ab136942 – LVV Hemorphin 7 ELISA Kit

Instructions for Use

For quantitative detection of LVV Hemorphin 7 in serum and tissue homogenates.

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 26 August 2015

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1. BACKGROUND

Abcam's LVV Hemorphin 7 *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of LVV Hemorphin 7 in serum and tissue homogenates. This kit it not intended for use with plasma samples.

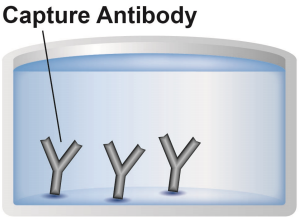
A polyclonal anti-Rabbit-IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with a solution of LVV Hemorphin 7 conjugated to biotin, followed by a solution of polyclonal antibody to LVV Hemorphin 7. The plate is washed to remove unbound reagents. A solution of streptavidin-HRP conjugate is then added. After further incubation the excess reagents are washed away and TMB substrate is added, which is catalyzed by HRP to generate a yellow color. A stop solution changes this color from yellow to blue, and the intensity of this blue coloration is inversely proportional to the amount of LVV Hemorphin 7 captured in the plate.

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

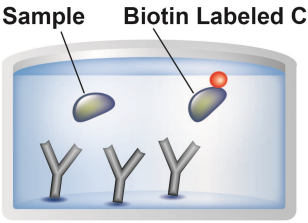
LVV-hemorphin 7 binds strongly to the Angiotensin IV (AT4) receptors in the brain. The AT4 receptor is an integral membrane amino peptidase also known as IRAP (insulin- regulated membrane amino peptidase). LVV-hemorphin 7 and AT4 are not substrates but rather inhibitors of the AT4 (IRAP) receptor. Both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

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.

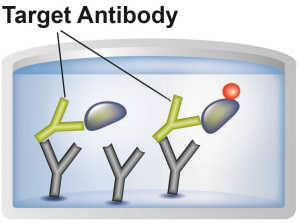
2. ASSAY SUMMARY



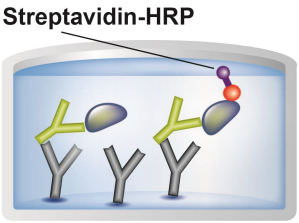
Prepare all reagents and samples as instructed.



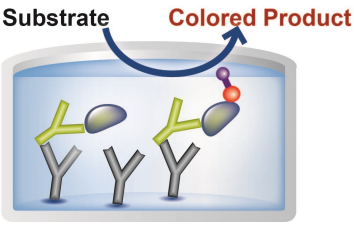
Add samples and Biotinylated antigen and to appropriate wells. Incubate at room temperature.



Add LVV Hemorphin7 polyclonal Antibody to appropriate wells. Incubate at room temperature.



Wash and add prepared labeled Streptavidin HRP-conjugate to appropriate wells. Incubate at room temperature.



Wash and add TMB substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance
- Some Solutions supplied in this kit are caustic; care should be taken with their use
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results
- Care should be taken handling the LVV Hemorphin 7 standard because of the known and unknown effects of LVV Hemorphin 7

4. STORAGE AND STABILITY

Store all components at +4°C immediately upon receipt, apart from the Standard, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-Rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4°C
Assay Buffer 16	30 mL	+4°C
LVV Hemorphin 7 Standard	2 Vials	-20°C
LVV Hemorphin 7 Antibody	6 mL	+4°C
LVV Hemorphin 7-biotin conjugate	6 mL	+4°C
Streptavidin-HRP Conjugate	12.5 µg	+4°C
20X Wash Buffer Concentrate	27 mL	+4°C
TMB Substrate	2 x 10 mL	+4°C
Stop Solution 2 (1N HCl)	10 mL	+4°C

6. MATERIALS REQUIRED, NOT SUPPLIED

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- Optional (for tissue samples): Liquid nitrogen, mortar & pestle, and concentrated HCl

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- This kit is not species specific. However, samples containing rabbit IgG will interfere in the assay due to the anti-Rabbit IgG coated plate.

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 1X Wash Buffer

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

9.2 1X Streptavidin-HRP

Reconstitute one vial of Streptavidin-HRP with 250 µL of distilled 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 1X Streptavidin-HRP by diluting the reconstituted stock 1:1000 in Assay Buffer 16. Example, for 96 wells add 20 µL of reconstituted Streptavidin-HRP to 20 mL of Assay Buffer 16. Mix thoroughly and gently. Do not store diluted Streptavidin-HRP.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Preparation of the Cyclic AMP standard should be prepared no more than 1 hour prior to use.

