MOUSE ALBUMIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 1000-1

ELISA for the Quantitative Determination of Mouse Albumin in Serum and Plasma

INTRODUCTION
Albumin is the most abundant protein found in serum (~ 40 mg/ml). It has a molecular weight of ~65,000 and plays a vital role in regulating the intravascular colloid osmotic pressure. It also serves as a carrier protein for steroid hormones and fatty acids. It is classified as a negative acute phase reactant because its serum levels can decrease by approximately 30% in response to disease, tissue injury, or inflammation (1-3).

PRINCIPLE OF THE TEST
The mouse albumin test kit is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-albumin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-albumin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in albumin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies, and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of albumin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS
Materials provided with the kit:
- Anti-mouse albumin antibody coated 96-well microtiter plate (provided as 12 detachable strips of 8)
- Enzyme conjugate reagent, 11 ml
- Reference standard (lyophilized)
- 10x Diluent, 50 ml
- 20x Wash solution, 50 ml
- TMB reagent (One-Step), 11 ml
- Stop solution (1N HCl), 11 ml

Materials required but not provided:
- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT
The unused kit should be stored at 2-8°C, and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS
All reagents should be allowed to reach room temperature (18- 25°C) before use.

DILUENT PREPARATION
The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION
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.

STANDARD PREPARATION
1. The mouse albumin standard is provided as a lyophilized stock. Add the volume of distilled or deionized water indicated on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable for at least one week at 2-8°C, but should be aliquoted and frozen at -20°C if use beyond this time is intended)***.
2. Label 7 polypropylene or glass tubes as 200, 100, 50, 25, 12.5, 6.25, and 3.13 ng/ml.
3. Into the tube labeled 200 ng/ml, pipette the volume of diluent detailed on the reference standard vial label. Then add the indicated volume of reconstituted standard and mix gently. This provides the 200 ng/ml standard.
4. Dispense 250 μl of diluent into the tubes labeled 100, 50, 25, 12.5, 6.25, and 3.13 ng/ml.
5. Pipette 250 μl of the 200 ng/ml albumin standard into the tube labeled 100 ng/ml and mix. This provides the working 100 ng/ml standard. Similarly prepare the 50, 25, 12.5, 6.25, and 3.13 ng/ml standards by serial dilution.

SAMPLE PREPARATION
Serum levels of albumin are ~ 40 mg/ml. Serum and plasma samples must therefore be significantly diluted prior to assay. We suggest an initial dilution of 800,000. This is best achieved by serial dilution:
1. Dispense 995 μl of 1 x diluent into two tubes and 475 μl into a third tube for each sample to be tested.
2. Dilute 5 μl of sample into one of the tubes containing 995 μl of diluent and mix gently. This provides a 200-fold diluted sample.
3. Dilute 5 μl of the 200-fold diluted sample into the second tube containing 995 μl of diluent and mix gently. This provides a 40,000-fold diluted sample.

4. Dilute 25 µl of the 40,000-fold diluted sample into the tube containing 475 µl of diluent and mix gently. This provides an 800,000-fold diluted sample.

Repeat the above procedure for each sample.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Aspirate the contents of the microtiter wells and wash the wells 5 times with 1x wash solution. This may be performed using either a plate washer (400 µl/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 µl of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
8. Wash as detailed in steps 4 to 5 above.
9. Dispense 100 µl of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
11. 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 the optical density at 450 nm with a microtiter plate reader within 5 minutes.

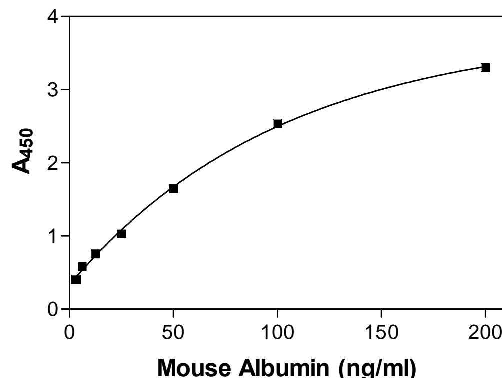
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of albumin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of albumin in the serum sample.
5. If available, PC graphing software may be used for the above steps.
6. If the A_{450} values of samples fall outside the standard curve samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against albumin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Albumin (ng/ml)	Absorbance (450 nm)
200.0	3.091
100.0	2.381
50.0	1.532
25.0	1.027
12.5	0.634
6.25	0.473
3.13	0.389



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. Aldred AR and Schreiber G. The negative acute phase proteins. pp 21-37. Acute Phase Proteins: Molecular Biology, Biochemistry and Clinical Applications. Eds. Mackiewicz A, Kushner I, and Baumann H. CRC Press (1993)
2. Eberini I, et.al., Proteins of rat serum IV. Time-course of acute-phase protein expression and its modulation by indomethacine. Electrophoresis 20: 846-853 (1999)
3. Whalen R, Voss SH and Boyer TD. Decreased expression levels of rat liver glutathione S-transferase A2 and albumin during the acute phase response are mediated by HNF1 (hepatic nuclear factor 1) and IL6DEX-NP. Biochem J. 377:763-768 (2004)

CHICKEN -1-ACID GLYCOPROTEIN (-1-AGP) ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2510-3

Enzyme Immunoassay for the Quantitative Determination of Chicken -1-Acid Glycoprotein Protein (-1-AGP)

INTRODUCTION

-1-AGP is an acute phase protein that is elevated in chicken serum as a result of injury, infection or disease. Studies have demonstrated that -1-AGP levels may increase five fold or more in chickens during the acute phase response[1-2]. As such, -1-AGP is a useful marker of injury and infection.

PRINCIPLE OF THE TEST

The chicken -1-AGP ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-chicken -1-AGP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-chicken -1-AGP antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in -1-AGP molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of -1-AGP is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-chicken -1-AGP antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized)
- 10x Diluent (25 ml)
- 20x Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips.
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer.
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The unused kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18- 25°C) before use.
2. Serum or plasma samples should be diluted ~10,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The chicken -1-AGP standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable for at least 7 days at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 8 polypropylene or glass tubes as 150, 75, 37.5, 18.8, 9.4, 4.7, 2.3 and 0 ng/ml
3. In the tube labeled 150 ng/ml, prepare the 150 ng/ml working standard as described on the label of the reconstituted reference standard vial.
4. Dispense 250 l of diluent into the remaining tubes.
5. Prepare the 75 ng/ml standard by diluting and mixing 250 l of the 150 ng/ml standard with 250 l of diluent in the tube labeled 75 ng/ml.
6. Similarly prepare the 37.5, 18.8, 9.4, 4.7, and 2.3 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Our studies find that -1-AGP may be present in chicken serum at concentrations of 0.2 to 1 mg/ml or greater. In order to obtain values within the range of the standard curve we suggest that samples initially be diluted 10,000 fold using the following procedure for each sample to be tested:

1. Dispense 495 l of 1x diluent into two tubes.
2. Pipette and mix 5 l of the serum/plasma sample into the first tube containing 495 l of diluent. This provides a 100 fold diluted sample.

3. Mix 5 l of the 100 fold diluted sample with the 495 l of diluent in the second tube. This provides a 10,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture using a plate washer or by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 and 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

CALCULATION OF RESULTS

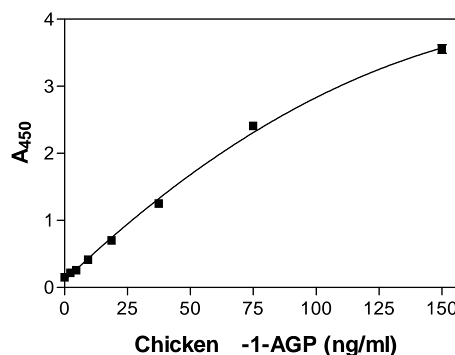
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of -1-AGP in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of -1-AGP in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 10,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against -1-AGP concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

-1-AGP (ng/ml)	Absorbance (450 nm)
150	3.555
75	2.428
37.5	1.250
18.8	0.701
9.4	0.416
4.7	0.260
2.3	0.218
0	0.150

Typical Chicken -1-AGP Standard Curve



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1. Holt PS and Gast RK, Comparison of the effects of infection with Salmonella enteritidis, in combination with an induced molt, on serum levels of the acute phase protein, 1 acid glycoprotein, in hens. Poultry Science 81:1295-1300 (2002)
2. Takimoto T, et al. Role of Chicken TL1A on inflammatory responses and partial characterization of its receptor. Journal of Immunology. 180:8327-8332 (2008)

RAT -1-ACID GLYCOPROTEIN (-1-AGP) ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2510-2

Enzyme Immunoassay for the Quantitative Determination of Rat -1-Acid Glycoprotein Protein (-1-AGP) in Serum

INTRODUCTION

-1-AGP is an acute phase protein that is elevated in serum as a result of injury, infection or disease. Studies performed at Life Diagnostics Inc., have demonstrated a ten fold increase in -1-AGP in rats with chronic disease.

PRINCIPLE OF THE TEST

The rat -1-AGP ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat -1-AGP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat -1-AGP antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in -1-AGP molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of -1-AGP is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

Anti-rat -1-AGP antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
Enzyme Conjugate Reagent, 11 ml
Reference standard (lyophilized), containing 2 g/ml rat -1-AGP when reconstituted as detailed on the vial label
10x Diluent (25 ml)
20x Wash Solution (50 ml)
TMB Reagent (One-Step) 11 ml
Stop Solution (1N HCl), 11 ml

Materials required but not provided:

Precision pipettes and tips.
Distilled or deionized water
Polypropylene or glass tubes
Vortex mixer.
Absorbent paper or paper towels
Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The unused kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18- 25°C) before use.
2. Serum or plasma samples should be diluted ~50,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The rat -1-AGP standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 g/ml rat -1-AGP stock ***(the reconstituted standard remains stable for at least 10 days at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 8 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0 ng/ml
3. Dispense 950 l of diluent into the tube labeled 100 ng/ml and 300 l of diluent into the remaining tubes.
4. Pipette 50 l of the 2 g/ml -1-AGP standard into the tube labeled 100 ng/ml and mix. This provides the working 100 ng/ml -1-AGP standard.
5. Prepare a 50 ng/ml standard by diluting and mixing 300 l of the 100 ng/ml standard with 300 l of diluent in the tube labeled 50 ng/ml. Similarly prepare the 25, 12.5, 6.25, 3.125, and 1.56 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Our studies find that -1-AGP may be present in rat serum at concentrations of 0.05 to 1 mg/ml or greater. In order to obtain values within the range of the standard curve we suggest that samples initially be diluted 50,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 l and 495 l of 1x diluent into separate tubes.
2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 998 l of diluent. This provides a 500 fold diluted sample.

3. Mix 5 l of the 500 fold diluted sample with the 495 l of diluent in the second tube. This provides a 50,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (350 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 and 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

CALCULATION OF RESULTS

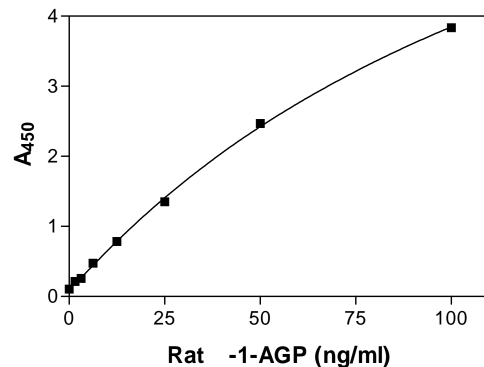
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of -1-AGP in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of -1-AGP in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 50,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against -1-AGP concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

-1-AGP (ng/ml)	Absorbance (450 nm)
100	3.836
50	2.467
25	1.351
12.5	0.786
6.25	0.474
3.13	0.258
1.56	0.214
0	0.106

Typical Rat -1-AGP Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

RAT ALBUMIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 1000-2

ELISA for the Quantitative Determination of Rat Albumin in Serum and Plasma

INTRODUCTION

Albumin is the most abundant protein found in serum (~ 40 mg/ml). It has a molecular weight of ~65,000 and plays a vital role in regulating the intravascular colloid osmotic pressure. It also serves as a carrier protein for steroid hormones and fatty acids. It is classified as a negative acute phase reactant because its serum levels can decrease by approximately 30% in response to disease, tissue injury, or inflammation (1-3).

PRINCIPLE OF THE TEST

The rat albumin test kit is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat albumin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat albumin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in albumin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies, and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of albumin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat albumin antibody coated 96-well microtiter plate (provided as 12 detachable strips of 8)
- Enzyme conjugate reagent, 11 ml
- Reference standard (lyophilized)
- 10x Diluent, 50 ml
- 20x Wash solution, 50 ml
- TMB reagent (One-Step), 11 ml
- Stop solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT

The unused kit should be stored at 2-8°C, and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18- 25°C) before use.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The rat albumin standard is provided as a lyophilized stock. Add the volume of distilled or deionized water indicated on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable for at least one week at 2-8°C, but should be aliquoted and frozen at -20°C if use beyond this time is intended)***.
2. Label 8 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 ng/ml.
3. Into the tube labeled 100 ng/ml, pipette the volume of diluent detailed on the reference standard vial label. Then add the indicated volume of reconstituted standard and mix gently. This provides the 100 ng/ml standard.
4. Dispense 250 µl of diluent into the tubes labeled 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 ng/ml.
5. Pipette 250 µl of the 100 ng/ml albumin standard into the tube labeled 50 ng/ml and mix. This provides the working 50 ng/ml standard. Similarly prepare the 25, 12.5, 6.25, 3.13, and 1.56 ng/ml standards by serial dilution.

