ELISA PRODUCT INFORMATION & MANUAL

LVV Hemorphin 7

NBP2-62158

Enzyme-linked Immunosorbent Assay for quantitative detection of Non-species specific LVV Hemorphin 7.
For research use only.
Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

LVV Hemorphin 7 ELISA kit

Catalog # **NBP2-62158**
96 Well Enzyme-linked Immunosorbent Assay Kit
For use with serum and tissue homogenates

Reagents require separate storage conditions.

Check our website for additional protocols, technical notes and FAQs.

For proper performance, use the insert provided with each individual kit received.

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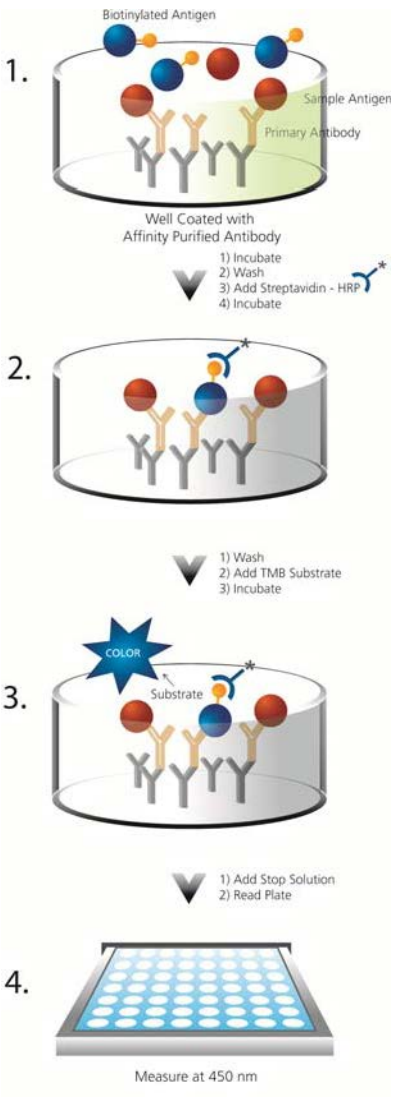


Introduction

The Novus Biologicals LVV Hemorphin 7 Enzyme-Linked Immunosorbent Assay (ELISA) kit is a complete kit for the quantitative determination of LVV Hemorphin 7 in serum and tissue homogenates, with results in three hours. Please read the entire kit insert before performing this assay. This kit is not intended for use with plasma samples.

Hemorphins are opioid peptides derived by proteolysis from hemoglobin [1]. Their sequences are identical in several mammalian species including human, sheep and bovine.

LVV-hemorphin 7 (LVVYPWTQRF) binds strongly to the Angiotensin IV (AT4) receptors in the brain [2,3]. The AT4 receptor is an integral membrane aminopeptidase also known as IRAP (insulin-regulated membrane aminopeptidase) [2,3].
LVV-hemorphin 7 and AT4 are not substrates but rather inhibitors of the AT4 (IRAP) receptor [2,3]. Both promote learning and memory and reverse amnesia in animal models [2,3].

Elevated serum levels of LVV Hemorphin 7 have also been documented in patients with some forms of breast cancers that are associated with an increased expression of cathespins B and D [4].

Principle

1. Standards and samples are added to wells coated with a GxR IgG antibody. A blue solution of LVV Hemorphin 7 conjugated to biotin is added, followed by a yellow solution of rabbit polyclonal antibody to LVV Hemorphin 7.
2. During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, the LVV Hemorphin 7 in the sample or conjugate. The plate is washed, leaving only bound LVV Hemorphin 7.
3. A solution of streptavidin conjugated to horseradish peroxidase is added to each well, to bind the biotinylated LVV Hemorphin 7. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is inversely proportional to the level of LVV Hemorphin 7 in the sample.

Materials Supplied

Do not mix components from different kit lots or use rea- gents beyond the expiration date of the kit.

The standard should be handled with care due to the known and unknown effects of the antigen.

1. **Goat anti-Rabbit IgG Microtiter Plate**
   One plate of 96 wells
   A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody
2. **Assay Buffer 16**
   30 mL
   Tris buffer containing proteins and preservative
3. **LVV Hemorphin 7 Standard**
   Two vials containing 40 ng lyophilized LVV Hemorphin 7
4. **LVV Hemorphin 7 Antibody**
   6 mL
   A yellow solution of polyclonal antibody to LVV Hemorphin 7
5. **LVV Hemorphin 7 Conjugate**
   6 mL
   A blue solution of biotinylated LVV Hemorphin 7
6. **Streptavidin-HRP**
   One vial containing 12.5 µg of lyophilized streptavidin conjugated to horseradish peroxidase.
7. **Wash Buffer Concentrate**
   27 mL
   Tris buffered saline containing detergents
8. **TMB Substrate**
   Two bottles containing 10mL each
   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide
9. **Stop Solution 2**
   10 mL
   A 1N solution of hydrochloric acid in water
10. **LVV Hemorphin 7 Assay Layout Sheet**
    1 each
11. **Plate Sealer**
    2 each

Protect substrate from prolonged exposure to light.

