ELISA PRODUCT INFORMATION & MANUAL

Angiotensin I

NBP2-62134

Enzyme-linked Immunosorbent Assay for quantitative detection of Non-species specific Angiotensin I.

For research use only.

Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

New Conjugate storage temperature

Angiotensin I ELISA kit

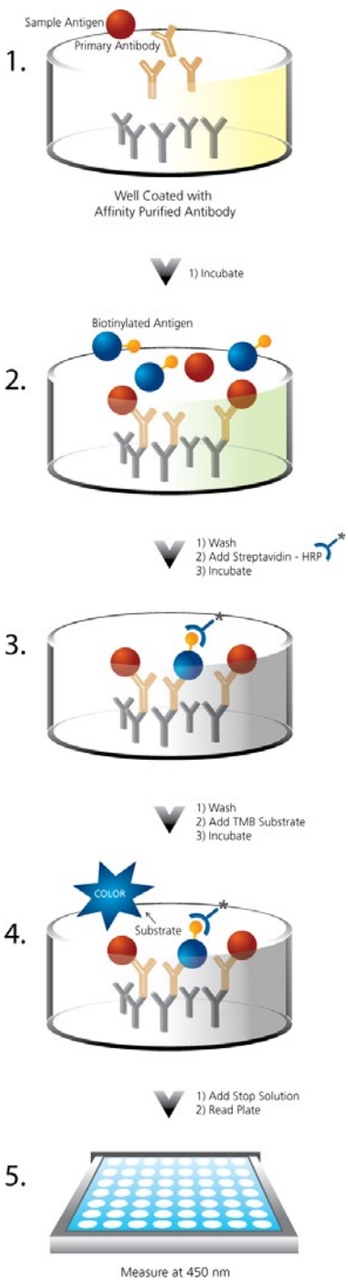
Catalog # NBP2-62134
96 Well Enzyme-linked Immunosorbent Assay Kit
For use with serum and plasma

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.

Table of Contents



Introduction

The Angiotensin I Enzyme Immunometric Assay (ELISA) kit is a complete kit for the quantitative determination of Angiotensin I in serum and plasma. Other matrices, such as urine and tissue, may be suitable but have not been validated. Please read the entire kit insert before performing this assay.

Angiotensins are small peptides derived from angiotensinogen. Several of the known angiotensins are established endocrine effectors in the regulation of blood pressure, but they are also known to have other functions locally, in several organs and tissues (paracrine) and at the cellular level (autocrine / intracrine)[1,2,3,4]. Angiotensin I (DRVYIHPFHL) has no known effector function but it is an immediate precursor of Angiotensin II (DRVYIHPF). Angiotensin II binds AT1 receptors, which promote vasoconstriction, sodium retention, release of aldosterone, release of Arg-vasopressin, cell proliferation, inflammation, fibrosis, anxiety, and cardiac hypertrophy. Angiotensin A (ARVYIHPF)[5], and Angiotensin III (RVYIHPF), also bind AT1 receptors. Angiotensin (1-7) (DRVYIHP) binds a different receptor called MAS-1 which has opposite effects (vasodilation, natriuresis, antiproliferation, NO release, PGE release, and apoptosis)[6]. Angiotensin IV (VYIHPF) binds yet another receptor called AT4 (IRAP), which promotes increase of blood flow, angiogenesis, and natriuresis, and which has also been implicated in memory formation and in the pathogenesis of Alzheimer's disease. The peptide LVV-hemorphin-7 (LVVYPWTQRF), which is not an angiotensin, also binds the AT4 receptor. There are other angiotensin peptides that have been identified, including Ang (1-9) (DRVYIHPFH), Ang (1-12) (DRVYIHPFHLVI), Ang V(3-7) (VYIHP), as well as several other shorter peptides that have undetermined functions.

Angiotensins can be present in very low concentrations in some biological samples. In such cases, dilution of samples to avoid "non-specific" interference by any present factors is not productive because the angiotensin analyte is also diluted to levels far below the minimum detection concentration. Thus, investigators have used several procedures for extracting angiotensins from biological samples prior to using them for immunoassays. See, for example: a) C18 extraction of Ang I and Ang II from plasma, eluted with ACN:H2O:AcOH (74:24:4)[7]; b) phenylsilylsilica cartridge extraction of Ang (1-7) from blood, eluted with methanol[8].

Angiotensins share common sequences, and in some cases they cannot be discriminated by immunoassays. Prior extraction and separation by HPLC may, thus, be required[9]. We have characterized cross-reactivities with several relevant peptides (see cross-reactivity table).