SAMPLE PREPARATION

Serum levels of albumin are ~ 40 mg/ml. Serum and plasma samples must therefore be significantly diluted prior to assay. We suggest an initial dilution of 800,000. This is best achieved by serial dilution:

1. Dispense 995 µl of 1 x diluent into two tubes and 475 µl into a third tube.
2. Dilute 5 µl of sample into one of the tubes containing 995 µl of diluent and mix gently. This provides a 200-fold diluted sample.
3. Dilute 5 µl of the 200-fold diluted sample into the second tube containing 995 µl of diluent and mix gently. This provides a 40,000-fold diluted sample.

4. Dilute 25 µl of the 40,000-fold diluted sample into the tube containing 475 µl of diluent and mix gently. This provides an 800,000-fold diluted sample.

Repeat the above procedure for each sample.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Aspirate the contents of the microtiter wells and wash the wells 5 times with 1x wash solution. This may be performed using either a plate washer (400 µl/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 µl of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
8. Wash as detailed in steps 4 to 5 above.
9. Dispense 100 µl of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
11. 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 the optical density at 450 nm with a microtiter plate reader within 5 minutes.

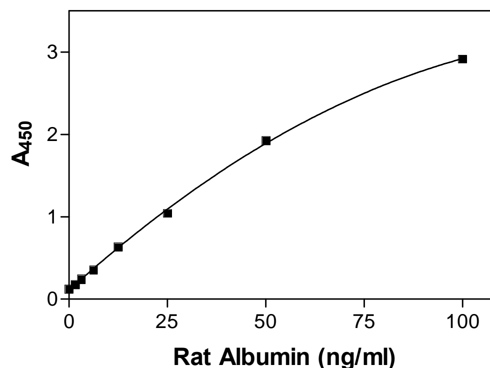
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of albumin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of albumin in the serum sample.
5. If available, PC graphing software may be used for the above steps.
6. If the A_{450} values of samples fall outside the standard curve samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against albumin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Albumin (ng/ml)	Absorbance (450 nm)
100	2.915
50	1.924
25	1.042
12.5	0.638
6.25	0.355
3.13	0.244
1.56	0.176
0	0.123



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. Aldred AR and Schreiber G. The negative acute phase proteins. pp 21-37. Acute Phase Proteins: Molecular Biology, Biochemistry and Clinical Applications. Eds. Mackiewicz A, Kushner I, and Baumann H. CRC Press (1993)
2. Eberini I, et.al., Proteins of rat serum IV. Time-course of acute-phase protein expression and its modulation by indomethacine. Electrophoresis 20: 846-853 (1999)
3. Whalen R, Voss SH and Boyer TD. Decreased expression levels of rat liver glutathione S-transferase A2 and albumin during the acute phase response are mediated by HNF1 (hepatic nuclear factor 1) and IL6DEX-NP. Biochem J. 377:763-768 (2004)

MONKEY -1-ACID GLYCOPROTEIN (-1-AGP) ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2510-4

Enzyme Immunoassay for the Quantitative Determination of Monkey -1-Acid Glycoprotein Protein (-1-AGP)

INTRODUCTION

-1-AGP is an acute phase serum protein. Studies at Life Diagnostics, Inc., have demonstrated that levels of -1-AGP are elevated 5-10 fold in serum of monkeys undergoing veterinary treatment. -1-AGP is a useful biomarker of tissue injury, inflammation and infection in monkeys.

PRINCIPLE OF THE TEST

The monkey -1-AGP ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-monkey -1-AGP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-monkey -1-AGP antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in -1-AGP molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of -1-AGP is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-monkey -1-AGP antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized)[1]
- 10x Diluent (25 ml)
- 20x Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips.
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer.
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
- Graph paper (PC graphing software is optional)

[1] Due to international import/export restrictions of monkey derived products, the -1-AGP standard supplied with this kit is of non monkey origin. The standard curve obtained with this material is identical to that obtained with monkey -1-AGP.

STORAGE OF TEST KIT AND INSTRUMENTATION

The unused kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18- 25°C) before use.
2. Serum or plasma samples should be diluted ~20,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The monkey -1-AGP standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable for at least 7 days at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 6 polypropylene or glass tubes as 100, 50, 25, 12.5, 6.25, and 3.13 ng/ml
3. In the tube labeled 100 ng/ml, prepare the 100 ng/ml working standard as described on the label of the reconstituted reference standard vial.
4. Dispense 250 l of diluent into the remaining tubes.
5. Prepare the 50 ng/ml standard by diluting and mixing 250 l of the 100 ng/ml standard with 250 l of diluent in the tube labeled 50 ng/ml.
6. Similarly prepare the 25, 12.5, 6.25 and 3.13 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Our studies find that -1-AGP is present in monkey serum at concentrations of 0.2 to 2 mg/ml. In order to obtain values within the range of the standard curve we suggest that samples initially be diluted 20,000 fold using the following procedure for each sample to be tested:

1. Dispense 198 l and 497.5 l of 1x diluent into two tubes.
2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 198 l of diluent. This provides a 100 fold diluted sample.

3. Mix 2.5 l of the 100 fold diluted sample with the 497.5 l of diluent in the second tube. This provides a 20,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture using a plate washer or by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 and 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

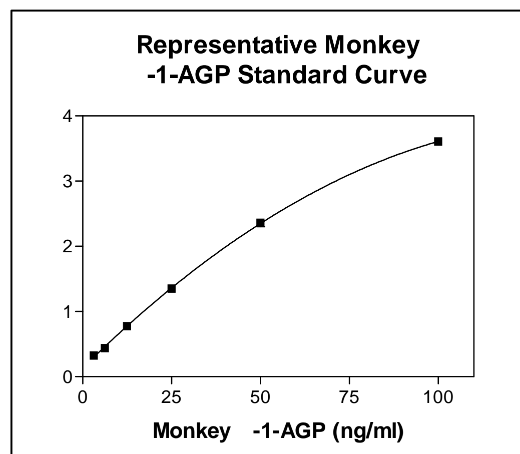
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of -1-AGP in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of -1-AGP in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at the suggested dilution of 20,000 samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against -1-AGP concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

-1-AGP (ng/ml)	Absorbance (450 nm)
100	3.608
50	2.360
25	1.353
12.5	0.774
6.25	0.439
3.13	0.326



RAT CERULOPLASMIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2610-2

Enzyme Immunoassay for the Quantitative Determination of Rat Ceruloplasmin in Serum or Plasma

INTRODUCTION

Ceruloplasmin is an acute phase protein that is elevated in serum as a result of injury, infection or disease. Studies at Life Diagnostics, Inc., indicate that ceruloplasmin levels increase 10-20 fold during chronic disease in rats. Measurement of ceruloplasmin provides a convenient marker of inflammation and disease.

PRINCIPLE OF THE TEST

The rat ceruloplasmin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat ceruloplasmin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat ceruloplasmin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in ceruloplasmin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of ceruloplasmin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat ceruloplasmin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Ceruloplasmin stock (lyophilized)
- 10x Diluent (25 ml)
- 20x Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips.
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer.
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-4
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~10,000 fold with diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The rat ceruloplasmin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 7.5 g/ml rat ceruloplasmin stock. *(the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if further use is intended).*
2. Label 8 polypropylene or glass tubes as 300, 150, 75, 37.5, 18.8, 9.4, 4.7 and 0 ng/ml.
3. Dispense 960 l of diluent into the tube labeled 300 ng/ml and 500 l of diluent into the tubes labeled 150, 75, 37.5, 18.8, 9.4, 4.7 and 0 ng/ml.
4. Prepare a 300 ng/ml standard by diluting and mixing 40 l of the reconstituted 7.5 g/ml standard with 960 l of diluent in the appropriately labeled tube.
5. Prepare a 150 ng/ml standard by diluting and mixing 500 l of the 300 ng/ml standard with 500 l of diluent in the tube labeled 150 ng/ml. Similarly prepare the 75, 37.5, 18.8, 9.4, 4.7 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Studies at Life Diagnostics, Inc., indicate that ceruloplasmin is present in normal rat serum at a concentration of ~ 0.5 mg/ml. In order to obtain values within the range of the standard curve we suggest that samples initially be diluted 10,000 fold using the following procedure for each sample to be tested:

1. Dispense 198 l and 297 l of diluent into separate tubes.

2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 198 l of diluent. This provides a 100 fold diluted sample.
3. Mix 3 l of the 100 fold diluted sample with the 297 l of diluent in the second tube. This provides a 10,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 l of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
8. Wash as detailed in 4 above.
9. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
10. Dispense 100 l of TMB Reagent into each well.
11. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
12. Stop the reaction by adding 100 l of Stop Solution to each well.
13. Gently mix. It is important to make sure that all the blue color changes to yellow.
14. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

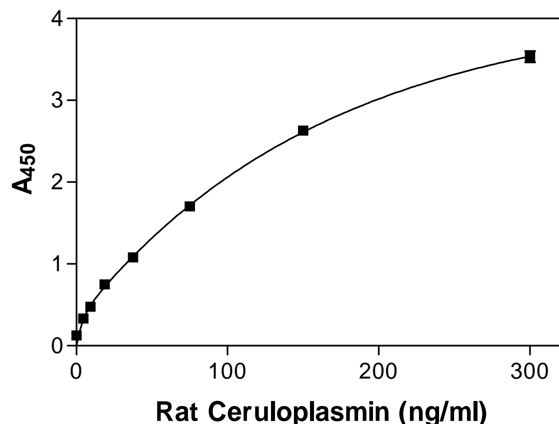
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of ceruloplasmin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of ceruloplasmin in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 10,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against ceruloplasmin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Ceruloplasmin (ng/ml)	Absorbance (450 nm)
300	3.533
150	2.627
75	1.702
37.5	1.080
18.8	0.748
9.4	0.474
4.7	0.331
0	0.124

Representative Rat Ceruloplasmin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

PIG C-REACTIVE PROTEIN (CRP) ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2210-6

Enzyme Immunoassay for the Quantitative Determination of Pig C-Reactive Protein (CRP) in Serum

INTRODUCTION

CRP is an acute phase protein that is elevated in serum as a result of injury, infection or disease. Baseline levels of CRP in pigs range from 5-30 g/ml. Levels may increase 10-25 fold during the acute phase response[1]. Measurement of CRP therefore provides a convenient marker of inflammation and disease.

PRINCIPLE OF THE TEST

The pig CRP ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses anti-pig CRP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-pig CRP antibodies for detection. The test sample is diluted and incubated in microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. CRP molecules are thereby sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of CRP is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-pig CRP coated 96-well plate (12 x 8-well strips)
- Enzyme Conjugate Reagent, 11 ml
- Pig CRP standard (lyophilized, 3 vials)
- 10x Pig CRP Diluent (25 ml)
- 20x CRP Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips.
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer.
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength with an optical density range of 0-4 OD
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT

The lyophilized standards must be stored at or below -20°C when the ELISA kit is received. The remainder of the kit should be stored at 2-8 C and should not be frozen. The microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~500 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water (the diluted diluent is referred to in the following text as 1x diluent).

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. Add 1 ml of 1x diluent to one of the pig CRP standard vials and mix gently until dissolved. Use the standard within 4 hours of reconstitution and discard after use.
2. Label 6 polypropylene or glass tubes: 150, 75, 37.5, 18.75, 9.38 and 4.67 ng/ml.
3. Prepare a 150 ng/ml working CRP standard as detailed on the standard vial label, by mixing the indicated volume of diluent and reconstituted standard in the tube labeled 150 ng/ml.
4. Dispense 250 l of diluent into the tubes labeled 75, 37.5, 18.75, 9.38 and 4.67 ng/ml.
5. Prepare a 75 ng/ml standard by diluting and mixing 250 l of the 150 ng/ml standard with 250 l of diluent in the tube labeled 75 ng/ml. Similarly prepare the 37.5, 18.75, 9.38 and 4.67 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: CRP is present in normal pig serum at concentrations of 5-30 g/ml and levels can increase 10 fold during infection. In order to obtain values within the range of the standard curve we <u>suggest</u> that samples initially be diluted 500 fold using the following procedure for each sample to be tested:

1. Dispense 998 l of 1x diluent into a polypropylene or glass tube.

2. Pipette and mix 2 l of the serum/plasma sample with the 998 l of 1x diluent. This provides a 500 fold diluted sample.
3. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that standards and samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture using a plate washer and wash the microtiter wells 5 times with 1x wash solution. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 l of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 45 minutes.
8. Wash as detailed in 4 above.
9. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
10. Dispense 100 l of TMB Reagent into each well.
11. Gently mix on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 20 minutes.
12. Stop the reaction by adding 100 l of Stop Solution to each well.
13. Gently mix. It is important to make sure that all the blue color changes to yellow.
14. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

CALCULATION OF RESULTS

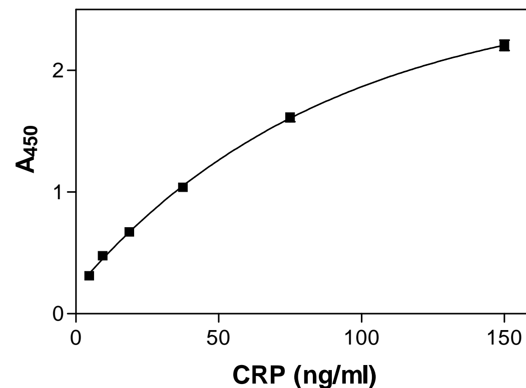
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of CRP in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of CRP in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 500, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against CRP concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

CRP (ng/ml)	Absorbance (450 nm)
150	2.206
75	1.615
37.5	1.038
18.75	0.671
9.38	0.477
4.67	0.312

Representative Pig CRP Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and adherence to good laboratory practice.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

ADDITIONAL INFORMATION

The concentration of pig CRP in the lyophilized standards provided with the kit was determined by reference to pig CRP prepared at Life Diagnostics Inc.

