Colorimetric ELISA kit for the sensitive detection of Ubiquitin C-terminal Hydrolase 1 (UCHL-1) in plasma, serum and CSF.

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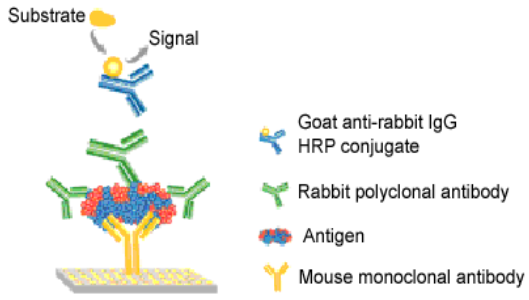
Introduction:

Ubiquitin C-terminal Hydrolase 1 (UCHL-1) was independently discovered by several research groups and so has several 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 UCHL-1 was neuron-specific in the CNS and it was claimed to represent 1-2% of total brain protein. Later work showed that the Pgp9.5 protein was an enzyme which cleaved ubiquitin from ubiquitinated proteins, allowing recycling of this vital protein, and resulting in the name UCHL-1. Genetic knockout of the UCHL-1 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 UCHL-1 is an essential enzyme.

This assay was developed and used to show that UCHL-1 was released in large amounts into the CSF of aneurysmal subarachnoid hemorrhage patients, larger amounts indicating both acute events and poorer outcomes (4). It has since been used to study UCHL-1 released into blood following various injury and disease states (e.g. 5). Much work from other groups suggests that blood UCHL-1 is a potentially valuable biomarker of CNS damage and disease states (6,7).

Principle of the Assay:

ELISA is a convenient method for measuring the level of protein targets within a single, small-volume sample. ELISA-UCHL-1 is a 2-site sandwich enzyme linked immunoassay in which EnCor provides a plate pre-coated with capture mouse monoclonal antibody against UCHL-1. The user incubates the samples on the plate and any UCHL1 binds to the capture antibody. The user then incubates with a solution containing rabbit polyclonal UCHL-1 detection antibodies, which bind to the captured UCHL-1. The detection antibody is then itself bound by goat anti-rabbit horse radish peroxidase (HRP) antibody conjugate. The user adds a developer solution which produces a colored product in wells containing HRP, the intensity of the color corresponding to the amount of UCHL-1 in the sample. Finally the user stops the reaction by adding H_2SO_4. The color changes from blue to yellow and can be quantified using a suitable ELISA plate reader at an absorbance wavelength of 450 nm.



Reagents Supplied:

Content	Store	Kit component	Quantity
Plate	4 ˚C	Precoated anti-UCHL-1 strip plate, MCA-BH7	8 X 12 wells
RPCA-UCHL-1	4 ˚C	Affinity purified rabbit polyclonal anti-UCHL-1 detection antibody (500X) Lot # 138-111215	1 X 15 µL
α–Rb-HRP	4 ˚C	Goat anti-rabbit IgG, HRP conjugated antibody (10,000X)	1 X 5 µL
Standard	-20˚C	UCHL-1-recombinant protein, 0.5 mg/mL, Lot #2615	1 X 10 µL
Blocker	4˚C	500 mg	1 X 500 mg
TBST	4 ˚C	Tris Buffered Saline-Tween-20, 10X stock	1 X 5 mL
TMB developer	4 ˚C	One step Ultra TMB developer solution, ready to use	1 X 12 mL

Additional Materials and Equipment (not provided):

- Microplate reader (450 nm)
- Appropriately sized tubes for reagent preparation
- Polypropylene microcentrifuge tubes for preparing dilutions
- Multichannel pipette
- Microplate shaker
- Automated plate washer
- Stop Solution (2N H_2SO_4)
- Deionized water
- Wash solution:TBST (TBS pH=7.6 plus 0.1% Tween-20)

Limitations of the Assay:

- For research use only. Not for use in diagnostic procedures.
- Absorbance values beyond the range of the standard curve are not valid.

Warning: Before starting the assay, read the instructions for use completely and carefully. Make sure that everything is understood!

Measure desired amounts of reagents by weighing or pipetting them out from original container!

Best Practices and Technical Hints

1. Do not mix or substitute reagents from different sources or different kit lots.

2. Dilute samples in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution. Two common pipetting methods used for ELISA are standard (forward) and reverse. Use standard (forward) pipetting for the preparation of sample dilutions, and reverse pipetting for the addition of diluted samples, controls and reagents. Careful pipetting is crucial for accurate test results. Become familiar with the pipette and both methods before running actual tests. Be sure to use the correct pipette and tip (volume capacity) for the volume being transferred.

