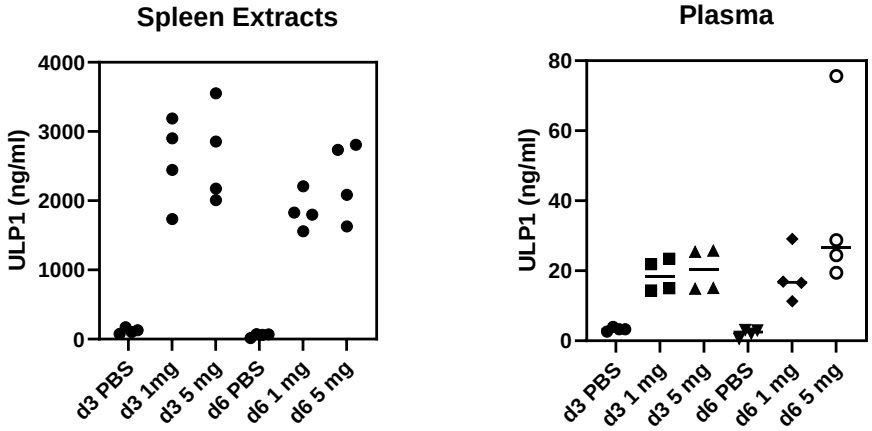
INTRODUCTION

Ubiquitin-like protein 1 (ULP1), also known and Interferon Stimulated Gene 15 (ISG15) is a 17 kDa protein that has multiple roles. It has extracellular cytokine activity, acts as an intracellular protein modifier, and is involved in antiviral responses. We developed the ULP1 ELISA as a tool to investigate viral infections in trout and salmon. As shown below, ULP1 levels are increased in spleen extracts and plasma on days 3 and 6 from trout injected with 1 or 5 mg/kg of poly I:C (a mimic of virus infection).



PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies that recognize ULP1 from Atlantic Salmon and Ranbow Trout. Unconjugated antibodies are coated on wells of a microtiter plate and used for capture. Horseradish Peroxidase (HRP) conjugated antibodies are used for detection. Standards and samples (100 µl) are incubated in the antibody coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If ULP1 molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If ULP1 is present, a blue color develops. Color development is stopped after 20-minutes by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of ULP1 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-ULP1 coated plate (12 x 8-well strips)
- Anti-ULP1 HRP conjugate stock
- ULP1 stock, 1 vial
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: SB50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the kit should at 4°C. It will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

[1] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

WASH SOLUTION

The Wash Solution (TBS50-20) is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DILUENT

The diluent (SB50-1) is formulated for measurement of ULP1 in Trout and Salmon tissue extracts, serum, or plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of recombinant ULP1 in a stabilizing matrix. Reconstitute it with 200 µl of deionized water, gently mix, and prepare the 5 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 5 ng/ml ULP1 standard into the tube labeled 2.5 ng/ml and mix. This provides the 2.5 ng/ml ULP1 standard.
4. Similarly prepare the 1.25 – 0.078 ng/ml standards by two-fold serial dilution.

If future use of the reconstituted stock is intended, it should be stored at or below -20°C in a sealed tube.

HRP CONJUGATE

The HRP conjugate stock must be diluted with diluent SB50-1 as described on the stock vial label about 5 minutes before use. Use 100 µl of the diluted HRP conjugate per well.

SAMPLES

The assay can be used to measure ULP1 in serum, plasma, and tissue extracts. Diluent SB50-1 must be used for dilution.

Serum and Plasma. In serum and plasma, we found it necessary to dilute samples at least 10-fold to avoid matrix effects. Please be aware that red blood cells contain significant levels of ULP1 that will be released by cell lysis. Therefore, when measuring ULP1 in serum or plasma, hemolyzed samples should not be used.

Tissue extracts. Thus far, we have measured ULP1 in extracts from spleen and anterior kidney. Extracts were prepared by placing 100 mg of tissue in 1.5 ml Safe-lock Eppendorf tubes containing 0.5 ml of 150 mM NaCl, 10 mM Tris.Cl, pH 7.4, and 0.1 ml of 1.6 mm stainless steel beads. Homogenization was performed for 1 minute at setting 12 in a cooled Bullet Blender. After homogenization, tubes were centrifuged for 4 minutes at 15,000 rpm. Supernatants were removed and saved for ULP1 measurement. When testing samples from fish injected with 1 or 5 mg/kg poly I:C, we found it necessary to dilute samples 1000-fold to obtain values within range of the standard curve. However, optimal dilutions must be determined by the end user. We recommend that ULP1 levels in tissue extracts be normalized to total protein, as determined by BCA protein assay.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 µl of standards and samples into appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
5. Dispense 100 µl of diluted HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 µl of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20 minutes stop the reaction by adding 100 µl of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

RESULTS

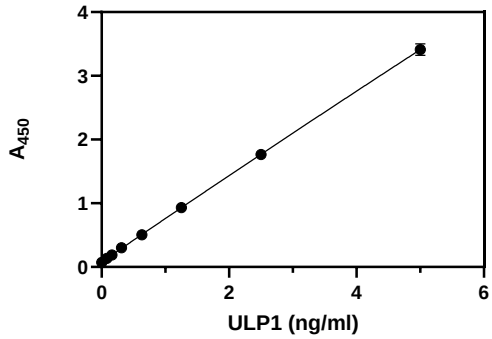
1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the ULP1 concentration. We suggest using a second order polynomial (quadratic) equation.
2. Derive the concentration of ULP1 in the samples.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
4. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

[2] If absorbance of the high standard is ≥4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

TYPICAL STANDARD CURVE

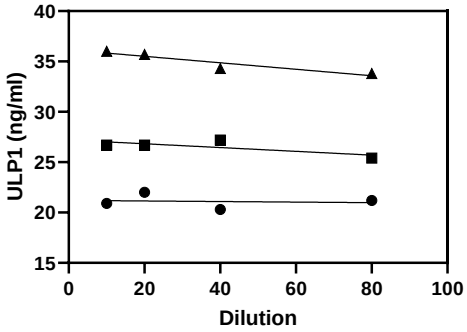
A typical standard curve is shown below. This curve is for illustration only.

ULP1 (ng/ml)	A_{450}
5	3.411
2.5	1.765
1.26	0.933
0.625	0.504
0.313	0.301
0.156	0.188
0.078	0.134
0	0.072



PERFORMANCE

Linearity: To assess the linearity of the assay, three Rainbow Trout serum with ULP1 concentrations of 21, 27, and 34 ng/ml were serially diluted from 10 to 80-fold to produce values within range of the assay.



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