

INTRODUCTION

Growth Differentiation Factor-15 (GDF-15) expression levels increase during infection, inflammation, and sepsis (refs 1 – 3). Elevated levels of GDF-15 are associated with appetite suppression. In studies at Life Diagnostics, Inc. we found levels of approximately 5 ng/ml in plasma from healthy Rainbow Trout. Levels of up to 1000 ng/ml were found in plasma from fish infected with *Flavobacterium psychrophilum* and *Yersinia ruckeri*.

PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies generated against recombinant Rainbow Trout GDF-15. It recognizes Rainbow Trout and Atlantic Salmon GDF-15. 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 diluted samples (100 μ l) are incubated in the antibody coated microtiter wells for one hour. After washing the wells, HRP-conjugate (100 μ l) is added and incubated for 45 minutes. If GDF-15 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 GDF-15 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 GDF-15 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-GDF-15 coated plate (12 x 8-well strips)
- HRP conjugate stock.
- GDF-15 stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: SB50-1, 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 standard stock vials at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit 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 plate¹. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 μ l 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.

WASH SOLUTION

The wash solution 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 is formulated for measurement of GDF-15 in trout and salmon serum or plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of pure recombinant Rainbow Trout GDF-15 in a stabilizing matrix. Reconstitute it with 200 μ l of deionized water, gently mix, and prepare the 250 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 125, 62.5, 31.25, 15.63, 7.81, 3.91, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 250 ng/ml GDF-15 standard into the tube labeled 125 ng/ml and mix. This provides the 125 ng/ml GDF-15 standard.
4. Similarly prepare the 62.5 – 3.91 ng/ml standards by two-fold serial dilution.

If future use is intended, the reconstituted standard stock should be stored in an ultralow freezer within 15 minutes of reconstitution.

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

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 μ l of the diluted HRP conjugate per well.

SAMPLES

We found GDF-15 levels ranging from approximately 5 ng/ml in plasma from healthy fish to 1000 ng/ml in plasma from trout infected with *Flavobacterium Psychrophilum* and *Yersinia Ruckeri*. Although optimal dilutions should be determined empirically, we recommend that samples be evaluated at a 4-fold dilution. A four-fold dilution can be achieved by mixing 37.5 μ l of sample with 112.5 μ l of SB50-1 (do not substitute other dilution buffers). To avoid matrix effects, do not test samples at dilutions less than four-fold.

Ideally, dilutions should be performed in polystyrene 96-well plates (not provided). This allows quick and easy transfer of diluted samples to the antibody-coated plate using 8 or 12-channel multi-pipettors.

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 μ l of standards and samples into the wells.
3. Incubate on a plate shaker at 150 rpm and 25°C for one hour.
4. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 μ l/well).
5. Dispense 100 μ l 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 5x with 1x wash solution using a plate washer (400 μ l/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 μ l 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 μ l 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 nm² with a plate reader within 5 minutes.

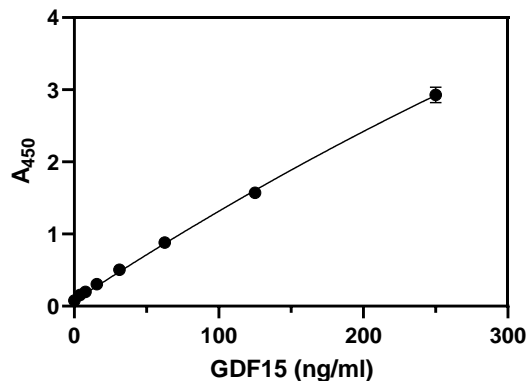
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the GDF-15 concentration. We suggest using a second order polynomial (quadratic) equation.
2. Derive the concentration of GDF-15 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.

TYPICAL STANDARD CURVE

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

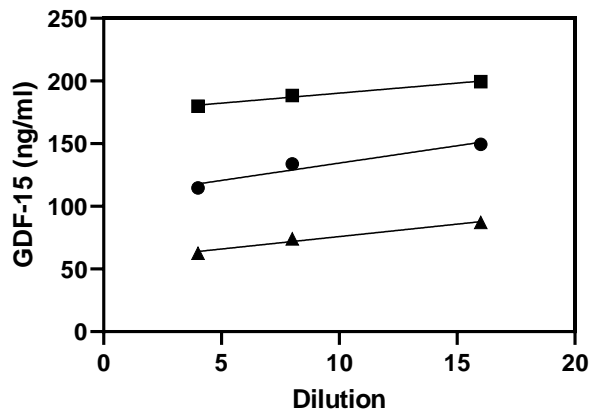
GDF-15 (ng/ml)	A ₄₅₀
250	2.929
125.00	1.570
62.50	0.882
31.25	0.504
15.63	0.304
7.81	0.199
3.91	0.156
0	0.080



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.

PERFORMANCE

Linearity: To assess the linearity of the assay, three rainbow trout plasma samples with GDF-15 concentrations of 189, 137, and 75 ng/ml were serially diluted to produce values within the dynamic range of the assay.



REFERENCES

1. Marancick D, et al. Whole-body transcriptome of selectively bred, resistant-, control-, and susceptible-line rainbow trout following experimental challenge with *Flavobacterium psychrophilum*. <https://www.frontiersin.org/articles/10.3389/fgene.2014.00453>
2. Pereiro P, et al. Conserved function of zebrafish (*Danio rerio*) Gdf15 as a sepsis tolerance mediator. *Developmental & Comparative Immunology*. 109 (2020) 10398. <https://doi.org/10.1016/j.dci.2020.103698>
3. Blanco MB, et al. Growth differentiation factor 15 (GDF-15) is a novel orexigen in fish. *Molecular and cellular Immunology* 50 (2020) 110720

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For technical assistance please email us at info@lifediagnosics.com