3. Standard (Forward) Pipetting :

    a. Put a new tip on a single-channel pipette and make sure that it is on tight.
    b. Press the plunger to the first stop.
    c. Draw the calibrated volume of sample into the tip and pause for one second with the tip still in the sample. Be careful not to place the tip too deeply into the sample.
    d. Touch the tip to the side of the sample container to remove any excess liquid on the outside of the tip.
    e. Dispense the sample into the measured diluent by depressing the plunger past the first stop to the second stop. Be careful not to place the tip too deeply into the sample diluent.
    f. After dispensing the sample into the diluent, rinse the pipette tip in the diluent by pushing the plunger up and down 2–3 times before ejecting the tip.
    g. Mix samples using vortex if they are in the tube, and tapping the plate if the samples added directly to the well.
    h. Eject the tip into a waste container.

4. Reverse pipetting using single or multichannel pipette.

    a. Put new tips on the pipette. Make sure they are on tight and straight.
    b. Press the plunger past the first stop and halfway to the second stop.
    c. Draw the liquid in a slow motion, being careful not to draw any air bubbles into the tips, check for consistency of volume in the tips.
    d. Touch the tips to the edge of the reagent reservoir to remove excess liquid on the outside of the tips. If the wells on your plate are empty, position the tips into the lower corner of each well, making contact with the plastic. If the wells on your plate contain liquid, position the tips above the liquid, making contact with the plastic.
    e. Slowly dispense the liquid into the wells by depressing the plunger to the first stop. Be careful not to splash liquid out of the wells, and make sure there are no drops left on the tips.

f. To repeat, hold the plunger at the first stop and continue with step "c".
g. Eject the tips into a waste container.

NOTE: Reverse pipetting uses more reagent/volume (=“dead volume”)

5. Measure desire amounts of reagents by weighing or pipetting them from original container.

6. Avoid prolonged exposure of HRP-conjugated antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.

7. Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results, and are particularly a problem during optical density determination.

8. When using an automated plate washer, rotating the plate 180 degrees between washing steps may improve assay precision.

9. Gently tap the plate against paper towel to remove residual fluid after washing.

10. The TMB developer solution should be at room temperature when added to the plate. Keeping time intervals consistent between adding developing buffer and reading the plate should improve inter-plate precision.

11. If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the wells. If assay results are above the top of the calibration curve, dilute samples, and repeat the assay.

When running partial plates, seal the unused portion of the plate with a plate seal or adhesive tape to avoid contaminating unused wells. After reading a partial plate, remove used strips, and return plate to its original bag with desiccant. Keep it at 4˚C.

Reagent Preparation:

Prepare wash solution -TBST:

Wash solution is 1X Tris buffered saline - tween (TBSt)
(10 mM Tris base, 150 mM NaCl, 0.1% Tween-20), for 1 L

- 1.21 g - Tris base
- 8.77 g - NaCl

Add deionized water to a final volume of 900 mL.
Adjust pH to 7.5 using concentrated HCl (careful!), then add

- 1 mL - Tween 20, mix well. Bring final volume to 1L with dH_2O.

Prepare stop solution:

Stop solution is 2N H_2SO_4 solution
to make 250 mL

- 26.7 mL concentrated H_2SO_4
- 223.3 mL H_2O

Add concentrated H_2SO_4 slowly to water. Stop solution can be stored at room temperature for up to 6 months.

Prepare dilution buffer (DB):

Combine 500 mg of blocker, 5 mL of 10X TBST (provided by EnCor), add dH_2O to have final volume of 50 mL, dissolve well. The buffer can be stored at 4°C for 2 days.

Dilute detection antibody:

Prepare the detection antibody solution immediately prior to use.
EnCor provides affinity purified rabbit polyclonal anti-UCHL-1 detection antibody (RPCA-UCHL1) at concentration of 0.15 mg/mL, which is 500X stock solution. The working solution is 1X. For 1 plate combine:

- 6 mL of dilution buffer (DB)
- 12 µL of RPCA-UCHL-1, vortex well

Make enough solution for an experiment and discard remainders after use.

Prepare anti rabbit-HRP solution:

Prepare the detection antibody solution immediately prior to use.
EnCor provides goat anti-rabbit IgG HRP conjugate antibody as a 10,000X stock solution. For 1 plate :

- add 2 µL of anti-rabbit-HRP to 20 mL of dilution buffer (DB).

Discard remainder of solution after use.

