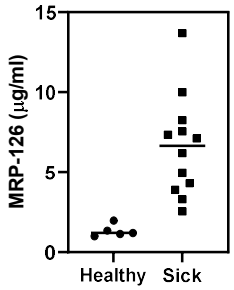
INTRODUCTION

MRP-126 (S100-A12) belongs to the calgranulin protein family. It is a homodimer that calcium dependently sequesters zinc, thereby inhibiting bacterial growth. Proteomic studies indicate that levels increase in plasma of health-compromised turtles (ref 1). Using Loggerhead turtle samples kindly provided by the Loggerhead Marinelife Center (Juno Beach, FL)[1], we found that plasma MRP-126 levels increase from ~1.3 μg/ml in healthy turtles up to 14 μg/ml in turtles with chronic debilitation.



PRINCIPLE OF THE ASSAY

The assay uses a polyclonal antibody for green turtle MRP-126. Unconjugated antibody is coated on wells of a microtiter plate and used for capture. Horseradish peroxidase (HRP) conjugated antibody is used for detection. Standards and diluted samples (100 μl) are incubated in the antibody coated wells for 45-minutes. After washing the wells, HRP-conjugate (100 μl) is added and incubated for 45 minutes. If MRP-126 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 MRP-126 is present, a blue color develops. Color development is stopped by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of MRP-126 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-MRP-126 coated plate (12 x 8-well strips)
- HRP conjugate, 11 ml
- MRP-126 stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD50-1, 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 standard vial 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 plate[2]. 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.

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.

[1] Samples were collected under Florida Fish and Wildlife Marine Turtle Permit 086.

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

DILUENT

The diluent is formulated for measurement of MRP-126 in turtle serum or plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

The stock is comprised of purified turtle MRP in a stabilizing matrix. It is provided in lyophilized form.

1. Reconstitute the stock with deionized water and prepare the 12.5 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 12.5 ng/ml MRP-126 standard into the tube labeled 6.25 ng/ml and mix. This provides the 6.25 ng/ml MRP-126 standard.
4. Similarly prepare the 3.125 – 0.195 ng/ml standards by two-fold serial dilution.

The reconstituted stock is stable for at least one day if stored in a sealed vial at room temperature.

HRP CONJUGATE

The HRP conjugate is provided ready to use. Use 100 µl per well.

SAMPLES

In studies at Life Diagnostics, we found MRP-126 levels ranging from ~1.3 to ~14 µg/ml in plasma. Optimal dilutions should be determined empirically. However, we suggest that samples initially be tested at a dilution of 2000-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. A 2000-fold dilution can be achieved as follows.

1. For each sample to be tested dispense 196 µl and 243.75 µl into separate tubes/wells.
2. Mix 4.0 µl of serum or plasma with 196 µl of diluent in the first tube/well. This gives a 50-fold dilution.
3. Mix 6.25 µl of the 50-fold diluted sample with 243.75 µl of diluent in the second tube/well. This provides a 2000-fold dilution.

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 the wells.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
4. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 µl/well).
5. Dispense 100 µl of HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
8. Dispense 100 µl of TMB into each well.
9. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
10. After 20-minutes, stop the reaction by adding 100 µl of Stop solution to each well.
11. Gently mix. It is important to make sure that all the blue color changes to yellow.
12. Read absorbance at 450 nm[3] with a plate reader within 5 minutes.

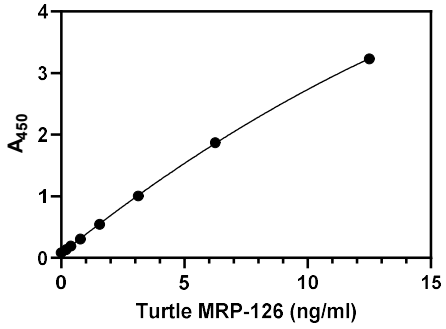
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the MRP-126 concentration.
2. Fit the standard curve using graphing software. We suggest using a second order polynomial (quadratic) equation.
3. Derive the concentration of MRP-126 in the samples.
4. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
5. 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.

MRP-126 (ng/ml)	A_{450}
12.5	3.232
6.25	1.871
3.125	1.006
1.563	0.545
0.781	0.304
0.391	0.190
0.195	0.135
0	0.086



[3] 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.

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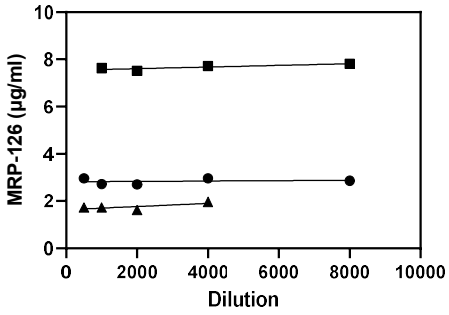
PERFORMANCE

Linearity: To assess the linearity of the assay, turtle plasma samples with MRP-126 concentrations of 7.67, 2.84, and 1.76 μg/ml were serially diluted to produce values within the dynamic range of the assay.



REFERENCES

1. Marancik DP, Perrault JR, Komoroske LM, Stoll JA, Kelley KN and Manire CA. Plasma proteomics of green turtles (Chelonia mydas) reveals pathway shifts and potential biomarker candidates associated with health and disease. Conservation Physiology, Volume 9, Issue 1, 2021, coab018, https://doi.org/10.1093/conphys/coab018

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