

# Product Information & ELISA Manual

Angiotensin II ELISA Kit (Colorimetric)  
NBP2-62135

Enzyme-linked Immunosorbent Assay  
for quantitative detection.

## Contact

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Novus kits are  
guaranteed for 6 months  
from date of receipt.

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therapeutic procedures.**



Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Please contact Novus Biologicals' Technical Support if necessary.

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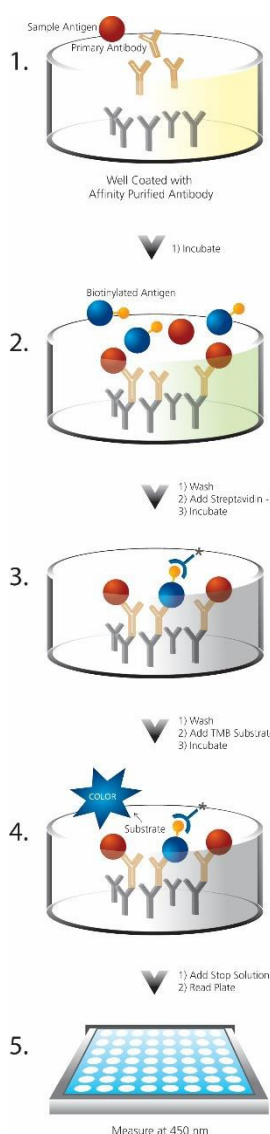
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## INTRODUCTION



The Angiotensin II ELISA Kit (Colorimetric) is a complete kit for the quantitative determination of Angiotensin II 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 / intra-crane)<sup>1,2,3,4</sup>. 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)<sup>5</sup>, 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)<sup>6</sup>. 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:H<sub>2</sub>O:AcOH (74:24:4)<sup>7</sup>; b) phenylsilylsilica cartridge extraction of Ang (1-7) from blood, eluted with methanol<sup>8</sup>.

Angiotensins share common sequences, and in some cases they cannot be discriminated by immunoassays. Prior extraction and separation by HPLC may, thus, be required<sup>9</sup>. 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 II is then added. The plate is incubated at room temperature.
2. A blue solution of Angiotensin II conjugated to biotin is added and incubated at room temperature. During this incubation the specific antibody binds, in a competitive manner, the Angiotensin II in the sample or conjugate. The plate is washed, leaving only bound Angiotensin II or conjugate.
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 unbound 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 II in the sample.



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



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



Protect substrate from prolonged exposure to light.



Stop solution is caustic. Keep tightly capped.

## MATERIALS SUPPLIED

### 1. Assay Buffer 16

27 mL

Tris buffer containing proteins and preservative

### 2. Angiotensin II Standard

One vial containing 1 µg lyophilized Angiotensin II

### 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 II Antibody

A yellow solution of polyclonal antibody to Angiotensin II

### 5. Angiotensin II Conjugate

A blue solution of biotinylated Angiotensin II

### 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

10 mL

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. Plate Sealer

2 each



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
9. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit
10. 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 II 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<sub>18</sub> 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 (~ 1 ml/min) by opening/closing empty stopcocks or by adjusting the vacuum seal.
- Vacuum line
- 1.5 mL polypropylene microfuge tubes
- Microfuge at 4°C
- 15 mL screw cap polypropylene tubes
- Floor or table top swinging-bucket centrifuge with proper rotor for 15 mL 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.**