REFERENCES

1. Martinez-Subiela S, et al. A time-resolved immunofluorometric assay for porcine C-reactive protein in whole blood. Luminescence. 22:171-176 (2007)

RABBIT C-REACTIVE PROTEIN (CRP) ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2210-5

Enzyme Immunoassay for the Quantitative Determination of Rabbit C-Reactive Protein (CRP) in Serum

INTRODUCTION

CRP is an acute phase protein in rabbits that is elevated in serum as a result of injury, infection or disease. It is reported that CRP levels can increase several hundred fold in rabbit serum during the acute phase response[1]. Measurement of CRP therefore provides a convenient marker of inflammation and disease.

PRINCIPLE OF THE TEST

The rabbit CRP ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rabbit CRP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rabbit CRP antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in CRP molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of CRP is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

Anti-rabbit CRP antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
Enzyme Conjugate Reagent, 11 ml
Reference standard (200 l, lyophilized), containing rabbit CRP (concentration and dilution instructions are listed on the vial label). **Store at -20°C**.
10x Diluent (25 ml)
20x Wash Solution (50 ml)
TMB Reagent (One-Step) 11 ml
Stop Solution (1N HCl), 11 ml

Materials required but not provided:

Precision pipettes and tips.
Polypropylene tubes
Vortex mixer.
Absorbent paper or paper towels
Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
A microtiter plate reader capable of measuring absorbance at 450 nm.
Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT

1. **The lyophilized reference standard should be stored at minus 20°C for optimum stability** (it can be safely shipped at 2-8°C).
2. The remainder of the kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air.
3. Test kits will remain stable for six months from the date of purchase, provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum samples should be diluted ~1000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

The wash solution is provided as a 20x stock. Prior to use dilute the contents of the bottle (50 ml) with 950 ml of of distilled or deionized water.

STANDARD PREPARATION

The rabbit CRP standard is comprised of lyophilized rabbit serum of known CRP concentration. The CRP content was determined by reference to purified rabbit CRP prepared at Life Diagnostics Inc.

1. Reconstitute the lyophilized rabbit CRP reference standard by addition of 200 l of de-ionized or distilled water. Mix gently several times over a period of 5-10 minutes. The concentration of CRP in the reconstituted stock is indicated on the vial label.
2. Label 8 polypropylene tubes as 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml
3. Into the tube labeled 250 ng/ml, pipette the volume of diluent detailed on the CRP reference standard vial label. Then add the indicated volume CRP standard (shown on the vial label) and mix gently. This provides the 250 ng/ml standard.
4. Dispense 250 l of 1x diluent into the tubes labeled 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml
5. Pipette 250 l of the 250 ng/ml CRP standard into the tube labeled 125 ng/ml and mix. This provides the working 125 ng/ml CRP standard.
6. Prepare a 62.5 ng/ml standard by diluting and mixing 250 l of the 125 ng/ml standard with 250 l of diluent in the tube labeled 62.5 ng/ml. Similarly prepare the 31.25, 15.6, 7.8 and 3.9 ng/ml standards by serial dilution.

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610-431-7707 – 610-431-7818 (Fax)
info@lifediagnostics.com – www.lifediagnostics.com

Please Note: The unused reconstituted reference standard should be aliquoted and stored frozen at or below -20°C (within 1 hour of reconstitution) if future use is intended.

SAMPLE PREPARATION

CRP is present in rabbit serum at concentrations ranging from less than 100 ng/ml to several hundred g/ml. In order to identify the optimum dilution for a particular sample set, we suggest that a limited number of samples be tested as singlets at a dilution of 1000 fold side by side with the 250 and 0 ng/ml standards. Based on these results, an appropriate dilution factor for the remaining samples might be estimated. A 1000 fold dilution of serum samples may be obtained as follows:

1. Using a precision micropipette, pipette and mix 1.0 l of the serum sample into a tube containing 999 l of 1x diluent. This provides a 1000 fold diluted sample.
2. Repeat this procedure for each sample to be tested.

Please dilute serum samples a minimum of 10 fold.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking the plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with wash solution. This may performed using either a plate washer or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash solution.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

CALCULATION OF RESULTS

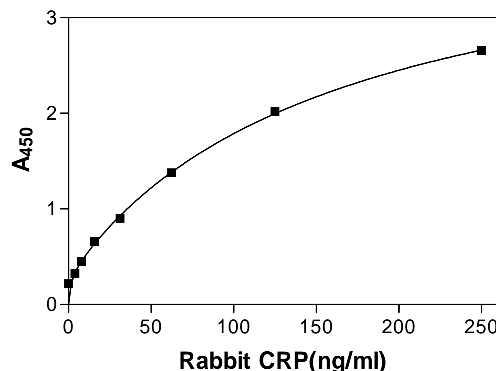
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of CRP in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of CRP in the serum sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of diluted samples fall outside the standard curve when tested at a dilution of 1000. Samples should be rediluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against CRP concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

CRP (ng/ml)	Absorbance (450 nm)
0	0.217
3.91	0.324
7.81	0.451
15.63	0.658
31.25	0.900
62.5	1.377
125	2.019
250	2.628

Typical Rabbit CRP Standard Curve



REFERENCES

1. Kushner I and Feldman G. Control of the acute phase response: Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit. J. Exp. Med. 148:466-477 (1978)

MONKEY C-REACTIVE PROTEIN (CRP) ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2210-4

Enzyme Immunoassay for the Quantitative Determination of Monkey C-Reactive Protein (CRP) in Serum

FOR RESEARCH USE ONLY

PLEASE READ ATTACHED MSDS BEFORE USING THE KIT

INTRODUCTION

CRP is an acute phase protein in monkeys that is elevated in serum as a result of injury, infection or disease. Normal levels of CRP range from 0-8.3 g/ml[1,2] and levels may increase one hundred fold or more during the acute phase response[1-4].

PRINCIPLE OF THE TEST

The monkey CRP ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-monkey CRP antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-monkey CRP antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in CRP molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of CRP is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-monkey CRP antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard containing monkey CRP (concentration and dilution instructions are listed on the vial label)
  <u>Store at -20°C</u>.
- 10x Diluent (25 ml)
- 20x Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips.
- Distilled or deionized water
- Polypropylene tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader capable of measuring absorbance at 450 nm
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT

The lyophilized reference standard should be stored at or below -20°C for optimum stability (it can be safely shipped at 2-8°C). The remainder of the kit should be stored at 2-8°C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

General Instructions

All reagents should be allowed to reach room temperature (18-25°C) before use.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION (PLEASE READ ATTACHED MSDS ON PAGE 4 FOR BIOHAZARD INFORMATION)

The monkey CRP standard is comprised of lyophilized Cynomolgus monkey serum of known CRP concentration. The CRP content was determined by reference to purified Cynomolgus monkey CRP prepared at Life Diagnostics Inc.

1. Reconstitute the lyophilized monkey CRP reference standard to a concentration of 2 g/ml by adding the volume of de-ionized or distilled water indicated on the vial label.
2. Label 8 polypropylene tubes as 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17 and 0 ng/ml.
3. Into the tube labeled 75 ng/ml, pipette the volume of 1x diluent detailed on the CRP reference standard vial label. Then add the indicated volume of CRP standard (shown on the vial label) and mix gently. This provides the 75 ng/ml standard.
4. Dispense 250 l of 1x diluent into the tubes labeled 37.5, 18.75, 9.38, 4.69, 2.34, 1.17 and 0 ng/ml.
5. Pipette 250 l of the 75 ng/ml CRP standard into the tube labeled 37.5 ng/ml and mix. This provides the working 37.5 ng/ml CRP standard.

6. Prepare an 18.75 ng/ml standard by diluting and mixing 250 l of the 37.5 ng/ml standard with 250 l of diluent in the tube labeled 18.75 ng/ml. Similarly prepare the 9.38, 4.69, 2.34, 1.17 ng/ml standards by serial dilution.

Please Note: The unused reconstituted reference standard should be aliquoted and stored frozen at or below -20°C (within 1 hour of reconstitution) if future use is intended.

SAMPLE PREPARATION

General Note: We find that CRP is present in normal pooled monkey serum at a concentration of ~5 g/ml and in-house studies indicate that acute phase concentrations can exceed 150 g/ml. We suggest that samples initially be diluted 1000 fold using the following procedure for each sample to be tested:

1. Dispense 999 l of 1x diluent into one tube for each sample to be tested.
2. Pipette 1.0 l of the serum sample into the tube containing 999 l of 1x diluent using a precision micro pipettor and mix. This provides a 1000 fold diluted sample.
3. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking the plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash solution.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual wash solution.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

CALCULATION OF RESULTS

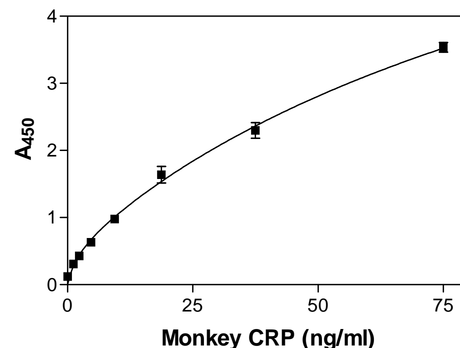
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of CRP in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of CRP in the serum sample.
5. If available, PC graphing software may be used for the above steps. We find that data usually fit well to a two site binding (hyperbola) equation.
6. If the OD_{450} values of samples fall outside, or at the extremes, of the standard curve when tested at a dilution of 1000, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against CRP concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

CRP (ng/ml)	Absorbance (450 nm)
75	3.538
37.5	2.299
18.75	1.638
9.38	0.978
4.69	0.633
2.34	0.430
1.17	0.309
0	0.122

Typical Monkey CRP Standard Curve



Warnings and Precautions

1. CAUTION: The lyophilized CRP standard contained in this kit was derived from Cynomolgus monkey serum. Please refer to the attached MSDS (page 4) prior to using the kit.
2. Avoid contact with 1N HCl. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. Do not pipette reagents by mouth
5. FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

References

1. Jinbo T, Hayashi S, Iguchi K, Shimuzu M, Matsumoto T, Naiki M, and Yamamoto S. Development of monkey C-Reactive protein method. Vet Immunol Immunopathol. 61:195-202 (1998)
2. Jinbo T, Ami Y, Suzaki Y, Kobune F, Ro S, Naiki M, Iguchi K, Yamamoto S. Concentrations of c-reactive protein in normal monkeys (Macac irus) and in monkeys inoculated with Bordella bronchiseptica R-5 and measles virus. Vet Res Commun. 23:265-74 (1999)
3. Hart BAT, Bank RA, De Roos JADM, Brok H, Jonker M, Theuns HM, Kakami J and Te Koppele JM. Colagen-induced arthritis in rhesus monkeys: evaluation of markers for inflammation and joint degradation. British J. Rheumatology 37:314-323 (1998)
4. Kingstrom J, Plyusnin A, Vaheri A and Lundkvist A. Wild-type Puumala Hanatavirus infection induces cytokines, C-reactive protein, creatinine, and nitric oxide in Cynomolgus Macaques. J. Virology. 76:444-449 (2002)

MATERIAL SAFETY DATA SHEET

PRODUCT: Monkey CRP Standard (component of kit 2210-4)

CUSTOMER INFORMATION

Please forward a copy of this abbreviated MSDS to your coordinator for review and filing. Please assure that this MSDS reaches the intended user of this material.

HAZARD INFORMATION

HANDLE THIS MATERIAL AND ITS DERIVATIVES AS A BIOHAZARD

Nonhuman primates can carry a variety of zoonotic diseases including B virus (*Cercopithecine Herpes Virus 1 or Herpesvirus simae)*, Measles, Influenza, Pox viruses (Monkeypox and Yaba virus), filoviruses such as Ebola virus, Gastrointestinal disease (*Salmonella, Shigella, Giardia, Entamoeba histolytica, Balantidium coli)*, Bacterial pneumonia (*Streptoccus pneumoniae*), and Tuberculosis (*Mycobacterium tuberculosis*). Zoonotic diseases are those that can be transmitted between species. It is important to note that a disease that does not cause serious health effects in one species may cause severe, life-threatening illness in another species.

Care must be taken by all personnel who handle this material to prevent potential exposure to zoonotic pathogens. Contact with this material may irritate the eyes, skin, or mucous membranes and potentially result in infection. In order to limit exposure, exercise all due caution and wear appropriate personal protective equipment when handling this material. Good laboratory and manufacturing procedures are essential for safe use. If eye exposure occurs, flush product from eyes with water for at least 15 minutes, see a physician. If skin exposure occurs, wash and scrub the exposed area thoroughly with soap, concentrated solution of detergent, povidone-iodine, or chlorhexidine and water, irrigate the area with running water for 15-20 minutes, see a physician.

FIRE AND SPILL INFORMATION

In case of fire use suitable extinguishing agent such as water, carbon dioxide, foam or dry chemical to suppress the surrounding fire. In case of spill collect material in a leak proof container and decontaminate the spilled material with a freshly made 1% bleach solution (a 1:5 dilution of household bleach) or similar disinfectant with virucidal properties, and dispose of according to Federal, State, and local regulations. Decontaminate the area of the spill with a freshly made 1% bleach solution (a 1:5 dilution of commercial bleach) or similar disinfectant with virucidal properties. Allow sufficient contact time (30 minutes) before final clean up of surfaces.