Stop solution is caustic. Keep tightly capped.

Reagents require separate storage conditions.

Storage

All components of this kit, **except the Standard**, should be stored at 4°C upon receipt. The Standard should be stored at **-20°C**. Shipping conditions may not reflect storage conditions.

Materials Needed but Not Supplied

1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 50 µL and 200 µL
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. Microplate shaker
7. Lint-free paper toweling for blotting
8. Microplate reader capable of reading at 450 nm

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

Sample Handling

The assay is suitable for the measurement of LVV Hemorphin 7 in serum and tissue homogenates. This kit is not species specific. However, samples containing rabbit IgG will interfere in the assay due to the GxR IgG coated plate. Prior to assay, frozen samples should be brought to 4°C and centrifuged, if necessary, to isolate residual debris.

The minimum recommended dilution to remove matrix interference from serum samples and tissue homogenates is 1:16. Due to differences in samples, users must determine the optimal sample dilution for their particular experiments.

Protocol for Tissue Homogenate

1. Collect tissue and store in liquid nitrogen.
2. Using a homogenizer, homogenize 4 grams of tissue in 20 mL of 10% acetic acid.
3. Centrifuge the homogenate at 1500 x g for 15 min. Place supernatant into a clean tube.
4. The supernatant may be divided into aliquots and stored at or below -20˚C, or used immediately in the assay.
5. Avoid repeated freeze-thaw cycles.

Protocol for Serum

1. Collect whole blood in appropriate serum tubes.
2. Incubate upright at room temperature for 30-45 minutes to allow clotting to occur.
3. Centrifuge at 1000 x g for 15 minutes at 4°C. Do not use brake.
4. Without disturbing the cell layer, place supernatant into a clean tube.
5. The supernatant may be divided into aliquots and stored at or below -20˚C, or used immediately in the assay.
6. Avoid repeated freeze-thaw cycles.

Sample Recoveries

Recombinant LVV Hemorphin 7 was spiked at high, medium and low concentrations to samples that had been diluted sufficiently to eliminate matrix interference. Endogenous LVV Hemorphin 7 was subtracted from the spiked values and the average recovery in the spiked matrices was compared to the recovery of identical spikes in the assay buffer. The mean and the range percent recovery at the three concentrations are indicated below for each matrix.

Sample Matrix	Dilution	Spike Concentration	Recovery of Spike (Range)
Human Serum (n=5)	1:16	5000 ng/mL	89% (73-101%)
		1500 ng/mL	103% (97- 107%)
		100 ng/mL	90% (63-112%)
Rat Heart Homogenate	1:16	5000 ng/mL	96%
		1500 ng/mL	99%
		100 ng/mL	91%

Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

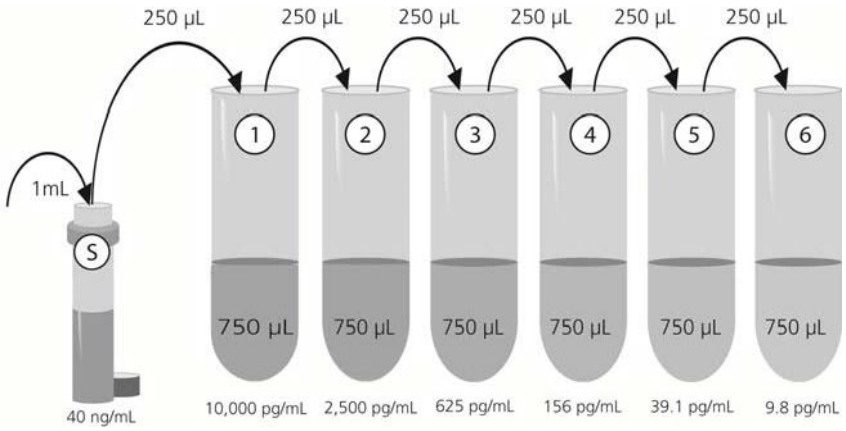
Reagent Preparation

1. **Wash Buffer**

   Prepare the wash buffer by diluting 5 mL of the supplied Wash Buffer Concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. **LVV Hemorphin 7 Standard**

   Reconstitute one vial of LVV Hemorphin 7 standard with 1 mL of the assay buffer. Vortex to ensure the entire cake is dissolved. Label six 12 x 75mm tubes #1 through
   #6. Pipet 750 µL of the assay buffer into tubes #1 through #6, remove 250 µL from the reconstituted vial and add to tube #1, this is standard #1. Vortex thoroughly. Add 250 µL from tube #1 to tube #2, vortex thoroughly. Continue this for tubes #3 through #6.