Principle

1. Standards and samples are added to wells coated with a goat anti-rabbit IgG antibody. A yellow solution of rabbit polyclonal antibody to Angiotensin I is then added and the plate is incubated at room temperature.
2. Following this primary incubation, a blue solution of Angiotensin I conjugated to biotin is added and incubated at room temperature. The antibody binds in a competitive manner, the Angiotensin I in the sample or conjugate. The plate is washed, leaving only bound Angiotensin I.
3. A solution of streptavidin conjugated to horseradish peroxidase is added to each well, to bind the biotinylated angiotensin. 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 Angiotensin I in the sample.

Materials Supplied

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

1. **Assay Buffer 16**
   30ml
   Tris buffer containing proteins and preservative

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

2. **Angiotensin I Standard**
   One vial containing 1µg lyophilized Angiotensin I

3. **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

4. **Angiotensin I Antibody**
   4 ml
   A yellow solution of polyclonal antibody to Angiotensin I

5. **Angiotensin I Conjugate**
   4 ml
   A blue solution of biotinylated Angiotensin I

6. **Streptavidin-HRP**
   One vial containing 12.5µg of lyophilized streptavidin conjugated to horseradish peroxidase.

7. **Wash Buffer Concentrate**
   27 ml
   Tris buffered saline containing detergents

Protect substrate from prolonged exposure to light.

8. **TMB Substrate**
   10 ml
   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide

Stop solution is caustic. Keep tightly capped.

9. **Stop Solution 2**
   10 ml
   A 1N solution of hydrochloric acid in water

10. **Angiotensin I Assay Layout Sheet**
    1 each

11. **Plate Sealer**
    2 each

Reagents require separate storage conditions.

Storage

All components of this kit, **except the Standard and Conjugate**, should be stored at 4°C upon receipt. The Standard and Conjugate 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 5µl and 1,000µl
3. Repeater pipet for dispensing 50µl and 200µl
4. Protease inhibitor cocktail (PIC)
5. Phenylmethylsulfonyl fluoride (PMSF)
6. Disposable beakers for diluting buffer concentrates
7. Graduated cylinders
8. Microplate shaker
9. Lint-free paper toweling for blotting
10. Microplate reader capable of reading at 450 nm
11. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.
12. Materials and reagents to extract the analyte from biological fluids or tissues, if needed (see Sample Handling section)

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 Angiotensin I in serum and plasma. Other matrices such as urine and tissue may be suitable, but have not been validated. 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.

Please note that recovery of peptides from extraction processes can be variable. It is important to optimize any process to obtain optimum recoveries. Extraction efficiencies can be determined by a number of methods, including the use of radioactive peptide, or by spiking into paired samples and determining the recovery of this known amount of added analyte. The protocol provided below is a suggestion and should not be considered an optimized protocol for any specific analyte.

Suggested Extraction Protocol

Materials

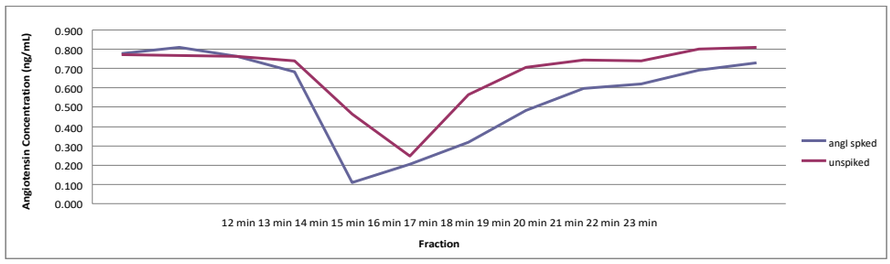
- 100 or 200 mg C_{18} columns
- Solutions
  - Column activation: Methanol
  - Column washing and binding: 1% Trifluoroacetic acid (TFA), 99% Water
  - Elution: 60% Acetonitrile (ACN), 1% TFA, 39% water
- Vacuum manifold with Luer syringe stopcocks to control the flow rate through the extraction columns. Control the flow rate (~ 1ml/min) by opening/closing empty stopcocks or by adjusting the vacuum seal.
- Vacuum line
- 1.5ml polypropylene microfuge tubes
- Microfuge at 4°C
- 15ml screw cap polypropylene tubes
- Floor or table top swinging–bucket centrifuge with proper rotor for 15ml tubes
- Lyophilizer (or a vacuum chamber, a dry-Ice ethanol trap and a powerful vacuum pump)
- Additional standard (excess provided) to determine extraction efficiency

Protocol for 100 mg columns. Adjust volumes accordingly if using 200 mg columns.