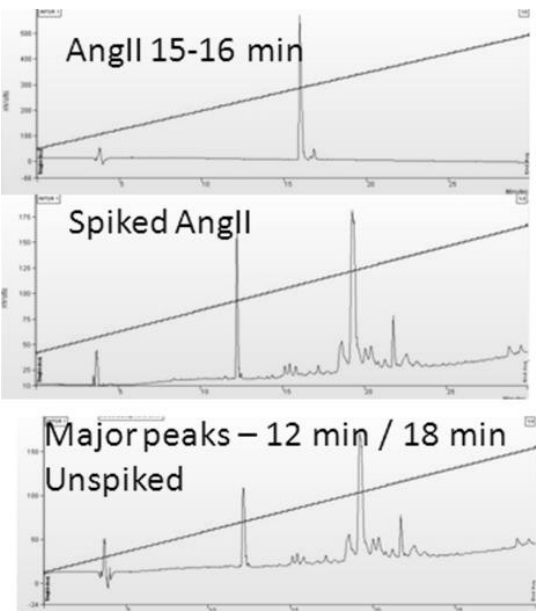
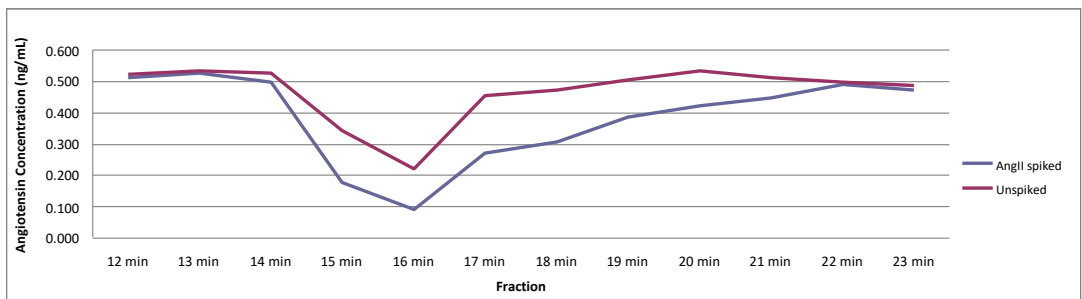
- 1) Prepare the samples
  - a) In a microfuge tube add 500  $\mu$ L 1% TFA to 500 $\mu$ l plasma sample.
  - b) Mix and centrifuge at maximum speed, cold (4°C), for 20 minutes.
  - c) Carefully remove the supernatant and store on ice until C<sub>18</sub> column is ready
- 2) Prepare the column
  - a) 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

Add 500  $\mu$ L the assay buffer to re-suspend the pellet at 1 X concentration and assay immediately.



## SAMPLE RECOVERIES

Angiotensin II was extracted from pooled human plasma, spiked with 50 ng/mL Angiotensin II, following the protocol described above. 75  $\mu$ L of the reconstituted eluate was further fractionated by HPLC. 25  $\mu$ L from each fraction was frozen, lyophilized and reconstituted with 100  $\mu$ L PBS. 50  $\mu$ L of the resuspended fractions were run in the assay.



Maximum inhibition was obtained with the 16 minute fraction for both spiked and unspiked samples. Angiotensin II elutes at 15-16 minutes, thus, the material detected by the kit in unspiked pooled human plasma co-elutes with AngII. It is therefore likely to be composed of specific angiotensin-like peptides. Note that AngII 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	2,619	52.2
50,000	100	500	345	69.0
50,000	1000	50	40	80.0



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

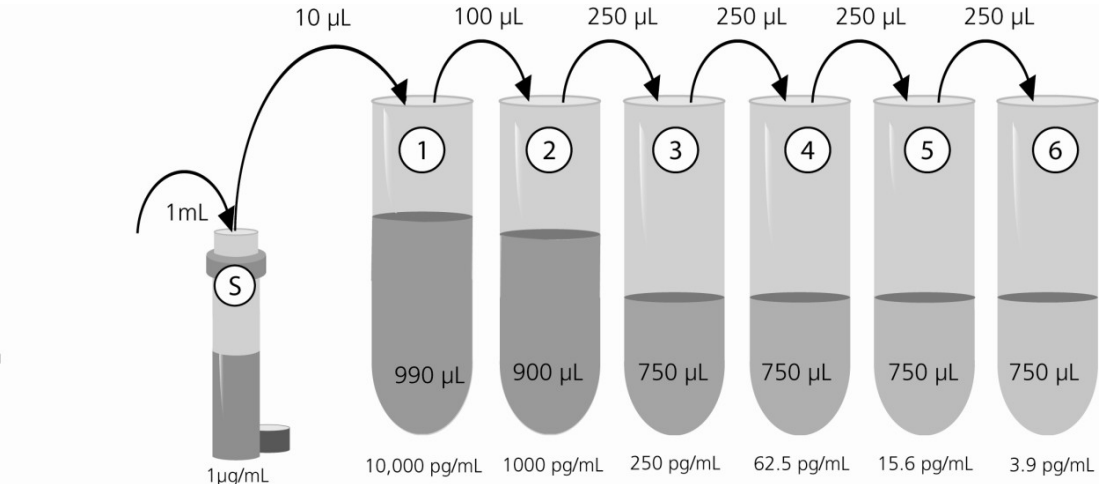
## REAGENT PREPARATION

### 1. Wash Buffer

Prepare the wash buffer by diluting the concentrate 20X into deionized water (example: 5 mL of the supplied Wash Buffer Concentrate with 95 mL of deionized water). The diluted wash buffer can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