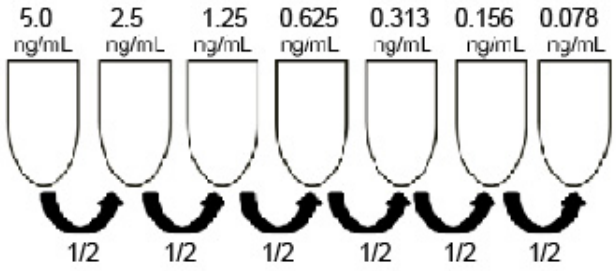
Protein Standards Preparation:

Prepare dilutions of protein standards:

EnCor supplies UCHL-1 standard (full length recombinant human protein) at the concentration 0.5 mg/mL that is 100,000-fold higher than the recommended the highest standard. We recommend 7- point calibration curve in duplicate with 2-fold serial dilution steps and a zero standard blank. Thaw the standard stock, and keep it on ice. Dilute standards in dilution buffer (DB), using suitable microcentrifuge tubes. To achieve the highest recommended UCHL-1 concentration of 5 ng/mL, perform serial dilutions: dilute 3 µL of UCHL-1-stock (0.5 mg/mL) in 147 µL of DB. Concentration in the tube #1 now is 10 µg/mL=10,000 ng/mL. Dilute 3 µL of Standard (10 µg/mL - tube #1) in 147 µL of DB. Concentration in the tube #2 now is 200 ng/mL. Dilute Standard (200 ng/mL - Tube #2) 40 times in DB (see table below), and you will have 5 ng/mL in the tube #3 - the recommended highest standard.

Data in the table below summarizes the preparation of UCHL-1 Standard of 5 ng/mL

Standard concentration ng/mL	Standard amount µL	Dilution Buffer µL	Dilution factor	Final concentration ng/mL	
Stock - 500,000	3	147	50	#1	10,000
10,000	3	147	50	#2	200
200	20	780	40	#3	5



Label 7 micro-tubes, one for each standard point (that is 5 ng/mL (#3 from dilutions described in the table, has 800 µL of 10 ng/mL), 5 ng/mL, 2.5; 1.25; 0.625; 0.3125; 0.156, and 0.078 ng/mL,). Add 300 µL of dilution buffer (DB) into each tube starting from the second one. Perform serial 1:2 dilutions starting from standard - 5 ng/mL by transferring 300 µL of it into 300 µL of DB in the next tube as shown on the picture above. Vortex well. That amount of diluted standards will be enough to assay up to 5 wells in the plate. Diluted UCHL-1 standards should be used immediately and cannot be stored and reused.

Sample Preparation:

Serum and Plasma:

Plasma samples prepared in heparin tubes commonly display additional clotting following thawing. Remove clots and all solid material by centrifugation. Similarly serum may sometimes contain small amounts of particulate material which can be removed by centrifugation. For those systems and assays using neat samples or lower dilution factors, the sample can be put directly into the wells of the coated plates. Follow the sequence below:

1. Add the dilution buffer (DB) to the plate.
2. Add the sample into the DB.
3. Mix by tapping the plate or repeating pipetting.

We recommend starting with diluted plasma or serum samples 1:2.5 times with DB (or 30 µL of dilution buffer plus 20 µL of serum or plasma sample) at a single concentration for pilot experiments. If strong UCHL-1 signals are detected samples may be run at higher dilutions. Typical pathological human plasma or serum samples show a single digit ng/mL amounts of UCHL-1. Always run samples in duplicate or triplicate. This will provide enough data for statistical validation of the results.

CSF samples:

Pathological human CSF samples are generally higher in UCHL-1 concentration. Start pilot experiments with 1:2.5 dilution of CSF in DB (In order to dilute samples directly in the plate, add 30 µL of dilution buffer to the well then add 20 µL of CSF sample, mix by tapping the plate). If strong UCHL-1 signals are detected samples may be diluted more (dilute CSF samples in the tube, then add 50 µL of diluted sample to the well.)
Always run samples in duplicate or triplicate. This will provide enough data for statistical validation of the results.

Other samples:

In principle this assay should be able to detect UCHL-1 in virtually any kind of appropriate biological fluid. In fact large amounts of UCHL-1 can be detected in the urine of patients with kidney compromise as a result of neonatal hypoxic ischemic encephalopathy and secondary to thoracic endovascular aortic repair operations (EnCor, in preparation).

Protocol:

1. Remove the plate from a plastic bag. It is ready to use.

2. Designate some wells of the plate for the standard curve. For example, add 50 µL/well of the 7 diluted standards starting from "A" wells and down the plate. Add 50 µL/well of DB to the last "H" wells as a blank.
   A standard curve must be prepared for each plate!