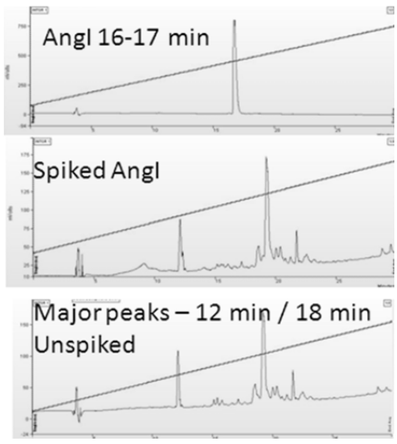
1) Prepare the samples
   a) In a microfuge tube add 500µL 1% TFA to 500µL plasma sample.
   b) Mix and centrifuge at maximum speed, cold (4°C), for 20 minutes.
   c) Carefully remove the supernatant and store on ice until C_{18} column is ready
2) Prepare the column
   a) Wash column with 0.5 ml Methanol
   b) Wash column 3 times with 1 ml 1 % TFA (use vacuum manifold, see above)
3) Binding
   a) Load prepared sample (1 ml) and let it flow through the column slowly (~ 2 min) (use vacuum manifold)
   b) Wash column 2 times with 1 ml 1 % TFA (use vacuum manifold)
4) Elution- Up to now all washes were discarded into the vacuum manifold. Now remove the columns from the vacuum manifold.
   a) Place columns on top of a labeled 15 ml polypropylene tube. The top column rims should prevent the columns from falling into the collection tubes.
   b) Add 1 ml 60% ACN 1% TFA and spin very slowly (300 – 500 rpm) ideally it should take several minutes to elute.
5) Lyophilization
   a) Cover the tubes with parafilm and punch small holes with a needle.
   b) Freeze in dry-ice and lyophilize overnight.
6) Reconstitution
   a) Add 500µl of the assay buffer to re-suspend the pellet at 1 X concentration and assay immediately.

Sample Recoveries

Angiotensin I was extracted from spiked pooled human plasma, spiked with 50ng/ml Angiotensin I, following the protocol described above. 75µl of the reconstituted eluate was further fractionated by HPLC. 25µl from each fraction was frozen, lyophilized and reconstituted with 100µl PBS. 50 µl of the resus- pended fractions were run in the assay.



Maximum inhibition was obtained with the 16 minute (spiked) and 17 minute (unspiked) fractions. AngI elutes at 16-17 minutes, thus, the material detected by the kit in unspiked pooled human plasma either co-elutes or elutes very close to AngI. It is therefore likely to be composed of specific angiotensin-like peptides. Note that AngI itself is not obviously detected by the HPLC (225 nm). Most of the signal is from materials eluted at 12 minutes, which do not interfere with the ELISA assay, and 18 minutes, which interfere by 50%.



Spiked Conc. (pg/ml)	Dilution Factor	Expected Conc. (pg/ml)	Determined Conc. (pg/ml)	% Recovery
50,000	10	5,000	3,286	66.0
50,000	100	500	448	90.0
50,000	1000	50	38	76.0

Reagent Preparation

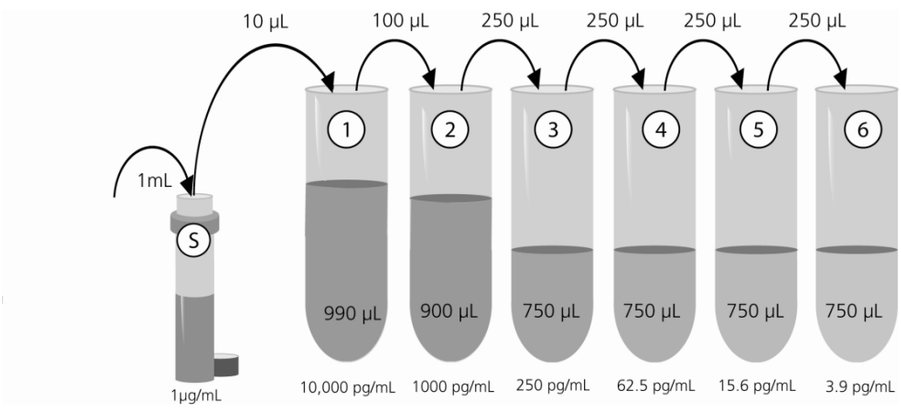
Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

1. Wash Buffer

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

2. Angiotensin I Standard

Reconstitute one vial of Angiotensin I standard with 1 ml of the assay buffer. Vortex to ensure the entire standard is dissolved. Label six 12 x 75mm tubes #1 through #6. Pipet 990µl of the assay buffer into tube #1, pipet 900µl of the assay buffer into tube #2. Pipet 750µl of assay buffer into tubes #3 through #6. Remove 10µl from the reconstituted vial and add to tube #1, this is standard #1. Vortex thoroughly. Add 100µl from tube #1 to tube #2. Vortex thoroughly. Add 250µl from tube #2 to tube #3. Vortex thoroughly. Continue this for tubes #4 through #6.