   Diluted standards should be used within 60 minutes of preparation.
   The concentrations of LVV Hemorphin 7 in the tubes are labeled above.

3. **Streptavidin-HRP**

   Reconstitute one vial of Streptavidin-HRP with 250 µL of deionized water and vortex thoroughly. Store at 4ºC for up to 3 months. For prolonged storage, aliquot and freeze at -20°C. Avoid repeated freeze/thaw cycles. Prepare the working concentration by diluting stock 1:1000 in the assay buffer. **Do not store diluted Streptavidin-HRP.**

Bring all reagents to room temperature for at least 30 minutes prior to opening.

Pre-rinse each pipet tip with reagent.
Use fresh pipet tips for each sample, standard, and rea- gent.

All standards and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid pos- sible contamina- tion.

Prior to the addi- tion of substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

Assay Procedure

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 150 µL of the assay buffer into the NSB (non-specific binding) wells.
2. Pipet 100 µL of the assay buffer into the Bo (0 ng/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #6 to the bottom of the appropriate wells.
4. Pipet 100 µL of the samples to the bottom of the appropriate wells.
5. Pipet 50 µL of the conjugate into each well except the Blank.
6. Pipet 50 µL of the antibody into each well except the Blank, and NSB wells.
7. Seal the plate. Incubate for 2 hour on a plate shaker (~500 rpm*) at room temperature.
8. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
9. Pipet 200 µL of streptavidin-HRP to each well except the Blank.
10. Seal the plate. Incubate for 30 minutes on a plate shaker (~500 rpm*) at room temperature.
11. Wash as above (Step 8).
12. Add 200 µL of the TMB substrate solution into each well.
13. Incubate for 30 minutes at room temperature without shaking.
14. Pipet 50 µL of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

* The optimal speed for each shaker will vary and may range from 120-700 rpm. The speed must be set to ensure adequate mixing of the wells, but not so vigorously that the contents of the wells splash out and contaminate other wells.

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Calculation of Results

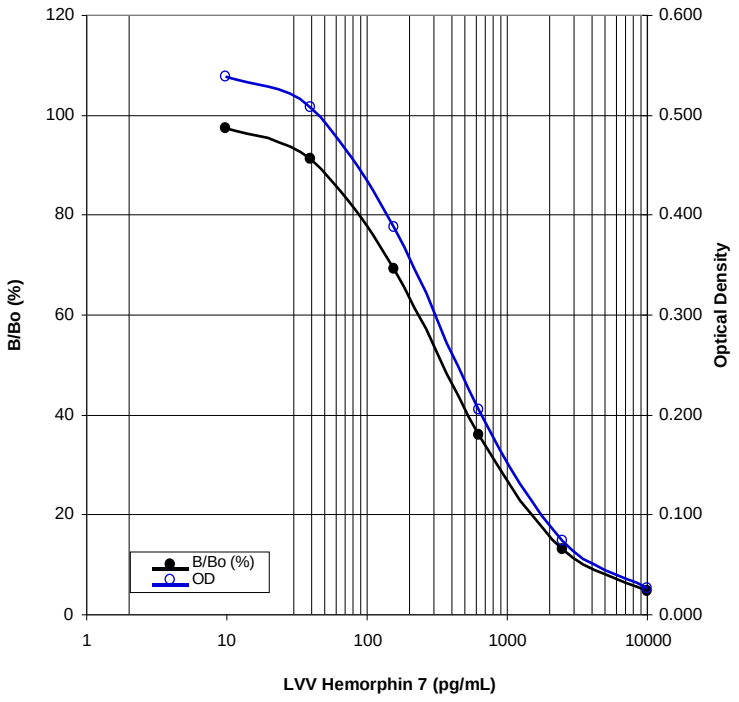
Several options are available for the calculation of the concentration of LVV Hemorphin 7 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Average Net OD	Percent Bound	LVV Hemorphin 7 (pg/mL)
Blank (mean)	(0.040)	---	---
NSB	0.039	0%	---
Bo	0.553	100%	0
S1	0.027	4.7%	10000
S2	0.074	13.1%	2500
S3	0.205	35.9%	625
S4	0.387	69.2%	156.3
S5	0.508	91.2%	39.1
S6	0.539	97.5%	9.8



Performance Characteristics

Specificity

The cross reactivities for a number of related compounds were determined by diluting the cross reactants to concentrations in the range of 0.1 pM to 500 nM. These samples were then measured in the assay.