### 2. Angiotensin II Standard

Reconstitute one vial of Angiotensin II standard with 1 mL of the supplied Assay Buffer. Vortex to ensure the entire standard is dissolved. Label six 12 x 75mm tubes #1 through #6. Pipet 990  $\mu$ L of Assay Buffer into tube #1, pipet 900  $\mu$ L of Assay Buffer into tube #2. Pipet 750  $\mu$ L of Assay Buffer into tubes #3 through #6. Remove 10  $\mu$ L from the reconstituted vial and add to tube #1, this is standard #1. Vortex thoroughly. Add 100  $\mu$ L from tube #1 to tube #2. Vortex thoroughly. Add 250  $\mu$ L from tube #2 to tube #3. Vortex thoroughly. Continue this for tubes #4 through #6. Dispense the remaining standard into useful-sized aliquots (i.e. 10uL) and store at -70°C. Aliquotted standards can be stored at -70°C for up to three months.



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

### 3. Streptavidin-HRP

Reconstitute one vial of Streptavidin-HRP with 250  $\mu$ L 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.**



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 the antibody, conjugate and 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 plate bag and seal. Store unused wells at 4°C.

1. Pipet 75  $\mu$ L of the assay buffer into the NSB (non-specific binding) wells.
2. Pipet 50  $\mu$ L of the assay buffer into the Bo (0 ng/mL standard) wells.
3. Pipet 50  $\mu$ L of Standards #1 through #6 to the bottom of the appropriate wells.
4. Pipet 50  $\mu$ L of the samples to the bottom of the appropriate wells.
5. Pipet 25  $\mu$ L 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 25  $\mu$ L 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 400  $\mu$ L 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 100  $\mu$ L 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 100  $\mu$ L of the substrate solution into each well.
14. Seal the plate. Incubate for 30 minutes at room temperature without shaking.
15. Pipet 100  $\mu$ L stop solution into each well.
16. 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 Angiotensin II in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program.

The concentration of Angiotensin II can be calculated as follows.

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average NSB OD}$$

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

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

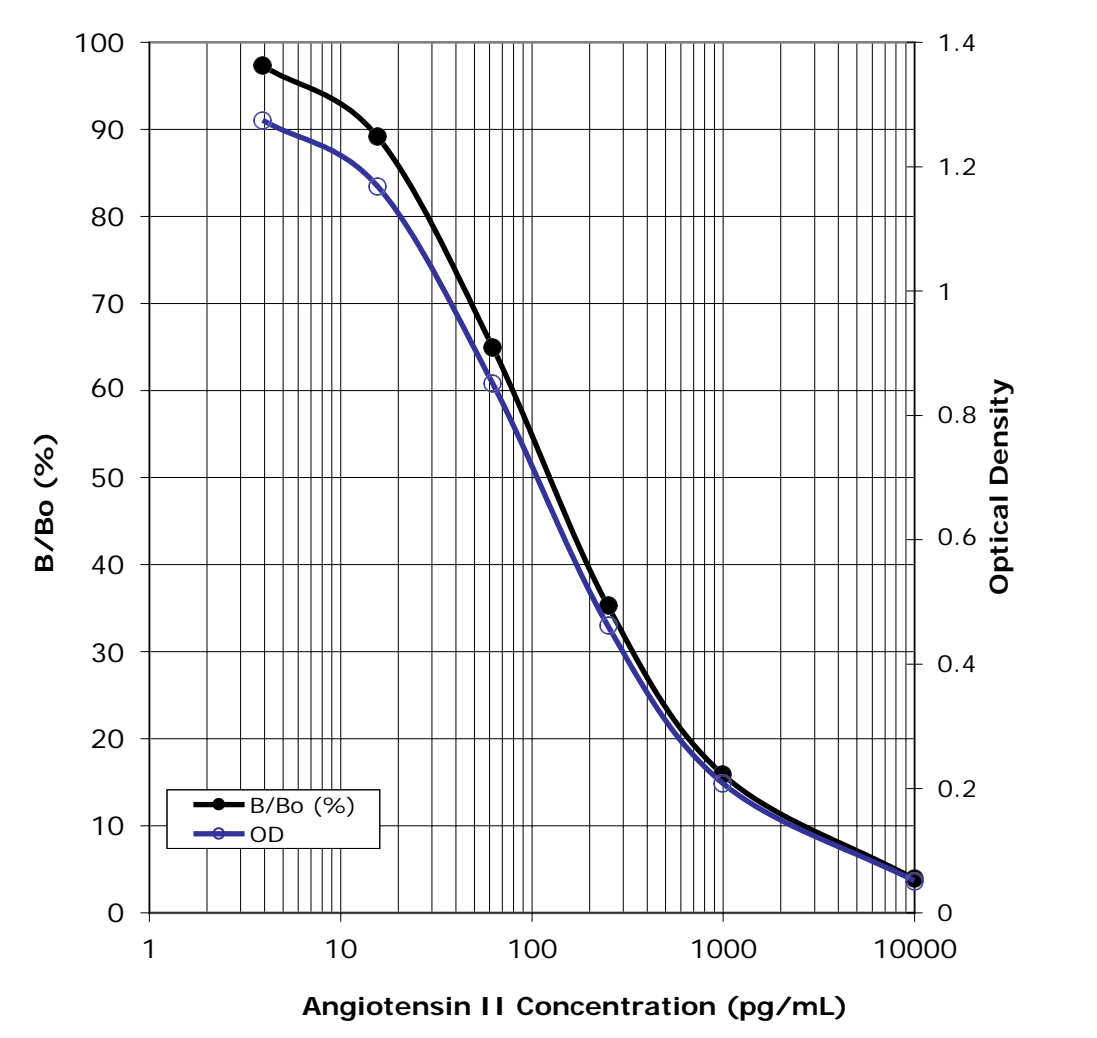
3. Plot the Percent Bound (B/Bo) versus concentration of Angiotensin II for the standards (linear y-axis log x-axis). Draw a best fit line through the points. The concentration of Angiotensin II of the unknowns can be determined by interpolation.