Diluted standards should be used within 60 minutes of preparation. The concentrations of Angiotensin I in the tubes are labeled above.

3. Streptavidin-HRP

Reconstitute one vial of Streptavidin-HRP with 250µl of deionized water and vortex thoroughly. Store at 4°C 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.**

Assay Procedure

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 reagent.

All standards and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid possible contamination.

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

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 75µl of the assay buffer into the NSB (non-specific binding) wells.
2. Pipet 50µl of the assay buffer into the Bo (0 ng/ml standard) wells.
3. Pipet 50µl of Standards #1 through #6 to the bottom of the appropriate wells.
4. Pipet 50µl of the samples to the bottom of the appropriate wells.
5. Pipet 25µl of the antibody into each well except the Blank, and NSB wells.
6. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm*) at room temperature.
7. Pipet 25µl of the conjugate into each well except the Blank.
8. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm*) at room temperature.
9. Empty the contents of the wells and wash by adding 400µl 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.
10. Pipet 100µl of the streptavidin-HRP conjugate to each well except the Blank.
11. Seal the plate. Incubate for 1 hour on a plate shaker (~500 rpm*) at room temperature.
12. Wash as above (Step 9).
13. Add 100µl of the substrate solution into each well.
14. Seal the plate. Incubate for 30 minutes at room temperature without shaking.
15. Pipet 100µl stop solution into each well.
16. After blanking the plate reader against the substrate blank, read optical density at 450nm. 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

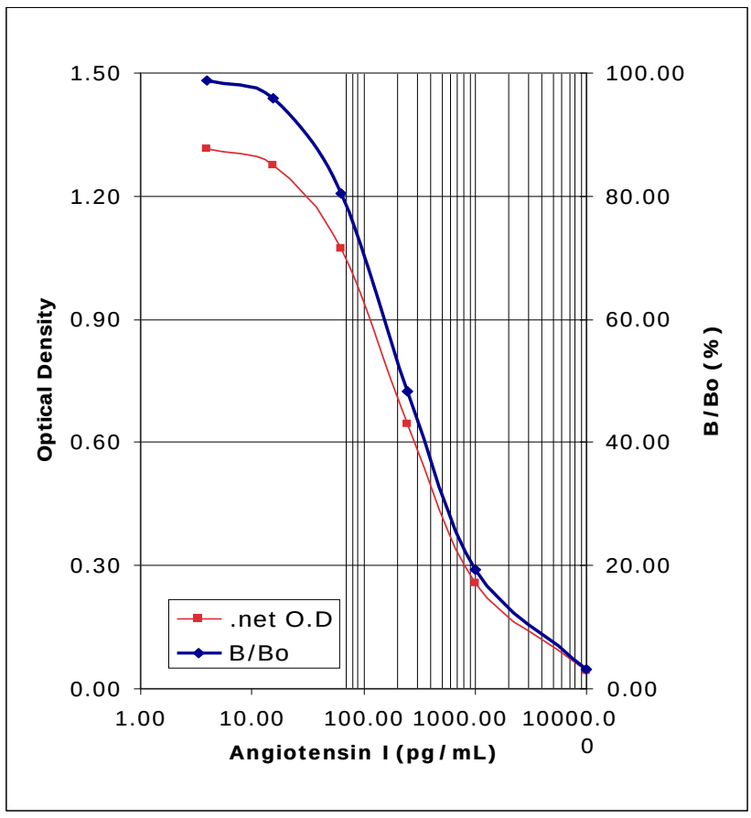
Several options are available for the calculation of the concentration of Angiotensin I in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program.

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	Percent	Angiotensin
Blank	(0.04)		
NSB	0.007	0	---
Bo	1.332	100	**0**
S1	0.043	3.21	**10000**
S2	0.258	19.39	**1000**
S3	0.644	48.33	**250**
S4	1.071	80.41	**62.5**
S5	1.275	95.78	**15.6**
S6	1.315	98.77	**3.9**



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 0.1pM to 500nM. These samples were then measured in the assay.