10.1 Reconstitute one vial of LVV Hemorphin 7 standard with 1 mL of Assay Buffer to yield a **Stock Standard** of 40 ng/mL. Vortex to ensure dissolution.

10.2 Label six tubes with numbers #1 – #6

10.3 Add 750 µL of appropriate diluent (Assay Buffer or Tissue Culture Media) to all 6 tubes.

10.4 Prepare a 10,000 pg/mL **Standard 1** by adding 250 µL of 40 ng/mL Stock Standard into tube #1. Vortex thoroughly.

10.5 Prepare **Standard 2** by transferring 250 µL from Standard 1 to tube #2. Vortex thoroughly.

10.6 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube #3. Vortex thoroughly.

10.7 Using the table below as a guide, repeat for tubes #4 through #6.

10.8 B_0 contains no protein and is the Blank Activity control

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Standard	250	750	40,000	10,000
2	Standard 1	250	750	10,000	2,500
3	Standard 2	250	750	2,500	625
4	Standard 3	250	750	625	156
5	Standard 4	250	750	156	39.1
6	Standard 5	250	750	39.1	9.8

11. SAMPLE COLLECTION AND STORAGE

- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles
- Treatment of cells and tissue with HCl will stop endogenous phosphodiesterase activity and allow for the direct measurement of these samples in the assay without evaporation or further processing
- Samples containing rabbit IgG will interfere with the assay
- Culture supernatants may be run directly in the assay after treatment with concentrated HCl (not the 0.1M HCl supplied). Add 10 µL concentrated HCl to each 1mL of culture media to be assayed. Centrifuge at ≥600 x g at room temperature
- Please note that some samples may contain high levels of Cyclic AMP and additional dilution may be required. Samples with low levels of Cyclic AMP may be assayed in the acetylated format or the samples may be concentrated
- If acetylating standards, then samples must be acetylated in the same format by adding10 µL of the Acetylating Reagent for each 200 µL of the sample

11.1 Protocol for Tissue Homogenate

11.1.1. Collect tissue and store in liquid nitrogen.

11.1.2. Using a homogenizer, homogenize 4 grams of tissue in 20 mL of 10% acetic acid.

11.1.3. Centrifuge the homogenate at 1,500 x g for 15 minutes. Place supernatant into a clean tube.

11.1.4. The supernatant may be divided into aliquots and stored at or below -20°C, or used immediately in the assay. Avoid repeated freeze thaw cycles.

11.2 Protocol for Serum

11.2.1 Collect whole blood in appropriate serum tubes.

11.2.2 Incubate upright at room temperature for 30-45 minutes to allow clotting to occur.

11.2.3 Centrifuge at 1,000 x g for 15 minutes at 4ºC. Do not use break.

11.2.4 Without disturbing the cell layer, place supernatant into a clean tube.

11.2.5 The supernatant may be divided into aliquots and stored at or below -20ºC, or used immediately in the assay. Avoid repeated freeze-thaw cycles.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be sealed in the plate packet and stored at 4°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

	1	2	3	4
A	B_s	Std 2	Std 6	
B	B_s	Std 2	Std 6	
C	NSB	Std 3	Sample 1	
D	NSB	Std 3	Sample 1	
E	B_0	Std 4	Sample 2	
F	B_0	Std 4	Sample 2	
G	Std 1	Std 5	etc	
H	Std 1	Std 5	etc	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:

B_s = Blank; contains substrate only.

TA = Total Activity; contains conjugate (5 µL) and substrate.

NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

B_0 = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay**

13.1 Add 150 µL appropriate diluent* into the NSB (non-specific binding) wells. (*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).

13.2 Add 100 µL appropriate diluent (Assay Buffer or tissue culture media) into the B_0 (0 pg/mL standard) wells.

13.3 Add 100 µL of prepared standards and 100 µL diluted samples to appropriate wells.

13.4 Add 50 µL of the LVV Hemorphin 7-biotin conjugate (blue) into NSB, B_0, standard and sample wells, i.e. not B_s wells.

13.5 Add 50 µL of the Polyclonal LVV Hemorphin 7 antibody (yellow) into B_0, standard and sample wells, i.e. not Bs, TA and NSB wells.

13.6 Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided.

13.7 Empty the contents of the wells and wash by adding 400 µL of 1X Wash Buffer to every well. Repeat the wash 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.

