to plot a best fit curve through the linear portion of the standard curve and overlay a linear trendline (Fig. 1, bottom right). This is generally achieved by removing one or more of the higher standard points. Once an R^2 value of ~0.99 is achieved, the resultant equation displayed on the graph can then be used to calculate the unknown concentrations by solving for x. The measured concentration of samples calculated from the standard curve must then be multiplied by their respective dilution factor, because samples have been diluted prior to the assay, e.g. 400 pg/ml (from standard curve) x 2 (dilution factor) = 1,200 pg/ml = 1.2 ng/ml.

Conc. pg/mL	OD450
5000	2.2425
2500	1.511
1250	0.9645
625	0.4875
312.5	0.31125
156.25	0.1285
78.125	0.063
0	0

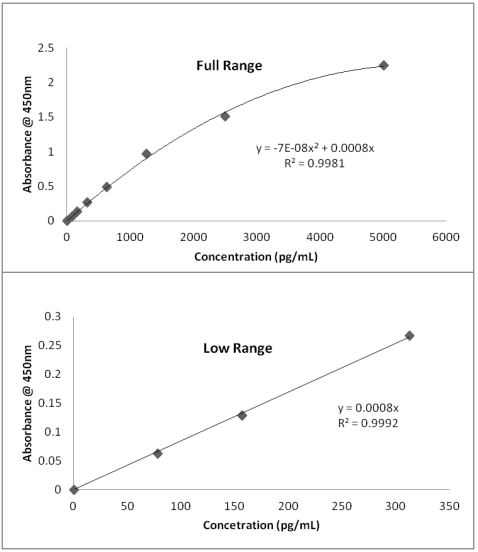


Figure 1. **Left:** A450 for the standard. **Top right**: graph of the total concentration range of the standard curve. **Bottom right**: plot of low concentration range of the standard curve.

11 Warranty

Data presented here were obtained using the protocol outlined here. Any modification or change in the protocol not suggested by EnCor Biotechnology Inc. may impact the results, in which event EnCor Biotechnology Inc. disclaims all warranties.