PERSONAL PROTECTIVE EQUIPMENT

Protective gloves, safety goggles, face shield, long sleeved lab coat or gown and access to a safety eyewash station are recommended. Protective clothing should be replaced if it is contaminated. Protective clothing should be removed on leaving the work area. Wash hands after removing gloves.

The information, data, and recommendations contained herein have been compiled from sources believed to be reliable and are believed to be accurate. Life Diagnostics, Inc. makes no warranty of any kind whatsoever with respect thereto and disclaims all liability from reliance thereon. This information is offered solely to you in advisement for the safe use and handling of this material. We reserve the right to revise this information periodically as new information becomes available.

RAT CLUSTERIN ELISA KIT
Life Diagnostics, Inc., Cat. No. 3300-2

ELISA FOR DETERMINATION OF RAT CLUSTERIN

STORAGE CONDITIONS

Store the kit at 2-8°C. Keep the microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

EXPIRATION

The kit expiration date (six months from the date of shipment) is indicated on the package label

BACKGROUND

Clusterin, also referred to as apolipoprotein J, sulfated glycoprotein-2, glycoprotein III and testosterone-repressed prostate message-2, is a glycoprotein of 70-80 kDa, composed of one -subunit and one -subunit, derived from proteolytic cleavage of a precursor peptide. Clusterin is expressed in many tissues and is found in serum, seminal fluid and urine. It has been identified as a potential biomarker of various forms of renal injury and prostate disease.

PRINCIPLE OF THE ASSAY

The clusterin ELISA uses two different affinity purified antibodies. One is used for solid phase immobilization (on the microtiter wells). The second is conjugated to horse radish peroxidase (HRP). The samples (diluted serum, plasma or urine) are mixed in the microtiter wells with HRP conjugated antibody and clusterin is sandwiched between the solid phase and HRP-conjugated antibodies. After incubation on a plate shaker for one hour at room temperature the wells are washed to remove unbound HRP-conjugated antibodies. A solution of tetramethylbenzidine (TMB), an HRP substrate, is then added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped by addition of 1N HCl changing the color to yellow. The concentration of clusterin is proportional to the absorbance at 450 nm.

REAGENTS AND MATERIALS PROVIDED

Anti clusterin-coated wells (1 plate, 96 wells)
Clusterin Stock: Lyophilized clusterin (reconstitute with 0.20 ml H_2O)
10x Diluent (25 ml)
20x Wash solution (50 ml)
Anti-clusterin HRP Conjugate (11 ml)
TMB Reagent (11 ml): HRP substrate solution
Stop Solution (11 ml): 1N HCl

MATERIALS REQUIRED BUT NOT PROVIDED

Distilled or deionized water
Pipettes: P-10, P-200 & P-1000 or equivalent
Disposable pipette tips
Plate reader capable of reading OD at 450 nm
Vortex mixer
Absorbent paper
Graph paper or appropriate PC graphing software
Polypropylene microcentrifuge tubes (1.5 ml)

WARNINGS AND PRECAUTIONS

Avoid contact with 1N HCl (stop solution). It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
Do not use reagents after expiration date and do not mix or use components from different kits.
Replace caps on reagents immediately. Do not switch caps.
Do not pipette reagents by mouth.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. Reconstitute the lyophilized clusterin stock by addition of 200 l of de-ionized or distilled water. Mix gently several times over a period of 5 minutes. The concentration of clusterin in the reconstituted stock is indicated on the vial label.
2. Label 8 polypropylene tubes as 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml.
3. Into the tube labeled 250 ng/ml, pipette the volume of 1x diluent detailed on the clusterin stock vial label. Then add the indicated volume of clusterin stock (shown on the clusterin stock vial label) and mix gently. This provides the 250 ng/ml standard.
4. Pipette 0.25 ml of clusterin diluent into the tubes labeled 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml.
5. Prepare a 125 ng/ml standard by diluting and mixing 0.25 ml of the 250 ng/ml standard with 0.25 ml of diluent in the tube labeled 125 ng/ml. Similarly prepare the 62.5, 31.25, 15.6, 7.8, 3.9 ng/ml standards by serial dilution.

NOTE: The reconstituted clusterin stock should be frozen immediately after use. It remains stable in frozen form for at least 1 month at -20°C and 6 months at -70°C. Discard the working 250 – 3.9 ng/ml standards after use.

SAMPLE COLLECTION AND PREPARATION

Serum or plasma should be prepared as quickly as possible after blood collection. If samples cannot be assayed immediately they should be frozen at –70°C and thawed only once prior to use. *Samples must not contain azide because this inactivates the HRP conjugate.*

Clusterin is generally present in normal rat serum or plasma plasma at a concentration of ~ 10 g/ml. In order to obtain values within the

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range of the standard curve we suggest that samples be diluted 100 fold by mixing 3 l of sample with 297 l of 1x diluent.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of samples into the appropriate wells (diluted samples must be added before the HRP conjugate).
3. Dispense 100 l of HRP conjugate into each well.
4. Incubate on an orbital shaker (150 rpm) at room temperature (18-25 C) for 60 minutes.
5. Remove the incubation mixture using a plate washer or by flicking the plate contents into a bio-waste container.
6. Wash and empty the microtiter wells 6 times with wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
7. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
8. Dispense 100 l of TMB Reagent into each well.
9. Incubate on an orbital shaker (150 rpm) at room temperature for 20 minutes.
10. Stop the reaction by adding 100 l of Stop Solution to each well.
11. Gently mix until all the blue color changes to yellow.
12. Read absorbance at 450 nm with a plate reader within 15 minutes. *Please Note: Due to plate reader differences, the high standard absorbance values may be out of range occasionally. If this occurs, absorbance values may be determined at 405 nm instead.*
13. If absorbance values exceed the high standard, the samples should be appropriately diluted and re-determined.

CALCULATION OF RESULTS

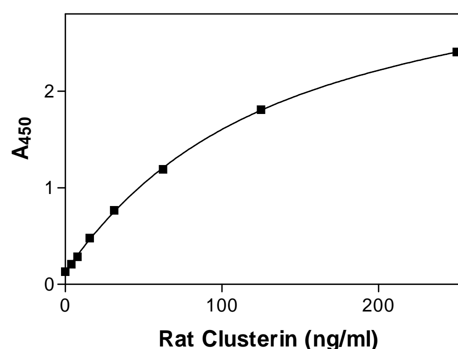
1. Calculate the mean absorbance value (A_{450}) for the standards and samples.
2. Construct a standard curve by plotting the A_{450} values obtained for each reference standard against its concentration in ng/ml on graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the A_{450} values for each sample, determine the corresponding concentration of clusterin (ng/ml) from the standard curve.
4. Multiply the derived clusterin value by the dilution factor to obtain the concentration in the original sample.
5. If available, graphing software may be used to analyze the data. Depending on the range of the standard curve used, we find that good fits of the data may be obtained with linear regression analysis or using a two-site binding model. Alternatively, standard curves may be generated using a point-to-point fit.

EXAMPLE OF STANDARD CURVE

Results of a typical standard curve with A_{450} plotted on the Y axis against clusterin concentrations on the X axis are shown below. NOTE: This standard curve is for the purpose of illustration only.

Clusterin (ng/ml)	Absorbance (450 nm)
250	2.407
125	1.810
62.5	1.190
31.25	0.768
15.6	0.481
7.8	0.287
3.9	0.210
0	0.133

Representative Rat Clusterin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
2. Urine samples can be assayed with this kit but we have not been able to fully validate the ELISA for use with urine due to a lack of positive control samples. Studies with clusterin-spiked urine samples indicate that best results are obtained if urine samples are either (i) diluted at least 50-fold in 1x diluent, or (ii) dialyzed against a 200-fold volume excess of de-ionized water and mixed with an equal volume of 2x diluent prior to assay. Either of these methods eliminates interfering factors. Use of this kit for measurement of urinary clusterin levels should be performed entirely at the discretion of the researcher.

RAT HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-2

Enzyme Immunoassay for the Quantitative Determination of Rat Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in rat serum as a result of injury, infection or disease. A recent report indicates that measurement of haptoglobin provides an excellent marker of inflammation/disease in rats[1]. The level of induction may be as much as ten fold.

PRINCIPLE OF THE TEST

The rat haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat haptoglobin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2 g/ml rat haptoglobin when reconstituted as detailed on the vial label
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The rat haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 g/ml rat haptoglobin stock ***(the reconstituted standard remains stable for at least 10 days at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 8 polypropylene or glass tubes as 62.5, 31.2, 15.6, 7.8, 3.9, 1.95, 0.98 and 0 ng/ml.
3. Dispense 968.8 l of diluent into the tube labeled 62.5 ng/ml and 300 l of diluent into the remaining tubes.
4. Pipette 31.25 l of the 2 g/ml haptoglobin standard into the tube labeled 62.5 ng/ml and mix. This provides the working 62.5 ng/ml haptoglobin standard.
5. Prepare a 31.25 ng/ml standard by diluting and mixing 300 l of the 62.5 ng/ml standard with 300 l of diluent in the tube labeled 31.25 ng/ml. Similarly prepare the 15.6, 7.8, 3.9, 1.95 and 0.98 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is generally present in normal rat serum at a concentration of ~ 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 100,000 fold using the following procedure for each sample to be tested:

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1. Dispense 998 l and 497.5 l of 1x diluent into separate tubes.
2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 998 l of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 l of the 500 fold diluted sample with the 497.5 l of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Rinse and flick the microtiter wells 6 times with *distilled or deionized water*. This is best performed by rinsing the cassette under a fast flowing faucet of house distilled or de-ionized water. The entire wash procedure should take no more than 15-20 seconds *(city water may be used at the investigators discretion).*
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes*.

CALCULATION OF RESULTS

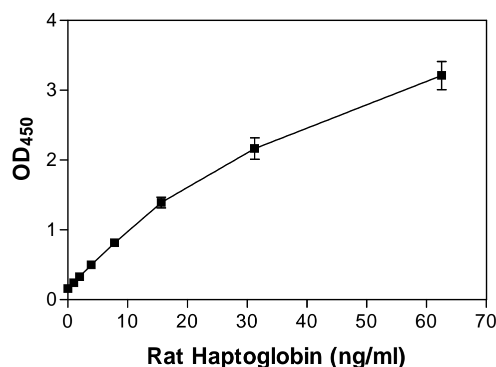
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
0	0.160
0.98	0.243
1.95	0.329
3.91	0.497
7.81	0.814
15.63	1.365
31.25	2.165
62.5	3.210

Representative Rat Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. PS Giffen et.al., Markers of experimental acute inflammation in the Wistar Han rat with particular reference to haptoglobin and C-reactive protein. Arch Toxicol. 77:392-402 (2003)

MOUSE HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-1

Enzyme Immunoassay for the Quantitative Determination of Mouse Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in mouse serum as a result of injury, infection or disease. Studies at Life Diagnostics, Inc., indicate that the level of induction may be as much as ten fold.

PRINCIPLE OF THE TEST

The mouse haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-mouse haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-mouse haptoglobin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-mouse haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2 g/ml mouse haptoglobin
- Wash Buffer (10x stock, 60 ml)
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-4.
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~25,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

The wash solution is provided as a 10x stock. Prior to use dilute the contents of the bottle (60 ml) with 540 ml of distilled or deionized water.

STANDARD PREPARATION

1. The standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 g/ml rat CRP stock ***(the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if future use is intended)***.
2. Label 8 polypropylene microcentrifuge tubes as 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95, and 0 ng/ml
3. Dispense 468.8 l of diluent into the tube labeled 125 ng/ml and 250 l of diluent into the remaining tubes.
4. Pipette 31.25 l of the 2 g/ml haptoglobin standard into the tube labeled 125 ng/ml and mix. This provides the working 125 ng/ml haptoglobin standard.
5. Prepare a 62.5 ng/ml standard by diluting and mixing 250 l of the 125 ng/ml standard with 250 l of diluent in the tube labeled 62.5 ng/ml. Similarly prepare the 31.2, 15.6, 7.8, 3.9, and 1.95 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is generally present in mouse serum at concentrations ranging from 0.1 – 2 mg/ml. In order to obtain values within the range of the standard curve samples should be diluted 25,000 fold. We suggest the following procedure for each sample to be tested:

1. Dispense 497.5 l and 248 l of 1x diluent into separate polypropylene tubes.

2. Pipette and mix 2.5 l of the serum/plasma sample into the tube containing 497.5 l of diluent. This provides a 200 fold diluted sample.
3. Mix 2.0 l of the 200 fold diluted sample with the 248 l of diluent in the second tube. This provides a 25,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container or using a plate washer.
5. Wash and empty the microtiter wells 4-5 times with 1x wash solution. This may performed using either a plate washer (350 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes*.

CALCULATION OF RESULTS

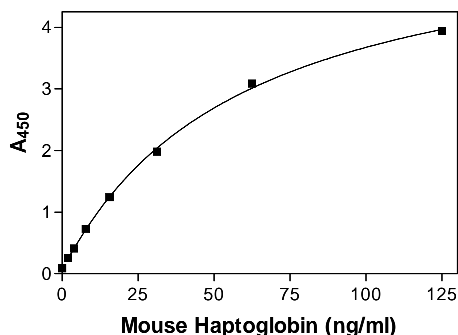
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 25,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
0	0.086
1.95	0.255
3.9	0.409
7.8	0.727
15.6	1.242
31.25	1.983
62.5	3.086
125	3.940

Typical Mouse Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

RAT FIBRINOGEN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 3200-2

Enzyme Immunoassay for the Quantitative Determination of Rat Fibrinogen in Plasma

INTRODUCTION

Fibrinogen is dimeric protein (mol wt, 340 kDa), that is synthesized in the liver and circulates in rat plasma at a concentration of approximately 3 mg/ml. It is significantly elevated during the acute phase response[1,2] and therefore serves as a useful marker of infection, disease and inflammation.