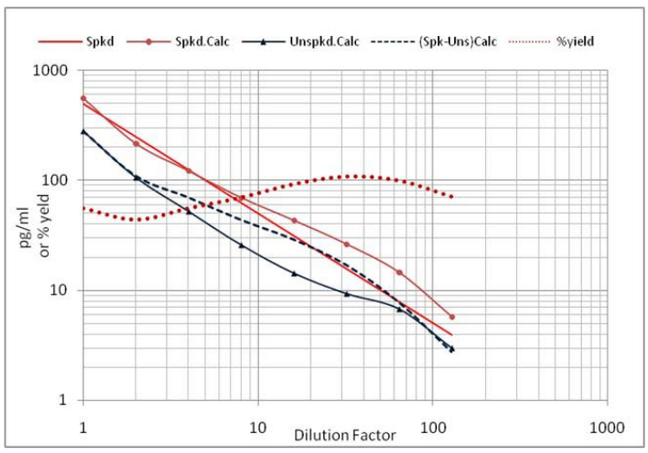
Analyte	Sequence	Percent cross reactivities in the range of 0.1pM - 500nM
Ang(1-12)	DRVYIHPFHLVI	0.025
Ang I	DRVYIHPFHL	100
Ang(1-9)	DRVYIHPFH	0.000
Ang II	DRVYIHPF	0.083
Ang(1-7)	DRVYIHP	0.000
Ang A	ARVYIHPF	0.182
Ang III	RVYIHPF	0.000
Ang IV	VYIHPF	0.000
LVV-hemorphin 7	LVVYPWTQRF	0.000
Bradykinin	RPPGFSPFR	0.000

Sensitivity

The sensitivity, defined as 2 standard deviations from the mean signal at zero, was determined from 6 independent standard curves. The sensitivity of the assay was determined to be 4.3pg/ml.

Dilutional Linearity

Pooled human plasma was spiked with 500pg/ml of Angiotensin I, serially diluted 1:2, and run in the assay. Unspiked pooled plasma was processed identically as a background control.



Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing Angiotensin I in a single assay.

pg/ml	%CV
163.6	8.3
53.2	7.3
25.1	10.0

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

pg/ml	%CV
182.2	7.9
48.1	10.0
24.4	7.6

References

1. WILSON C. Can Med Assoc J. 1964 Oct 31;91:964-70. RECENT ADVANCES IN HYPERTENSION.
2. Brown JJ, Lever AF, Davies DL, Robertson JI. Postgrad Med J. 1966 Mar;42(485):153-76. Renin and angiotensin. A survey of some aspects.
3. Halima I, Bald W, Serban DN. J Renin Angiotensin Aldosterone Syst. 2005 Dec;6(3):121-31. Review. Angiotensin peptides and their pleiotropic actions.
4. Ribeiro-Oliveira A Jr, Nogueira AI, Pereira RM, Boas WW, Dos Santos RA, Simões e Silva AC. Vasc Health Risk Manag. 2008;4(4):787-803. Review. Te renin-angiotensin system and diabetes: an update.
5. Jankowski V, Vanholder R, van der Giet M, Tölle M, Karadogan S, Gobom J, Furkert J, Oksche A, Krause E, Tran TN, Tepel M, Schuchardt M, Schlüter H, Wiedon A, Beyermann M, Bader M, Todiras M, Zidek W, Jankowski J. Arterioscler Thromb Vasc Biol. 2007 Feb;27(2):297-302. Epub 2006 Nov 30. Mass-spectrometric identification of a novel angiotensin peptide in human plasma.
6. Varagic J, Trask AJ, Jessup JA, Chappell MC, Ferrario CM. J Mol Med. 2008 Jun;86(6):663-71. Epub 2008 Apr 25. Review. New angiotensins.
7. Hodge G, Ye VZ, Duggan KA. Exp Physiol. 2002 Jan;87(1):1-8. Salt-sensitive hypertension resulting from nitric oxide synthase inhibition is associated with loss of regulation of Angiotensin I in the rat.
8. Nussberger J, Brunner DB, Nyfeler JA, Linder L, Brunner HR. Clin Chem. 2001 Apr;47(4):726-9. Measurement of immunoreactive angiotensin-(1-7) heptapeptide in human blood.
9. Nagata S, Kato J, Sasaki K, Minamino N, Eto T, Kitamura K. Biochem Biophys Res Commun. 2006 Dec 1;350(4):1026-31. Epub 2006 Oct 5. Isolation and identification of proangiotensin-12, a possible component of the renin-angiotensin system.

Notes

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Novus Biologicals is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Novus Biologicals' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Novus Biologicals, within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NONINFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

Several Novus Biologicals products and product applications are covered by US and foreign patents and patents pending.