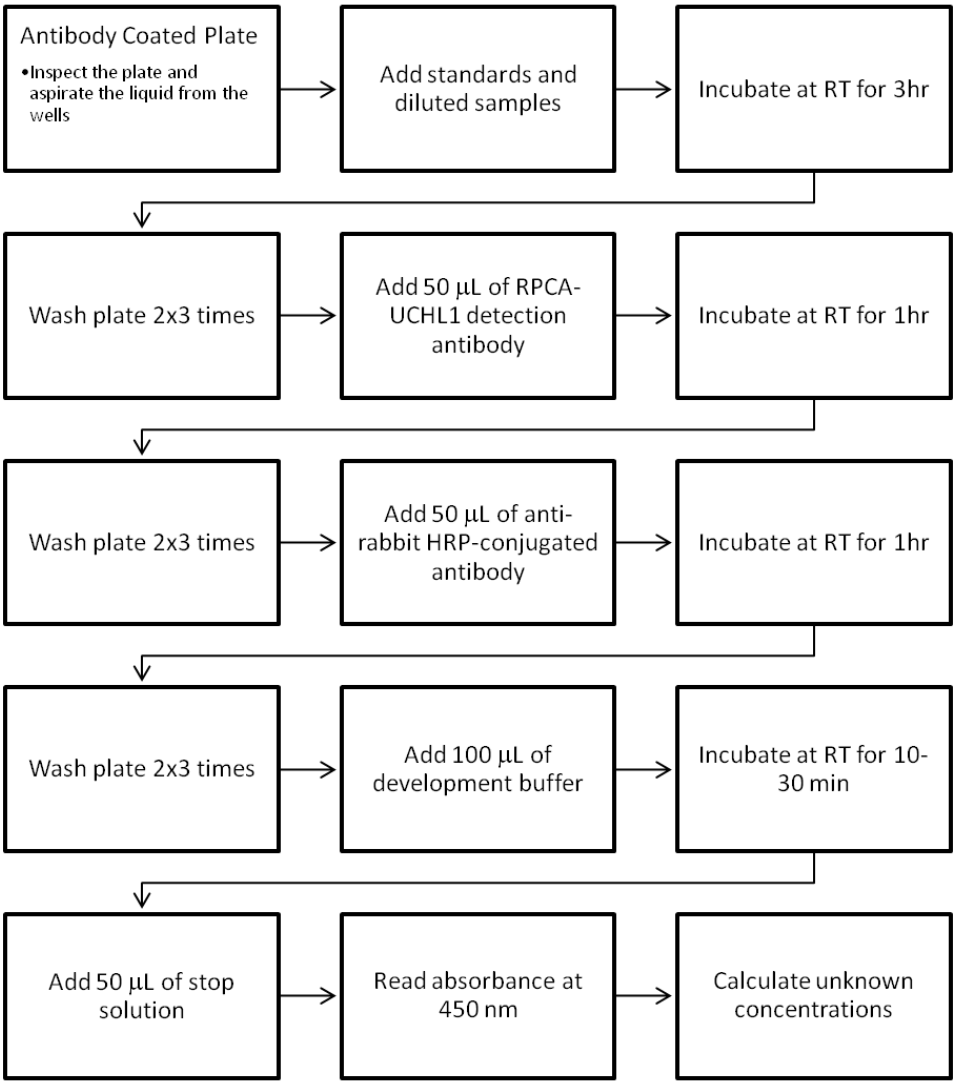
12 References

1. Doran JF, Jackson P, Kynoch PA, Thompson RJ. Isolation of PGP 9.5, a new human neurone-specific protein detected by high-resolution two-dimensional electrophoresis. J Neurochem. 40:1542-7 (1983).
2. Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science. 1989 246:670-3 (1989).
3. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell 111:209-18 (2002).
4. Day IN, Thompson RJ. UCHL1 (PGP 9.5): Neuronal biomarker and ubiquitin system protein. Prog Neurobiol. 2009 Oct 30.
5. Lewis, S. B., Wolper R., Miralia, L., Wang, Y., Yang, C. and Shaw, G. Identification and preliminary characterization of Ubiquitin C terminal Hydrolase 1 (UCHL1) as a biomarker of neuronal loss in aneurysmal subarachnoid hemorrhage. J. Neurosci. Res. J. Neurosci Res. 8:1475-1484 (2010).

13 Sample Worksheet

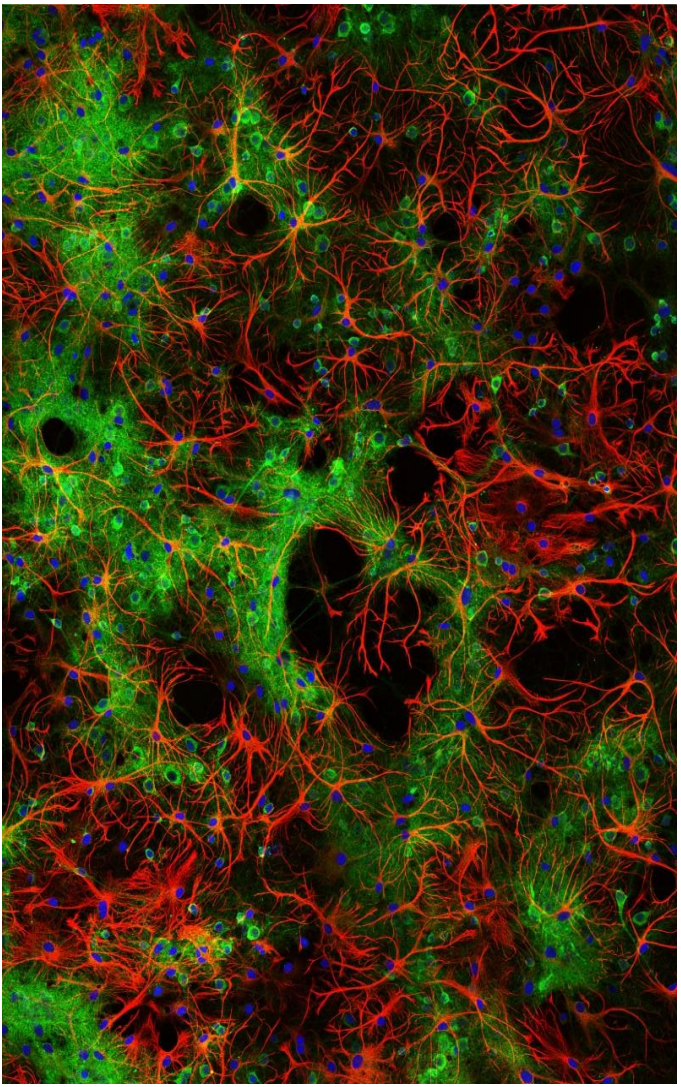
	Strips 1+2	Strip 3+4	Strip 5+6	Strip 7+8	Strip 9+10	Strip 11+12
A	ST 5	QC High	Sample 7	Sample 15	Sample 23	Sample 31
B	ST 2.5	QC Low	Sample 8	Sample 16	Sample 24	Sample 32
C	ST 1.25	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
D	ST .625	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
E	ST .3125	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
F	ST .156	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
G	ST 0.078	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
H	Blank	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38

14 Assay Procedure Summary



Notes

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												



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