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	Angiotensin II (pg/mL)
Blank (mean)	(0.04)	---	---
NSB	0.01	0	---
Bo	1.31	100	0
S1	0.051	3.9	10000
S2	0.208	15.9	1000
S3	0.461	35.2	250
S4	0.851	64.9	62.5
S5	1.169	89.2	15.6
S6	1.275	97.3	3.9
Unknown 1	0.251	19.2	721.3
Unknown 2	0.514	39.2	221.1



## 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.

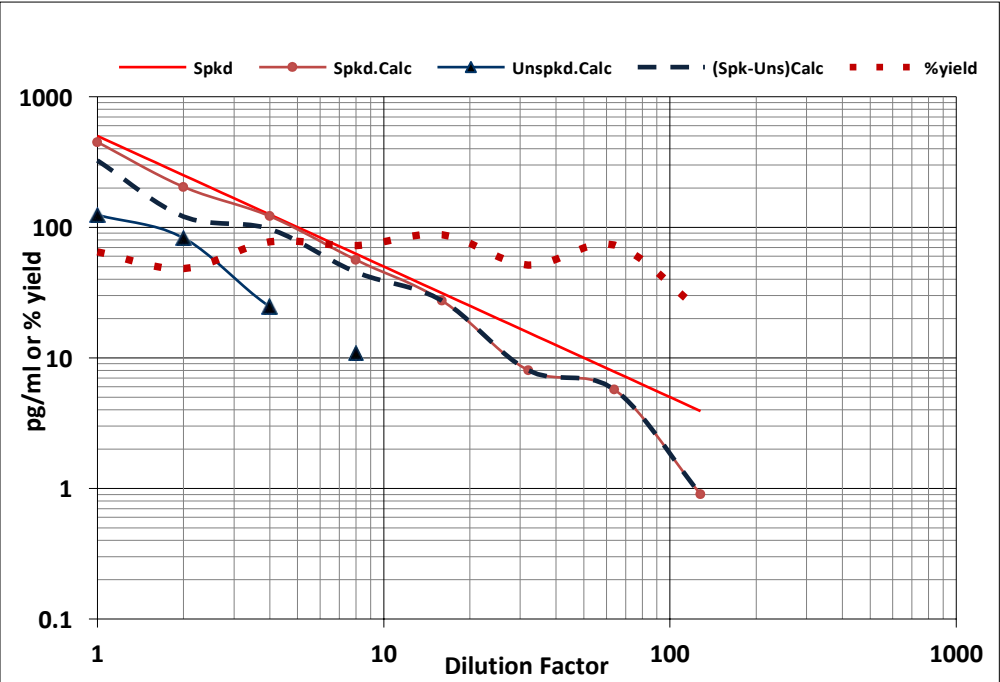
Analyte	Sequence	Percent cross reactivities in the range of 0.1 pM - 500 nM
Ang(1-12)	DRVYIHPFHLVI	0.548
Ang I	DRVYIHPFHL	0.319
Ang(1-9)	DRVYIHPFH	0.103
Ang II	DRVYIHPF	100
Ang(1-7)	DRVYIHP	0.053
Ang A	ARVYIHPF	100
Ang III	RVYIHPF	100
Ang IV	VYIHPF	100
LVV- hemorphin 7	LVVYPWTQRF	0.067
Bradykinin	RPPGFSPFR	0.022