Analyte	Sequence	Percent cross reactivities in the range of 0.1 pM - 500 nM
LVV Hemorphin 7	LVVYPWTQRF	100
Leu-Valorphin-Arg (LVV Hemorphin-6)	VVYPWTQR	<0.03
Valorphin	VVYPWTQ	<0.03
Ang(1-12)	DRVYIHPFHLVI	<0.03
Ang I	DRVYIHPFHL	<0.03
Ang(1-9)	DRVYIHPFH	<0.03
Ang II	DRVYIHPF	<0.03
Ang(1-7)	DRVYIHP	<0.03
Ang A	ARVYIHPF	<0.03
Ang III	RVYIHPF	<0.03
Ang IV	VYIHPF	<0.03

Sensitivity

The sensitivity, defined as 2 standard deviations from the mean signal at zero, was determined from 6 independent standard curves. The standard deviation was determined from 12 zero standard replicates. The sensitivity of the assay was determined to be 6.1 pg/mL.

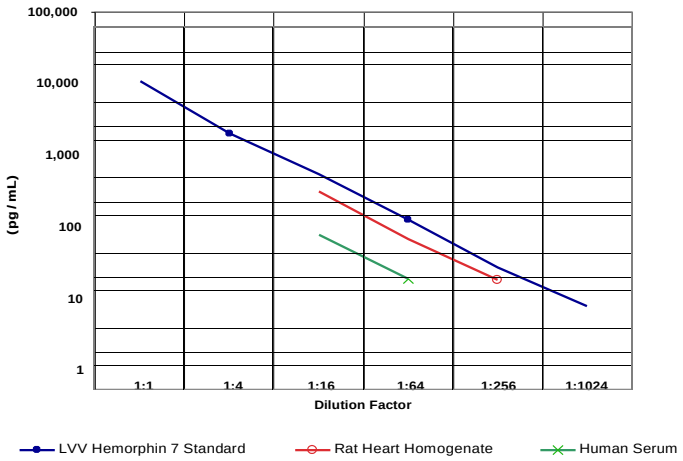
Dilutional Linearity

Human samples containing LVV Hemorphin 7 were serially diluted 1:4 in the kit assay buffer and measured in the assay. The results are shown in the table below.

Dilution	% of Expected	
	Serum	**Tissue Homogenate**
1:16	98%	93%
1:64	100%	88%
1:256	---	100%
1:1024	---	---

Parallelism

Dose-response curves from human plasma and serum diluted into assay buffer were com- pared to the LVV Hemorphin 7 standard curve. The parallel response indicates the standard effectively mimics the native protein.



Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing LVV Hemorphin 7 in a single assay.

pg/mL	%CV
708.1	4.1
305.5	8.8
71.5	20.9

Inter-assay precision was determined by measuring buffer controls of varying LVV Hemorphin 7 concentrations in multiple assays over several days.

pg/mL	%CV
707.0	9.2
334.1	9.5
72.3	16.5

References

1. Jinsmaa Y, Yoshikawa M. Biosci Biotechnol Biochem. 2002 May;66(5):1130-2. Release of hemorphin-5 from human hemoglobin by pancreatic elastase.

2. Albiston AL, Fernando R, Ye S, Peck GR, Chai SY. Biol Pharm Bull. 2004 Jun;27(6):765-7. Alzheimer's, angiotensin IV and an aminopeptidase.

3. Chai SY, Yeatman HR, Parker MW, Ascher DB, Thompson PE, Mulvey HT, Al- biston AL. BMC Neurosci. 2008 Dec 3;9 Suppl 2:S14. Development of cognitive enhancers based on inhibition of insulin-regulated aminopeptidase.

4. Cohen M, Fruitier-Arnaudin I, Sauvan R, Birnbaum D, Piot JM. Clin Chim Acta. 2003 Nov;337(1-2):59-67. Serum levels of Hemorphin-7 peptides in patients with breast cancer.

Notes

Notes

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

Warranty

Novus Biologicals makes no warranty of any kind, expressed or implied, which extends beyond the description of the product in this brochure, except that the material will meet our specifications at the time of delivery. Novus Biologicals makes no guarantee of results and assumes no liability for injuries, damages or penalties resulting from product use, since the conditions of handling and use are beyond our control.