PRINCIPLE OF THE TEST

The rat fibrinogen ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat fibrinogen antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat fibrinogen antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 30 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in fibrinogen molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of fibrinogen is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

Anti-rat fibrinogen antibody 96-well plate (provided as 12 detachable strips of 8)
Enzyme Conjugate Reagent, 11 ml
Reference standard (lyophilized), containing 2 g/ml rat fibrinogen when reconstituted as detailed on the vial label
20x Wash Solution, 50 ml
10x Diluent (25 ml)
TMB Reagent (One-Step) 11 ml
Stop Solution (1N HCl), 11 ml

Materials required but not provided:

Precision pipettes and tips
Distilled or deionized water
Polypropylene or glass tubes
Vortex mixer
Absorbent paper or paper towels
Micro-Plate incubator/shaker mixing speed of ~150 rpm
Plate washer
Plate reader with an optical density range of 0-4 at 450nm
Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.
3. Optimum results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

WASH SOLUTION PREPARATION

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.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The rat fibrinogen standard is provided as a lyophilized stock. Add the volume of distilled or deionized water indicated on the vial label and mix gently until dissolved to obtain a 2 g/ml stock ***(the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if additional use is intended)***.
2. Label 8 polypropylene or glass tubes as 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 0 ng/ml.
3. Dispense 525 l of diluent into the tube labeled 250 ng/ml and 300 l of diluent into the remaining tubes.
4. Pipette 75 l of the 2 g/ml fibrinogen standard into the tube labeled 250 ng/ml and mix. This provides the working 250 ng/ml fibrinogen standard.
5. Prepare a 125 ng/ml standard by diluting and mixing 300 l of the 250 ng/ml standard with 300 l of diluent in the tube labeled 125 ng/ml.
6. Similarly prepare the 62.5, 31.25, 15.6, 7.8 and 3.9 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Fibrinogen is present in normal rat plasma at a concentration of ~ 3 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be

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diluted 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 l and 497.5 l of 1x diluent into separate tubes.
2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 998 l of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 l of the 500 fold diluted sample with the 497.5 l of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution using a plate washer (400 l/well). The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
9. Wash as detailed in 4 to 6 above.
10. Dispense 100 l of TMB Reagent into each well.
11. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
12. Stop the reaction by adding 100 l of Stop Solution to each well.
13. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
14. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

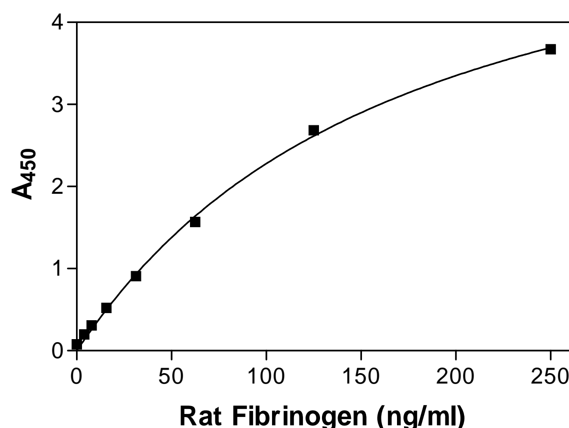
1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of fibrinogen in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of fibrinogen in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000 samples should be diluted appropriately and re-tested.

Typical Standard Curve

A typical standard curve with optical density readings at 450nm on the Y axis against fibrinogen concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Fibrinogen (ng/ml)	Absorbance (450 nm)
250.0	3.673
125.0	2.684
62.5	1.568
31.25	0.907
15.63	0.523
7.81	0.308
3.91	0.197
0.0	0.079

Typical Rat Fibrinogen Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. G Schreiber et. al., The acute phase response in the rodent. Ann N Y Acad Sci. 557:61-85 (1989)
2. A Larrson, J Bjork and C Lundberg. Nephelometric Determination of rat fibrinogen as a marker of inflammatory response. Vet Immunol Immunopathol 59:163-169 (1997)

MOUSE CLUSTERIN ELISA KIT
Life Diagnostics, Inc., Cat. No. 3300-1

ELISA FOR DETERMINATION OF MOUSE CLUSTERIN

STORAGE CONDITIONS
Store the kit at 2-8°C. Keep the microtiter plate in a sealed bag with desiccant to minimize exposure to damp air.

EXPIRATION
The kit expiration date (six months from the date of shipment) is indicated on the package label

BACKGROUND
Clusterin, also referred to as apolipoprotein J, sulfated glycoprotein-2, glycoprotein III and testosterone-repressed prostate message-2, is a glycoprotein of 70-80 kDa, composed of one α-subunit and one β-subunit, derived from proteolytic cleavage of a precursor peptide. Clusterin is expressed in many tissues and is found in serum, seminal fluid and urine. It has been identified as a potential biomarker of various forms of renal injury and prostate disease.

PRINCIPLE OF THE ASSAY
The clusterin ELISA uses two different affinity purified antibodies. One is used for solid phase immobilization (on the microtiter wells). The second is conjugated to horse radish peroxidase (HRP). The samples (diluted serum, plasma or urine) are mixed in the microtiter wells with HRP conjugated antibody and clusterin is sandwiched between the solid phase and HRP-conjugated antibodies. After incubation on a plate shaker for one hour at room temperature the wells are washed to remove unbound HRP-conjugated antibodies. A solution of tetramethylbenzidine (TMB), an HRP substrate, is then added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped by addition of 1N HCl changing the color to yellow. The concentration of clusterin is proportional to the absorbance at 450 nm.

REAGENTS AND MATERIALS PROVIDED
- Anti clusterin-coated wells (1 plate, 96 wells)
- Clusterin Stock: Lyophilized clusterin (reconstitute with 0.20 ml H_2O)
- 10x Diluent (25 ml)
- 20x Wash solution (50 ml)
- Anti-clusterin HRP Conjugate (11 ml)
- TMB Reagent (11 ml): HRP substrate solution
- Stop Solution (11 ml): 1N HCl

MATERIALS REQUIRED BUT NOT PROVIDED
- Distilled or deionized water
- Pipettes: P-10, P-200 & P-1000 or equivalent
- Disposable pipette tips
- Plate reader capable of reading OD at 450 nm
- Vortex mixer
- Absorbent paper
- Graph paper or appropriate PC graphing software
- Polypropylene microcentrifuge tubes (1.5 ml)

WARNINGS AND PRECAUTIONS
- Avoid contact with 1N HCl (stop solution). It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
- Do not use reagents after expiration date and do not mix or use components from different kits.
- Replace caps on reagents immediately. Do not switch caps.
- Do not pipette reagents by mouth.

DILUENT PREPARATION
The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION
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.

STANDARD PREPARATION
1. Reconstitute the lyophilized clusterin stock by addition of 200 μl of de-ionized or distilled water. Mix gently several times over a period of 5 minutes. The concentration of clusterin in the reconstituted stock is indicated on the vial label.
2. Label 8 polypropylene tubes as 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml.
3. Into the tube labeled 250 ng/ml, pipette the volume of 1x diluent detailed on the clusterin stock vial label. Then add the indicated volume of clusterin stock (shown on the clusterin stock vial label) and mix gently. This provides the 250 ng/ml standard.
4. Pipette 0.25 ml of clusterin diluent into the tubes labeled 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml.
5. Prepare a 125 ng/ml standard by diluting and mixing 0.25 ml of the 250 ng/ml standard with 0.25 ml of diluent in the tube labeled 125 ng/ml. Similarly prepare the 62.5, 31.25, 15.6, 7.8, 3.9 ng/ml standards by serial dilution.

NOTE: The reconstituted clusterin stock should be frozen immediately after use. It remains stable in frozen form for at least 1 month at -20°C and 6 months at -70°C. Discard the working 250 – 3.9 ng/ml standards after use.

SAMPLE COLLECTION AND PREPARATION
Serum or plasma should be prepared as quickly as possible after blood collection. If samples cannot be assayed immediately they

should be frozen at –70°C and thawed only once prior to use. ***Samples must not contain azide because this inactivates the HRP conjugate.***

Clusterin is generally present in normal mouse serum or plasma at a concentration of ~ 10 µg/ml. In order to obtain values within the range of the standard curve we suggest that samples be diluted 100 fold by mixing 3 µl of sample with 297 µl of 1x diluent.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. **Dispense 100 µl of samples into the appropriate wells (diluted samples must be added before the HRP conjugate).**
3. Dispense 100 µl of HRP conjugate into each well.
4. Incubate on an orbital shaker (150 rpm) at room temperature (18-25°C) for 60 minutes.
5. Remove the incubation mixture using a plate washer or by flicking the plate contents into a bio-waste container.
6. Wash and empty the microtiter wells 6 times with wash solution. This may performed using either a plate washer (400 µl/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
7. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
8. Dispense 100 µl of TMB Reagent into each well.
9. Incubate on an orbital shaker (150 rpm) at room temperature for 20 minutes.
10. Stop the reaction by adding 100 µl of Stop Solution to each well.
11. Gently mix until all the blue color changes to yellow.
12. Read absorbance at 450 nm with a plate reader within 15 minutes. ***Please Note: Due to plate reader differences, the high standard absorbance values may be out of range occasionally. If this occurs, absorbance values may be determined at 405 nm instead.***
13. If absorbance values exceed the high standard, the samples should be appropriately diluted and re-determined.

CALCULATION OF RESULTS

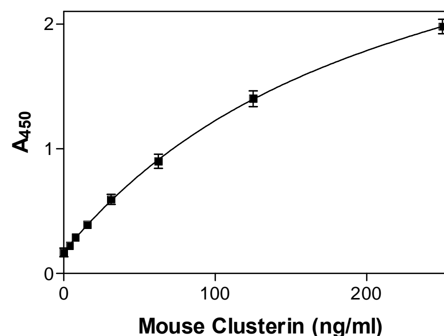
1. Calculate the mean absorbance value (A_{450}) for the standards and samples.
2. Construct a standard curve by plotting the A_{450} values obtained for each reference standard against its concentration in ng/ml on graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the A_{450} values for each sample, determine the corresponding concentration of clusterin (ng/ml) from the standard curve.
4. Multiply the derived clusterin value by the dilution factor to obtain the concentration in the original sample.
5. If available, graphing software may be used to analyze the data. Depending on the range of the standard curve used, we find that good fits of the data may be obtained with linear regression analysis or using a two-site binding model. Alternatively, standard curves may be generated using a point-to-point fit.

EXAMPLE OF STANDARD CURVE

Results of a typical standard curve with A_{450} plotted on the Y axis against clusterin concentrations on the X axis are shown below. **NOTE:** This standard curve is for the purpose of illustration only.

Clusterin (ng/ml)	Absorbance (450 nm)
250	1.980
125	1.401
62.5	0.900
31.25	0.594
15.6	0.391
7.8	0.289
3.9	0.221
0	0.169

Representative Mouse Clusterin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
2. Urine samples can be assayed with this kit but we have not been able to fully validate the ELISA for use with urine due to a lack of positive control samples. Studies with clusterin-spiked urine samples indicate that best results are obtained if urine samples are either (i) diluted at least 50-fold in 1x diluent, or (ii) dialyzed against a 200-fold volume excess of de-ionized water and mixed with an equal volume of 2x diluent prior to assay. Either of these methods eliminates interfering factors. Use of this kit for measurement of urinary clusterin levels should be performed entirely at the discretion of the researcher.

Revision Date: 08/19/11

DOG HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-3

Enzyme Immunoassay for the Determination of Dog Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in dog serum as a result of inflammation[1]. Serum levels also increase approximately two fold during pregnancy[2]. Measurement of haptoglobin provides a convenient marker of inflammation/disease in dogs.

PRINCIPLE OF THE TEST

The dog haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-dog haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-dog haptoglobin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-dog haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2 μg/ml dog haptoglobin when reconstituted as detailed on the vial label
- Wash Buffer (20x stock, 50 ml)
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT

The kit should be stored at 2-8°C and the microtiter strips should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.

WASH SOLUTION PREPARATION

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.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The dog haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 μg/ml dog haptoglobin stock ***(the reconstituted standard remains stable for at least 1 day at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 8 polypropylene or glass tubes as 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95 and 0 ng/ml.
3. Dispense 937.5 μl of diluent into the tube labeled 125 ng/ml and 300 μl of diluent into the remaining tubes.
4. Pipette 62.5 μl of the 2 μg/ml haptoglobin standard into the tube labeled 125 ng/ml and mix. This provides the working 125 ng/ml haptoglobin standard.
5. Prepare a 62.5 ng/ml standard by diluting and mixing 300 μl of the 125 ng/ml standard with 300 μl of diluent in the tube labeled 62.5 ng/ml. Similarly prepare the 31.25, 15.6, 7.8, 3.9 and 1.95 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal dog serum at a concentration of ~ 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 µl and 497.5 µl of 1x diluent into separate tubes.
2. Pipette and mix 2 µl of the serum/plasma sample into the tube containing 998 µl of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 µl of the 500 fold diluted sample with the 497.5 µl of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (350 µl/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 µl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 µl of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
13. Stop the reaction by adding 100 µl of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes*.