3. Add 50 µL of diluted samples per well, or dilute them directly in the wells (see "Sample preparation"). Incubate the plate for 3 hours at room temperature or overnight at 4˚C with mild shaking (seal the plate).

4. Wash the plate 2X4 times with TBSt using an automated plate washer, rotate the plate 180 degrees between wash steps.

5. Add 50 µL of detection antibody solution (RPCA-UCHL-1, see "Dilute detection antibody") to each well. Incubate the plate for 2-3 hours at room temperature with gentle shaking.

6. Wash the plate 2X4 times with TBSt using an automated plate washer, rotate the plate 180 degrees between wash steps.

7. Add 100 µL of anti-rabbit-HRP (see "Prepare anti-rabbit-HRP solution") to each well. Incubate the plate for 1 hour at room temperature with gentle shaking.

8. Bring TMB solution to room temperature.

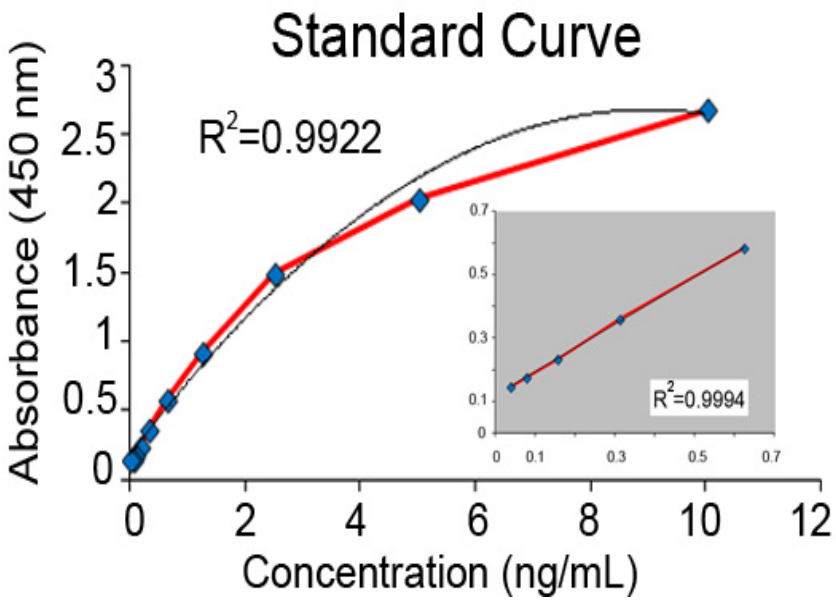
9. Wash the plate 2X4 times with TBSt using an automated plate washer, rotate the plate 180 degrees between wash steps.

10. Add 100 µL of TMB developing solution. Incubate the plate until you reach the desired color change; the highest standard should be dark blue, while blank should stay colorless. Incubation time is 5-20 minutes.

11. Stop reaction with 50 µL of stop solution; 2N H_2SO_4. The color changes from blue to yellow. No incubation in stop solution is required before reading the plate.

12. Read plate on an ELISA plate reader at 450 nm. There are many types of plate reader; refer to the manufacturer's instructions for details of operation.

Performance characteristics of the UCHL-1 - ELISA



Concentration ng/mL	OD-450 AVG
10	2.6735
5	2.0395
2.5	1.4925
1.25	0.9215
0.625	0.5835
0.3125	0.361
0.1563	0.235
0.0781	0.176
0.0391	0.1465
0	0.1433

Fig.1 Representative plot of the UCHL-1 standard curve (9 calibration points of recombinant human UCHL1 protein) obtained using the UCHL-1-ELISA kit. The inset is an enlarged portion of the lower range (0 - 0.625 ng/mL) of the standard curve. The trendlines fitted to the full range plot is polynomial while that for the lower range is linear. Both trendlines are shown in black color. R-squared values are displayed on both charts.

Measuring Range:

The above standard curve covers the interval 0.039 ng/mL - 10 ng/mL UCHL-1. The optimal range is 7 calibration points from 0.078 ng/mL - 5 ng/mL, as at 10 ng/mL saturation is observed. Extrapolation beyond the curve will not provide accurate results. Samples displaying signal outside the curve should be further diluted and remeasured.

Sensitivity:

The intra-assay variability %CV < 6; The inter-assay variability %CV < 12

Incubation time: ~7 hours

Shelf life:

When stored at an appropriate temperature, the product can be used within 6 months from date of production. The UCHL-1 standard and sample dilution concentrate (10X) should always be stored at -20˚C.

References:

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