13.8 Add 200 µL of 1X Streptavidin-HRP to all wells except B_s.

13.9 Incubate the plate at room temperature on a plate shaker for 30 minutes at ~500 rpm. The plate may be covered with the plate sealer provided.

13.10 Wash as step 13.7.

13.11 Add 200 µL TMB substrate solution to each well.

13.12 Incubate for 30 minutes at room temperature without shaking.

13.13 Add 50 µL of Stop Solution to all wells.

13.14 Blank the plate reader against the B_s wells, read the O.D. absorbance at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the blank wells, manually subtract the mean optical density of the blank wells from all readings.

14. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

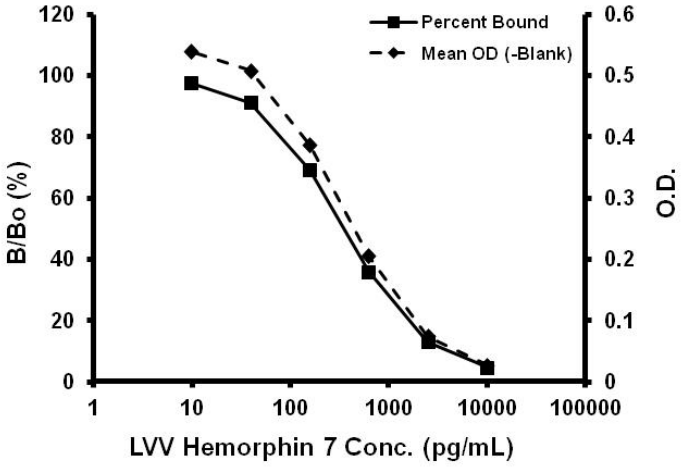
14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of LVV Hemorphin for the standards. The concentration of LVV Hemorphin in the unknowns can be determined by interpolation of net OD values.

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	Average Net OD	Percent Bound (%)	LVV Hemorphin 7 (pg/mL)
B_s (mean)	(0.040)	-	-
NSB	0.039	0	-
B_0	0.553	100	0
Standard 1	0.027	4.7	10,000
Standard 2	0.074	13.1	2,500
Standard 3	0.205	35.9	625
Standard 4	0.387	69.2	156.3
Standard 5	0.508	91.2	39.1
Standard 6	0.539	97.5	9.8

16. TYPICAL SAMPLE VALUES

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.

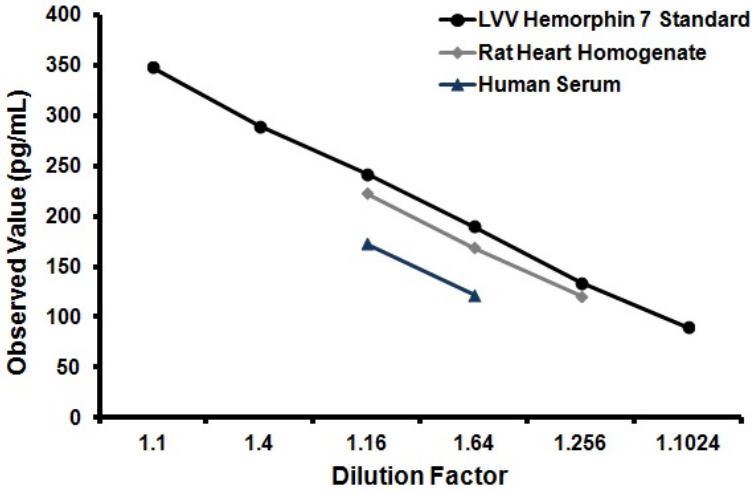
LINEARITY OF DILUTION –

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 compared 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.

Intra-Assay

LVV Hemorphin 7 (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.

Inter-Assay

LVV Hemorphin 7 (pg/mL)	%CV
707.0	9.2
334.1	9.5
72.3	16.5

17. ASSAY SPECIFICITY

CROSS REACTIVITY –

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	Percent cross reactivities in the range of 0.1 pM - 500 nM
LVV Hemorphin 7	100
Leu-Valorphin-Arg (LVV Hemorphin-6)	<0.003
Valorphin	<0.003
Ang(1-12)	<0.003
Ang I	<0.003
Ang(1-9)	<0.003
Ang II	<0.003
Ang(1-7)	<0.003
Ang A	<0.003
Ang III	<0.003
Ang IV	<0.003

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed

19. NOTES

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