CALCULATION OF RESULTS

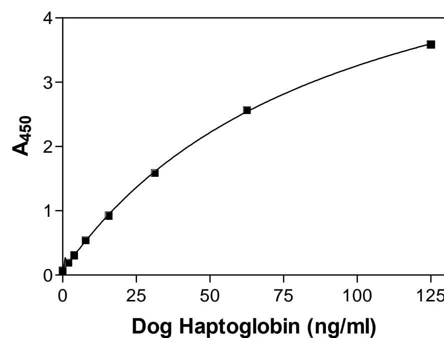
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the A_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
125	3.590
62.5	2.567
31.25	1.592
15.63	0.928
7.81	0.541
3.91	0.310
1.95	0.192
0	0.068

Representative Dog Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Do not use grossly hemolyzed samples. Serum hemoglobin concentrations of 0.1 mg/ml have no effect but concentrations of 1 mg/ml cause an approximate 20% decrease in apparent haptoglobin levels.
2. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. Solter, PF. et. al. Haptoglobin and ceruloplasmin as determinants of inflammation in dogs. Am J Vet Res. 52:1738-42 (1991)
2. Vannucchi, CI, Mirandola, RM and Oliviera, CM. Acute-phase protein profile during gestation and diestrous: proposal for an

early pregnancy test in bitches. Animal Reproduction Science.
74: 87-99 (2002)

REVISION DATE: 08/19/11

CAT HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-8

Enzyme Immunoassay for the Determination of Cat Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein, the concentrations of which can increase two to ten fold in cat serum as a result of disease[1]. Measurement of haptoglobin provides a convenient marker of inflammation and disease in cats.

PRINCIPLE OF THE TEST

The cat haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-cat haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-cat haptoglobin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-cat haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized)
- Wash Buffer (20x stock, 50 ml)
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT

The kit should be stored at 2-8°C and the microtiter strips should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18-25°C) before use.

WASH SOLUTION PREPARATION

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.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The cat haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the reference standard vial label and mix gently until dissolved. ***(The reconstituted standard remains stable for at least 1 day at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 8 polypropylene or glass tubes as 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0 ng/ml.
3. Prepare the 25 ng/ml standard as described on the reference standard vial label.
4. Dispense 300 µl of diluent into the tubes labeled 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0 ng/ml.
5. Pipette 300 µl of the 25 ng/ml haptoglobin standard into the tube labeled 12.5 ng/ml and mix. This provides the working 12.5 ng/ml haptoglobin standard.
6. Similarly prepare the 6.25, 3.125, 1.56, 0.78, and 0.39 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal cat serum at a concentration of ~ 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 µl and 497.5 µl of 1x diluent into separate tubes.

Life Diagnostics, Inc., 909 Old Fern Hill Road, West Chester, PA 19380
610-431-7707 – 610-431-7818 (Fax)
info@lifediagnostics.com – www.lifediagnostics.com

2. Pipette and mix 2 µl of the serum/plasma sample into the tube containing 998 µl of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 µl of the 500 fold diluted sample with the 497.5 µl of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This should preferentially be performed using a plate washer (400 µl/well). If a plate washer is not available use a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 µl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 µl of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
13. Stop the reaction by adding 100 µl of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

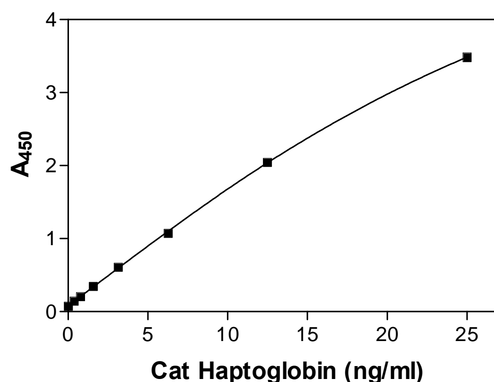
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the A_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
25	3.590
12.5	2.567
6.25	1.592
3.13	0.928
1.56	0.541
0.78	0.310
0.39	0.192
0	0.068

Typical Cat Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Do not use grossly hemolyzed samples. Serum hemoglobin concentrations of 0.1 mg/ml have no effect but concentrations of 1 mg/ml cause an approximate 20% decrease in apparent haptoglobin levels.
2. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. Kajikawa, T et.al., Changes in concentrations of serum amyloid A protein, a1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. Veterinary Immunology and Immunopathology 68:91-98 (1999)

Revision Date: 08/19/11

BOVINE HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-7

Enzyme Immunoassay for the Quantitative Determination of Bovine Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in bovine serum as a result of injury, infection or disease. Normal serum levels of bovine haptoglobin range from ~ 25 – 50 g/ml[1] and increase up to 50 fold during the acute phase response[2,3]. Haptoglobin therefore serves as an excellent acute phase marker in cattle.

PRINCIPLE OF THE ASSAY

The bovine haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-bovine haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-bovine haptoglobin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-bovine haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2.5 g/ml bovine haptoglobin when reconstituted as detailed on the vial label
- Wash Buffer (10x stock, 60 ml)
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~2,000 fold with 1x diluent in order to obtain values within the standard range.

WASH SOLUTION PREPARATION

The wash solution is provided as a 10x stock. Prior to use dilute the contents of the bottle (60 ml) with 540 ml of distilled or deionized water.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The bovine haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2.5 g/ml bovine haptoglobin stock ***(the reconstituted standard should be aliquoted and frozen at -20°C within 2 hours of reconstitution if additional use is intended)***.
2. Label 7 polypropylene or glass tubes as 250, 125, 62.5, 31.25, 15.6, 7.8, and 0 ng/ml.
3. Dispense 450 l of 1x diluent into the tube labeled 250 ng/ml and 300 l of 1x diluent into the remaining tubes.
4. Pipette 50 l of the 2.5 g/ml haptoglobin standard into the tube labeled 250 ng/ml and mix. This provides the working 250 ng/ml haptoglobin standard.
5. Prepare a 125 ng/ml standard by diluting and mixing 300 l of the 250 ng/ml standard with 300 l of 1x diluent in the tube labeled 125 ng/ml. Similarly prepare the 62.5, 31.25, 15.6, and 7.8 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal bovine serum at a concentration of 25 - 50 g/ml and can increase to over 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples initially be diluted 2,000 fold using the following procedure for each sample to be tested:

1. Dispense 195 l and 392 l of 1x diluent into separate tubes.
2. Pipette and mix 5 l of the serum/plasma sample into the tube containing 195 l of diluent. This provides a 40 fold diluted sample.
3. Mix 8 l of the 40 fold diluted sample with the 392 l of diluent in the second tube. This provides a 2,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This should preferably be performed using a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
9. Wash as detailed in 4 to 6 above.
10. Dispense 100 l of TMB Reagent into each well.
11. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
12. Stop the reaction by adding 100 l of Stop Solution to each well.
13. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
14. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes. ***In the event that the standards or samples exceed the absorbance range of your plate reader, optical density may be determined at 405 nm.***

CALCULATION OF RESULTS

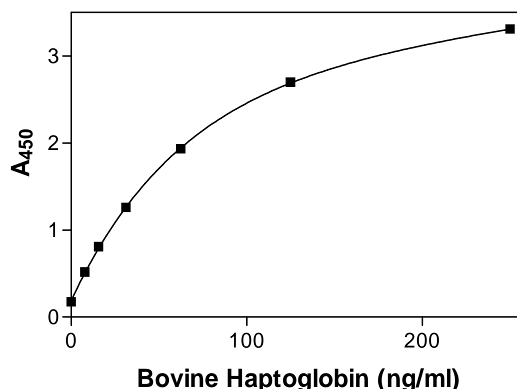
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 2,000, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
250	3.308
125	2.697
62.5	1.931
31.25	1.258
15.6	0.805
7.8	0.515
0	0.173

Representative Bovine Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. SM Salonen et.al., Quantitative determination of bovine serum haptoglobin in experimentally induced Escherichia coli mastitis. Res Vet Sci. 60:88-91 (1996)
2. M Morimatsu et.al., Bovine Haptoglobin: single radial immunodiffusion assay of its polymeric forms and dramatic rise in acute-phase sera. Vet Immunol Immunopathol. 33:365-372 (1992)
3. DL Godson et.al., Serum haptoglobin as an indicator of the acute phase response in bovine respiratory disease. Vet Immunol Immunopathol. 51:277-92 (1996).

MONKEY HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-5

Enzyme Immunoassay for the Quantitative Determination of Monkey Haptoglobin

INTRODUCTION

Haptoglobin is a hemoglobin binding protein that is elevated in serum during the acute phase response. Studies at Life Diagnostics, Inc., have demonstrated that levels of haptoglobin are elevated approximately 5-fold in serum of monkeys undergoing veterinary treatment. Haptoglobin is a useful biomarker of tissue injury, inflammation and infection in monkeys.

PRINCIPLE OF THE TEST

The monkey haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-monkey haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-monkey haptoglobin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

Anti-monkey haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 x 8-well strips)
Enzyme Conjugate Reagent, 11 ml
Reference standard (lyophilized)[1]
10x Diluent (25 ml)
20x Wash Solution (50 ml)
TMB Reagent (One-Step) 11 ml
Stop Solution (1N HCl), 11 ml

Materials required but not provided:

Precision pipettes and tips.
Distilled or deionized water
Polypropylene or glass tubes
Vortex mixer.
Absorbent paper or paper towels
Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an OD range of 0-4 OD
Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The unused kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18- 25°C) before use.
2. Serum or plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The monkey haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable for at least 7 days at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended)***.
2. Label 5 polypropylene or glass tubes as 25, 12.5, 6.25, 3.13 and 1.56 ng/ml.
3. In the tube labeled 25 ng/ml, prepare the 25 ng/ml working standard as described on the label of the reconstituted reference standard vial.
4. Dispense 250 l of diluent into the remaining tubes.
5. Prepare the 12.5 ng/ml standard by diluting and mixing 250 l of the 25 ng/ml standard with 250 l of diluent in the tube labeled 12.5 ng/ml.
6. Similarly prepare the 6.25, 3.13 and 1.56 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Our studies find that haptoglobin is present in monkey serum at concentrations of 0.3 to 2 mg/ml. In order to obtain values within the range of the standard curve we suggest that samples initially be diluted 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 l and 497.5 l of 1x diluent into separate tubes.

[1] Due to international import/export restrictions of monkey derived products, the haptoglobin standard supplied with this kit is of non monkey origin. The standard curve obtained with this material is identical to that obtained with monkey haptoglobin.

2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 998 l of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 l of the 500 fold diluted sample with the 497.5 l of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture using a plate washer or by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 and 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

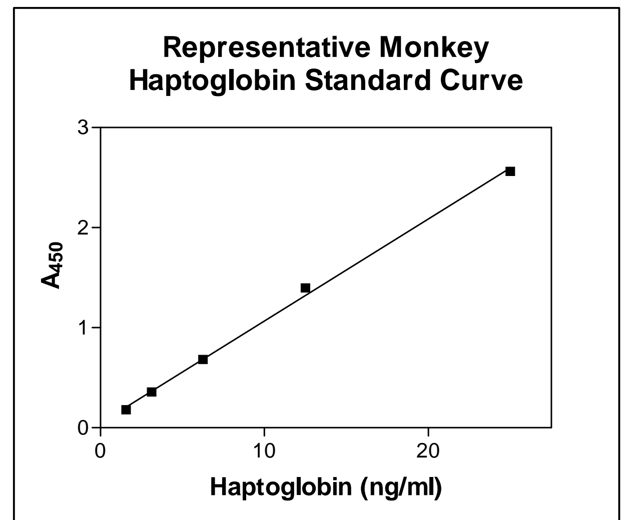
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at the suggested dilution of 100,000 samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
25	2.562
12.5	1.397
6.25	0.682
3.13	0.355
1.56	0.178



PIG HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-4

Enzyme Immunoassay for the Determination of Pig Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in pig serum as a result of inflammation and infection[1,2]. Measurement of haptoglobin therefore provides a convenient marker of inflammation/disease in pigs.

PRINCIPLE OF THE TEST

The pig haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-pig haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-pig haptoglobin antibodies for detection. Serum or plasma is denatured and subsequently diluted. The diluted sampleis incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

Anti-pig haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
Enzyme Conjugate Reagent, 11 ml
Reference standard (lyophilized), containing 300 g/ml pig haptoglobin when reconstituted as detailed on the vial label
Wash Buffer (20x stock, 50 ml)
Denaturing buffer (25 ml)
10x Diluent (25 ml)
TMB Reagent (One-Step) 11 ml
Stop Solution (1N HCl), 11 ml

Materials required but not provided:

Precision pipettes and tips
Distilled or deionized water
Polypropylene or glass tubes
Vortex mixer
Absorbent paper or paper towels
Micro-Plate incubator/shaker mixing speed of ~150 rpm
Plate reader with an optical density range of 0-4 at 450nm
Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT

The kit should be stored at 2-8 C and the microtiter strips should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.

WASH SOLUTION PREPARATION

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.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The pig haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 300 g/ml pig haptoglobin stock.
2. Dilute 10 l of the reconstituted 300 g/ml pig haptoglobin with 190 l of denaturing buffer. Incubate at room temperature for at least 10 minutes.
3. Dilute 20 l of the denatured haptoglobin with 0.98 ml of 1x diluent. This provides the working 300 ng/ml standard.
4. Label 5 polypropylene or glass tubes as 150, 75, 37.5, 18.75, and 0 ng/ml.
5. Dispense 500 l of diluent into the labelled tubes.
6. Prepare a 150 ng/ml standard by diluting and mixing 500 l of the 300 ng/ml standard with 500 l of diluent in the tube labeled 150 ng/ml. Similarly prepare the 75, 37.5, and 18.75 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal pig serum at a concentration of ~ 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 8,000 fold using the following procedure for each sample to be tested:

1. Dispense 190 l of denaturing buffer and 997.5 l of 1x diluent into separate tubes.

2. Pipette and mix 10 l of the serum/plasma sample into the tube containing 190 l of denaturing buffer. This provides a 20 fold diluted, denatured sample.
3. Mix 2.5 l of the 20 fold diluted sample with the 997.5 l of 1 x diluent in the second tube. This provides an 8000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (350 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes*.