### 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 4.6 pg/ml.

### Dilutional Linearity

Pooled human plasma was spiked with 500 pg/mL of Angiotensin II, 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 II in a single assay.

pg/mL	%CV
719.9	4.7
221.9	5.2
120.4	7.3

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

pg/mL	%CV
721.3	15.9
221.1	7.5
116.8	6.0

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**NOTES**

**NOTES**

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## Angiotensin II ASSAY LAYOUT SHEET for use with NBP2-62135

### • TABLE FOR MAKING STANDARDS 1-6:

Reconstitute lyophilized standard with 1 ml Assay Buffer, vortex gently to dissolve. Then proceed to making standards.

Std	Assay Buffer Vol. (μl)	Standard Vol. Added (μl)	Angiotensin II Conc. (pg/ml)
1	990	10	10000
2	900	100, Std. 1	1000
3	750	250, Std. 2	250
4	750	250, Std. 3	62.5
5	750	250, Std. 4	15.6
6	750	250, Std. 5	3.91

### • ASSAY PROTOCOL FLOW CHART Vol. Added (μl):

Well ID:	Blank A1, B1	NSB C1, D1	Bo A3, B3	Stds E1-H2	Samples A3-H12
Assay Buffer	—	75 μl	50 μl	—	—
Std. and/or Sample	—	—	—	50 μl	50 μl
Angiotensin II Antibody	—	—	25 μl	25 μl	25 μl
Incubate 1 hr. @ RT, shaking (sealed)	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Angiotensin II Conjugate	—	25 μl	25 μl	25 μl	25 μl
Incubate 1 hr. @ RT, shaking (sealed)	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Aspirate & Wash 4 x 400 μl	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
SA-HRP	—	100 μl	100 μl	100 μl	100 μl
Incubate 1hr. @ RT, shaking (sealed)	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Aspirate & Wash 4 x 400 μl	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
TMB Substrate	100 μl	100 μl	100 μl	100 μl	100 μl
Incubate 30 min. @ RT, (sealed)	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Stop Solution	100 μl	100 μl	100 μl	100 μl	100 μl
Read @ 450 nm	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒

Angiotensin I Plate Layout:

A1 Blank	A2 Std 3	A3 S0	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1 Blank	B2 Std 3	B3 S0	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1 NSB	C2 Std 4	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 NSB	D2 Std 4	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 Std 1	E2 Std 5	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 Std 1	F2 Std 5	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1 Std 2	G2 Std 6	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1 Std 2	H2 Std 6	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Kit Lot No. \_\_\_\_\_ Exp. Date \_\_\_\_\_  
 Date \_\_\_\_\_ Tech. \_\_\_\_\_

1<sup>st</sup> Incub.: Start Time \_\_\_\_\_ Temp. \_\_\_\_\_  
 End Time \_\_\_\_\_ Temp. \_\_\_\_\_

2<sup>nd</sup> Incub.: Start Time \_\_\_\_\_ Temp. \_\_\_\_\_  
 End Time \_\_\_\_\_ Temp. \_\_\_\_\_

3<sup>rd</sup> Incub.: Start Time \_\_\_\_\_ Temp. \_\_\_\_\_  
 End Time \_\_\_\_\_ Temp. \_\_\_\_\_

4<sup>th</sup> Incub.: Start Time \_\_\_\_\_ Temp. \_\_\_\_\_  
 End Time \_\_\_\_\_ Temp. \_\_\_\_\_