CALCULATION OF RESULTS

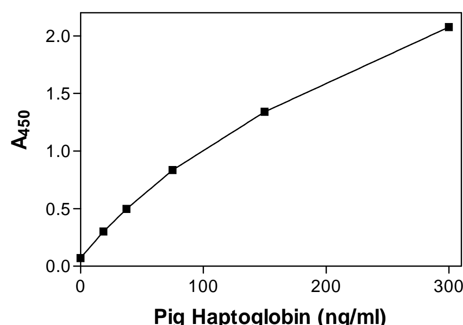
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the A_{450} values of samples fall outside the 300 – 18.75 ng/ml standard range when tested at a dilution of 16,000, samples should be re-diluted appropriately and re-tested. In the event that alternative dilutions are to be tested, prepare them by diluting from the denatured sample (prepared as described above) into 1x diluent. A minimum final dilution of 1000 fold must be achieved, i.e., at least a 50 fold dilution from the 20 fold diluted denatured sample into 1x diluent.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
300	2.077
150	1.341
75	0.835
37.5	0.498
18.75	0.301
0	0.071

Representative Pig Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Do not use grossly hemolyzed samples. Serum hemoglobin concentrations of 0.1 mg/ml have no effect but concentrations of 1 mg/ml cause an approximate 20% decrease in apparent haptoglobin levels.
2. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. Chin, H-H. et. al. Serum acute phase proteins and swine health status. Can J Vet Res. 67:283-90 (2003)
2. Enemark HL. et. al. Pathogenicity of Cryptosporidium parvum – evaluation of an animal infection model. Vet Parasitol. 113:35-57 (2003)

HORSE HAPTOGLOBIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2410-9

Enzyme Immunoassay for the Determination of Horse Haptoglobin in Serum or Plasma

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated up to nine fold in horse serum as a result of inflammation and infection[1,2]. Measurement of haptoglobin therefore provides a convenient marker of inflammation and disease in horses.

PRINCIPLE OF THE TEST

The horse haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-horse haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-horse haptoglobin antibodies for detection. Serum or plasma is denatured and subsequently diluted. The diluted sample is incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in haptoglobin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-horse haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 100 μg/ml horse haptoglobin when reconstituted as detailed on the vial label
- Wash Buffer (20x stock, 50 ml)
- Denaturing buffer (25 ml)
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional).

STORAGE OF THE KIT

1. For optimum stability store the lyophilized standard at or below -20°C when the ELISA kit is received
2. The remainder of the kit should be stored at 2-8°C and the microtiter strips should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18-25°C) before use.

WASH SOLUTION PREPARATION

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.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The horse haptoglobin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 100 μg/ml horse haptoglobin stock.
2. Dilute 10 μl of the reconstituted 100 μg/ml horse haptoglobin with 190 μl of denaturing buffer. Incubate at room temperature for at least 10 minutes.
3. Dilute 20 μl of the denatured haptoglobin with 0.98 ml of 1x diluent. This provides the working 100 ng/ml standard.
4. Label 5 polypropylene or glass tubes as 50, 25, 12.5, 6.25 and 3.13 ng/ml.
5. Dispense 500 μl of diluent into the labeled tubes.
6. Prepare a 50 ng/ml standard by diluting and mixing 500 μl of the 100 ng/ml standard with 500 μl of diluent in the tube labeled 50 ng/ml. Similarly prepare the 25, 12.5, 6.25 and 3.13 ng/ml standards by serial dilution.

NOTE: The reconstituted haptoglobin standard should be frozen immediately after use. It remains stable in frozen form for at least 6 months at -70°C. Discard the working 100 – 3.13 ng/ml standards after use.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal horse serum at a concentration of ~ 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that

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samples be diluted 32,000 fold using the following procedure for each sample to be tested:

1. Dispense 197.5 µl of denaturing buffer and 997.5 µl of 1x diluent into separate tubes.
2. Pipette and mix 2.5 µl of the serum/plasma sample into the tube containing 197.5 µl of denaturing buffer. This provides an 80 fold diluted, denatured sample. ***Please note: the sample must be diluted at least 20-fold in the denaturing buffer.***
3. Allow the samples to incubate in denaturing buffer for at least 10 minutes at room temperature.
4. Mix 2.5 µl of the 80 fold diluted sample with the 997.5 µl of 1 x diluent in the second tube. This provides a 32,000 fold dilution of the sample.
5. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 µl/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 µl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 µl of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
13. Stop the reaction by adding 100 µl of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 5 minutes*.

CALCULATION OF RESULTS

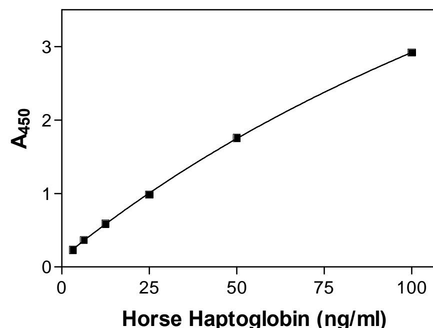
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the A_{450} values of samples fall outside the 100 – 3.13 ng/ml standard range when tested at a dilution of 32,000, samples should be re-diluted appropriately and re-tested. In the event that alternative dilutions are to be tested, prepare them by diluting from the denatured sample (prepared as described above) into 1x diluent. A minimum final dilution of 1000 fold must be achieved, i.e., at least a 50 fold dilution from the 20 fold diluted denatured sample into 1x diluent.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
100	2.919
50	1.756
25	0.988
12.5	0.590
6.25	0.365
3.13	0.233

Typical Horse Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. Tiara, T. et. al. Equine haptoglobin: isolation, characterization, and the effects of ageing, delivery and inflammation on its serum concentration. J Vet Med Sci. 54:435-42 (1992)

2. Kent , JE and Goodall, J. Assessment of an immunoturbidimetric method for measuring equine serum haptoglobin concentrations. Equine Vet J. 23:59-66 (1991)

REVISION DATE: 08/19/11

MOUSE -MACROGLOBULIN ELISA
Life Diagnostics, Inc., Catalog Number: 2810-1

Enzyme Immunoassay for the Quantitative Determination of Mouse -Macroglobulin in Serum or Plasma

INTRODUCTION

-Macroglobulin is a serum proteinase inhibitor that consists of two major (Mr 163,000 and 35,000) and one minor (Mr 185,000) polypepide chains[1]. It is a negative acute phase reactant, the levels of which decrease in mouse serum or plasma as a result of inflammation[2]. It has also been demonstrated that mouse -macroglobulin levels increase significantly with age, after gonadectomy and during pregnancy.

PRINCIPLE OF THE TEST

The mouse -macroglobulin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-mouse -macroglobulin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-mouse -macroglobulin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in -macroglobulin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and absorbance is measured at 450 nm. The concentration of -macroglobulin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-mouse -macroglobulin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized)
- Diluent (30 ml)
- 20x Wash solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450 or 405 nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT

The kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~50,000 fold with diluent in order to obtain values within the standard range.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The mouse -macroglobulin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved ***(the reconstituted standard remains stable at 2-8°C for several hours but should be aliquoted and frozen at or below -20°C after reconstitution if use beyond this time is intended)***.
2. Label 7 polypropylene tubes as 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 0 ng/ml.
3. Add 250 l of diluent into the tubes labeled 62.5, 31.2, 15.6, 7.8, 3.9 and 0 ng/ml.
4. Mix the volumes of diluent and reconstituted mouse -macroglobulin standard detailed on the standard vial label in the tube labeled 125 ng/ml.
5. Prepare a 62.5 ng/ml standard by diluting and mixing 250 l of the 125 ng/ml standard with 250 l of diluent in the tube labeled 62.5 ng/ml. Similarly prepare the 31.25, 15.6, 7.8, and 3.9 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: -Macroglobulin is present in normal mouse serum at a concentration of ~ 2.5 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 50,000 fold using the following procedure for each sample to be tested.

1. Dispense 998 l of water and 297 l of diluent into separate tubes.
2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 998 l of water. This provides a 500 fold diluted sample.

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3. Mix 3 l of the 500 fold diluted sample with the 297 l of diluent in the second tube. This provides a 50,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (standards and samples should be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking the plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 and 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes. ***Please Note: If the A_{450} of the high standard(s) exceeds the limits of the plate reader, absorbance of all wells may be determined at 405 nm instead.***

CALCULATION OF RESULTS

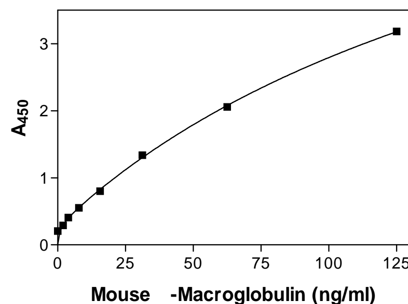
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of -macroglobulin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of -macroglobulin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 50,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against -macroglobulin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

-Macroglobulin (ng/ml)	Absorbance (450 nm)
125	3.140
62.5	2.046
31.25	1.419
15.6	0.848
7.8	0.552
3.9	0.395
0	0.167

Representative Mouse -Macroglobulin Standard Curve



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1. NW Hudson et al. Mouse -macroglobulin: Structure, function and a molecular model. Biochem J. 248:837-845 (1987)
2. K Yamamoto et al. Concentrations of murinoglobulin and -macroglobulin in the mouse serum: variations with age, sex, strain, and experimental inflammation. Biochem Int. 10:463-469 (1985)

RAT MURINOGLOBULIN ELISA
Life Diagnostics, Inc., Catalog Number: 2710-2

Enzyme Immunoassay for the Quantitative Determination of Rat Murinoglobulin in Serum or Plasma

INTRODUCTION

Murinoglobulin is a negative acute phase reactant, the levels of which decrease in rat serum or plasma as a result of injury, infection or disease. In a rat adjuvant induced arthritis model murinoglobulin levels decrease by approximately eight fold[1]. Murinoglobulin therefore provides an excellent marker of inflammation/disease in rats.

PRINCIPLE OF THE TEST

The rat murinoglobulin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat murinoglobulin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat murinoglobulin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 30 minutes. This results in murinoglobulin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of murinoglobulin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat murinoglobulin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (1 vial, lyophilized) containing 2 g/ml rat murinoglobulin when reconstituted as detailed on the vial label
- 10x Diluent (25 ml)
- 20x Wash solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450 or 405 nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT

The kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The rat murinoglobulin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 g/ml rat murinoglobulin stock ***(the reconstituted standard remains stable for at least 2 days at 2-8°C but should be aliquoted and frozen at or below -20°C after reconstitution if use beyond this time is intended)***.
2. Label 7 polypropylene or glass tubes as 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 0 ng/ml.
3. Dispense 937.5 l of diluent into the tube labeled 125 ng/ml and 300 l of diluent into the remaining tubes.
4. Pipette 62.5 l of the 2 g/ml murinoglobulin standard into the tube labeled 125 ng/ml and mix. This provides the working 125 ng/ml murinoglobulin standard.
5. Prepare a 62.5 ng/ml standard by diluting and mixing 300 l of the 125 ng/ml standard with 300 l of diluent in the tube labeled 62.5 ng/ml. Similarly prepare the 31.25, 15.6, 7.8, and 3.9 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Murinoglobulin is present in normal rat serum at a concentration of ~ 6 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 l and 497.5 l of 1x diluent into separate tubes.

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info@lifediagnostics.com – www.lifediagnostics.com

2. Pipette and mix 2 l of the serum/plasma sample into the tube containing 998 l of diluent. This provides a 500 fold diluted sample.
3. Mix 2.5 l of the 500 fold diluted sample with the 497.5 l of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (350 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 30 minutes.
9. Wash as detailed in 4 and 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. It is important to make sure that all the blue color changes to yellow.
15. Read the optical density at 450 nm with a microtiter plate reader within 15 minutes. ***Please Note: If the A_{450} of the high standard(s) exceeds the limits of the plate reader, absorbance may be determined at 405 nm.***

CALCULATION OF RESULTS

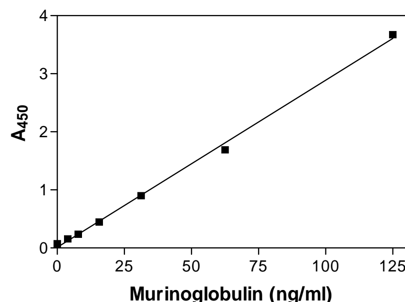
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of murinoglobulin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of murinoglobulin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000. Samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against murinoglobulin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Murinoglobulin (ng/ml)	Absorbance (450 nm)
125	3.673
62.5	1.689
31.25	0.899
15.63	0.446
7.81	0.235
3.95	0.156
0	0.072

Representative Rat Murinoglobulin Standard Curve



REFERENCES

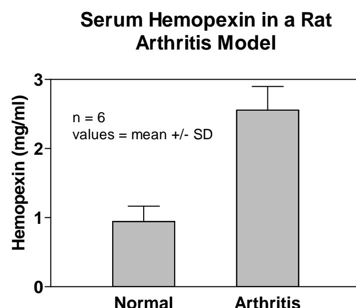
1. K Lonberg-Holm et.al., Three high molecular weight protease inhibitors of rat plasma: isolation, characterization, and acute phase changes. J Biol Chem. 262:438-445 (1987)

RAT HEMOPEXIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2910-2

Enzyme Immunoassay for the Quantitative Determination of Rat Hemopexin in Serum or Plasma

INTRODUCTION

Hemopexin is an acute phase protein that is elevated 2-3 fold in rat serum and plasma as a result of inflammation[1,2]. As illustrated in the figure below, studies at Life Diagnostics Inc. indicate that hemopexin is elevated approximately 2.5 fold in serum of arthritic rats. Hemopexin provides a convenient marker of inflammation and tissue injury in the rat.



PRINCIPLE OF THE TEST

The rat hemopexin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat hemopexin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat hemopexin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in hemopexin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of hemopexin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

Anti-rat hemopexin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
Enzyme Conjugate Reagent, 11 ml
Reference standard (lyophilized) containing 2 g/ml rat hemopexin
10x Diluent (25 ml)
20x Wash Solution (50 ml)
TMB Reagent (One-Step) 11 ml
Stop Solution (1N HCl), 11 ml

Materials required but not provided:

Precision pipettes and tips
Distilled or deionized water
Polypropylene or glass tubes
Vortex mixer
Absorbent paper or paper towels
Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-4.
Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~25,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. Add the volume of distilled or de-ionized water indicated on the lyophilized rat hemopexin standard vial label to the standard vial and mix gently until dissolved. This provides a 2 g/ml stock ***(the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if future use is intended)***.
2. Label 8 polypropylene microcentrifuge tubes as 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml
3. Dispense 875 l of 1x diluent into the tube labeled 250 ng/ml and 300 l of diluent into the remaining tubes.

4. Pipette 125 l of the 2 g/ml hemopexin standard into the tube labeled 250 ng/ml and mix. This provides the working 250 ng/ml hemopexin standard.
5. Prepare a 125 ng/ml standard by diluting and mixing 300 l of the 250 ng/ml standard with 300 l of diluent in the tube labeled 125 ng/ml. Similarly prepare the 62.5, 31.25, 15.6, 7.8, and 3.9 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Hemopexin is generally present in rat serum at concentrations ranging from 1-3 mg/ml. In order to obtain values within the range of the standard curve samples should be diluted 25,000 fold. We suggest the following procedure for each sample to be tested:

1. Dispense 497.5 l and 248 l of 1x diluent into separate polypropylene tubes.
2. Pipette and mix 2.5 l of the serum/plasma sample into the tube containing 497.5 l of diluent. This provides a 200 fold diluted sample.
3. Mix 2.0 l of the 200 fold diluted sample with the 248 l of diluent in the second tube. This provides a 25,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container or using a plate washer.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes*.

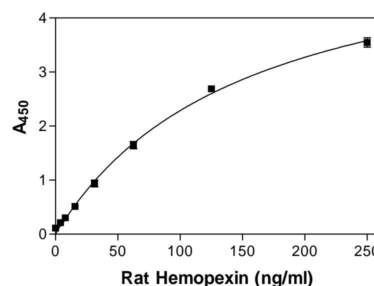
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of hemopexin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of hemopexin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 25,000, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against hemopexin concentrations on the X axis is shown below. This curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Hemopexin (ng/ml)	Absorbance (450 nm)
250	3.546
125	2.687
62.5	1.649
31.25	0.938
15.6	0.512
7.8	0.301
3.9	0.210
0	0.112



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

References

1. I Miller et.al. Proteins of rat serum: III. Gender-related differences in protein concentration under baseline conditions and upon experimental inflammation as evaluated by two-dimensional electrophoresis. Electrophoresis. 20:836-845 (1999)
2. S Marainkovic et.al. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins in vivo. J Immunol. 142:808-812 (1989)

MOUSE HEMOPEXIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2910-1

Enzyme Immunoassay for the Quantitative Determination of Mouse Hemopexin in Serum or Plasma

INTRODUCTION

Hemopexin is an acute phase protein that is elevated in mouse serum and plasma as a result of inflammation and infection[1,2]. The level of induction may be as much as 3-4 fold. Hemopexin therefore provides a convenient marker of inflammation and tissue injury in the mouse.

PRINCIPLE OF THE TEST

The mouse hemopexin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-mouse hemopexin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-mouse hemopexin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in hemopexin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of hemopexin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-mouse hemopexin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized) containing 2 g/ml mouse hemopexin
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-4.
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~25,000 fold with 1x diluent in order to obtain values within the standard range.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. Add the volume of distilled or de-ionized water indicated on the lyophilized mouse hemopexin standard vial label to the standard vial and mix gently until dissolved. This provides a 2 g/ml stock ***(the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if future use is intended)***.
2. Label 6 polypropylene microcentrifuge tubes as 100, 50, 25, 12.5, 6.25, and 0 ng/ml
3. Dispense 950 l of 1x diluent into the tube labeled 100 ng/ml and 300 l of diluent into the remaining tubes.
4. Pipette 50 l of the 2 g/ml hemopexin standard into the tube labeled 100 ng/ml and mix. This provides the working 100 ng/ml hemopexin standard.
5. Prepare a 50 ng/ml standard by diluting and mixing 300 l of the 100 ng/ml standard with 300 l of diluent in the tube labeled 50 ng/ml. Similarly prepare the 25, 12.5, and 6.25 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Hemopexin is generally present in mouse serum at concentrations ranging from 0.2 – 1 mg/ml. In order to obtain values within the range of the standard curve samples should be diluted 25,000 fold. We suggest the following procedure for each sample to be tested:

1. Dispense 497.5 l and 248 l of 1x diluent into separate polypropylene tubes.
2. Pipette and mix 2.5 l of the serum/plasma sample into the tube containing 497.5 l of diluent. This provides a 200 fold diluted sample.

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3. Mix 2.0 l of the 200 fold diluted sample with the 248 l of diluent in the second tube. This provides a 25,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container or using a plate washer.
5. Wash and empty the microtiter wells 5 times with distilled or deionized water. This may performed using either a plate washer (400 l/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
7. Add 100 l of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
11. Dispense 100 l of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
13. Stop the reaction by adding 100 l of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes*.

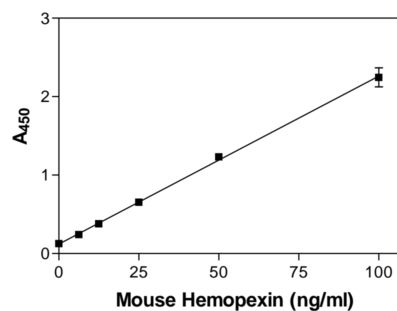
CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of hemopexin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of hemopexin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the standard curve (6.25 – 100 ng/ml) when tested at a dilution of 25,000 samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against hemopexin concentrations on the X axis is shown below. This curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Hemopexin (ng/ml)	Absorbance (450 nm)
100	2.245
50	1.230
25	0.653
12.5	0.380
6.25	0.241
0	0.125



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. X Duan et al. Immunodepletion of albumin for two-dimensional gel detection of new mouse acute-phase protein and other plasma proteins. Proteomics. 5:3991-4000 (2005)
2. R Wait et.al. Reference maps of mouse serum acute-phase proteins: Changes with LPS-induced inflammation and apolipoprotein A-I and A-II transgenes. Proteomics. 5:4245-4253 (2005)

RAT ALPHA-2-MACROGLOBULIN ELISA TEST KIT
Life Diagnostics, Inc., Catalog Number: 2810-2

ELISA for the Quantitative Determination of Rat Alpha-2-Macroglobulin in Serum or Plasma

INTRODUCTION

Alpha-2-macroglobulin is an acute phase protein that is elevated in serum or plasma as a result of injury, infection or disease. It functions as a broad range inhibitor of proteases such as trypsin and is belived to provide a protective role during the acute phase response. Studies at Life Diagnostics, Inc. indicate that alpha-2-macroglobulin levels increase 75-150 fold during chronic disease in rats. Measurement of alpha-2-macroglobulin provides an excellent marker of inflammation and disease.

PRINCIPLE OF THE TEST

The rat alpha-2-macroglobulin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat alpha-2-macroglobulin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat alpha-2-macroglobulin antibodies for detection. The test sample is first mixed with trypsin and incubated for one hour at room temperature. The trypsin reaction is then stopped by addition of trypsin inhibitor. Trypsin treatment normalizes reactivity of the alpha-2-macroglobulin isoforms in non-acute phase and acute phase serum. The sample is then appropriately diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in alpha-2-macroglobulin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of alpha-2-macroglobulin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat alpha-2-macroglobulin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Trypsin (lyophilized, 1ml)
- Trypsin inhibitor (lyophilized, 1 ml)
- Enzyme Conjugate Reagent, 11 ml
- Alpha-2-macroglobulin stock (lyophilized)
- 10x Diluent (25 ml)
- 20x Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips.
- Distilled or deionized water
- Polypropylene tubes
- Vortex mixer
- Absorbent paper or paper towels
- Plate shaker with an approximate mixing speed of 150 rpm
- Plate washer
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-4
- Graph paper (PC graphing software is optional)

STORAGE OF TEST KIT AND INSTRUMENTATION

The kit should be stored at 2-8 C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Optimum results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

TRYPSIN / TRYPSIN INHIBITOR PREPARATION

Trypsin and trypsin inhibitor are provided as lyophilized stocks. Prior to use reconstitute each vial with 1 ml of distilled or deionized water. Store the reconstituted stock at -20°C.

STANDARD PREPARATION

1. The rat alpha-2-macroglobulin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 g/ml rat alpha-2-macroglobulin stock ***(the***

Life Diagnostics, Inc., P.O. Box 5205, West Chester, PA 19380
610-431-7707 – 610-431-7818 (Fax)
info@lifediagnostics.com – www.lifediagnostics.com

reconstituted standard should be aliquoted and frozen at or below -20°C if further use is intended).

2. Label 7 polypropylene or glass tubes as 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 ng/ml.
3. Dispense 450 l of diluent nto the tube labeled 500 ng/ml and 300 l of diluent into the tubes labeled 250, 125, 62.5, 31.25, 15.6, and 7.8 ng/ml.
4. Prepare a 500 ng/ml standard by diluting and mixing 150 l of the reconstituted 2 g/ml standard with 450 l of diluent in the appropriately labeled tube.
5. Prepare a 250 ng/ml standard by diluting and mixing 300 l of the 500 ng/ml standard with 300 l of diluent in the tube labeled 250 ng/ml. Similarly prepare the 125, 62.5, 31.25, 15.6, and 7.8 ng/ml standards by serial dilution.

SAMPLE PREPARATION

A. Trypsin Treatment

1. For each sample to be tested, pipet 18 l of trypsin into a polypropylene microcentrifuge tube.
2. Add 2 l of the serum or plasma test sample and mix.
3. Incubate for 1 hour at room temperature.
4. Add 20 l of Trypsin Inhibitor and mix.
5. At this point the samples may be tested as described below or they may be frozen at or below -20°C for future use.
6. Please note that as a result of the trypsin/trypsin inhibitor treatment the samples are diluted 20-fold.

B. Dilution

Studies at Life Diagnostics, Inc., indicate that alpha-2-macroglobulin is present in normal rat serum at concentrations of approximately 25 g/ml and that levels can increase to approximately 5 mg/ml in acute phase serum. In order to ensure that values of test samples fall within the range of the standard curve we suggest that each sample be tested at dilutions of 1000 and 50,000. These dilutions may be achieved as follows:

1000 Fold Dilution

1. Pipette and mix 8 l of each trypsin treated serum/plasma sample into a tube containing 392 l of diluent. This provides a 1000 fold diluted sample relative to the original serum/plasma sample.

50,000 Fold Dilution

1. Pipette and mix 10 l of the 1000-fold diluted serum/plasma sample into a tube containing 490 l of diluent. This provides a 50,000 fold diluted sample.
2. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 l of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 400 l of 1x wash solution using a plate washer. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 l of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 45 minutes.
8. Wash as detailed in 4-5 above.
9. Dispense 100 l of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25 C) for 20 minutes.
11. Stop the reaction by adding 100 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 the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

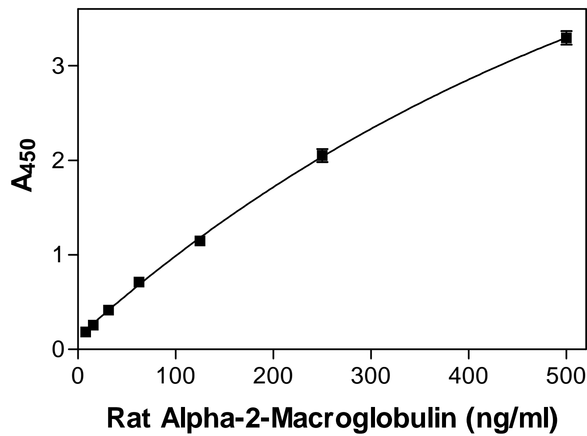
1. Calculate the average absorbance values (A_{450}) for each set of reference standards and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of alpha-2-macroglobulin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of alpha-2-macroglobulin in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD_{450} values of samples fall outside the limits of the standard curve samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against alpha-2-macroglobulin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Alpha-2-macroglobulin (ng/ml)	Absorbance (450 nm)
500	3.296
250	2.051
125	1.149
62.5	0.714
31.25	0.414
15.6	0.256
7.8	0.186

Typical Alpha-2-Macroglobulin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice. **PLEASE READ THE ENTIRE KIT INSERT BEFORE STARTING THE ASSAY**.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.