

ab136935 – Angiotensin A ELISA Kit

Instructions for Use

For quantitative detection of Angiotensin A in plasma and serum.

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

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1. BACKGROUND

Abcam's Angiotensin A *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Angiotensin A in plasma and serum.

A goat anti-rabbit IgG polyclonal antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with a solution of Angiotensin A conjugated to biotin, followed by a solution of polyclonal antibody to Angiotensin A. 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 Angiotensin A captured in the plate.

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). Angiotensin I has no known effector function but it is an immediate precursor of Angiotensin II. 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, and Angiotensin III, also bind AT1 receptors. Angiotensin (1-7) binds a different receptor called MAS-1 which has opposite effects (vasodilation, natriuresis, antiproliferation, NO release, PGE release, and apoptosis). Angiotensin IV 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, which is not an angiotensin, also binds the AT4 receptor. There are other angiotensin peptides that

have been identified, including Ang (1-9), Ang (1-12), Ang V(3-7), as well as several other shorter peptides that have undetermined functions.

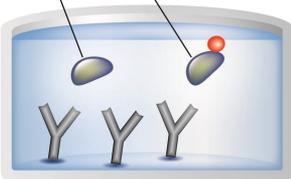
2. ASSAY SUMMARY

Capture Antibody



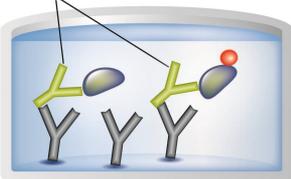
Prepare all reagents and samples as instructed.

Sample Biotin Labeled Conjugate



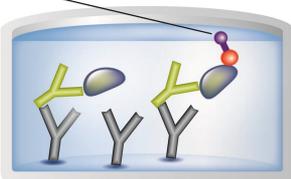
Add samples and Biotinylated antigen and to appropriate wells. Incubate at room temperature.

Target Antibody



Add Angiotensin I polyclonal Antibody to appropriate wells. Incubate at room temperature.

Streptavidin-HRP



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

Substrate Colored Product



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.

- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in 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

4. STORAGE AND STABILITY

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

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition
Goat anti-Rabbit IgG Microplate (12 x 8 wells)	96 Wells	2-8°C
Angiotensin A Biotin Conjugate	1 Vial	2-8°C
Angiotensin A Antibody	1 Vial	2-8°C
Angiotensin A Standard	2 x 40 ng	-20°C
Assay Buffer 16	30 mL	2-8°C
20X Wash Buffer Concentrate	27 mL	2-8°C
TMB Substrate	2 x 10 mL	2-8°C
Stop Solution 2	10 mL	2-8°C
Streptavidin-HRP	12.5 µg	2-8°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 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
- Protease inhibitor cocktail (PIC)
- Phenylmethylsulfonyl fluoride (PMSF)
- Deionized or distilled water

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

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
- 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 Streptavidin-HRP**

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

9.2 **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.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Reconstitution of the Angiotensin A standard should be prepared no more than 1 hour prior to the experiment. Diluted standards should be used within 60 minutes of preparation.

- 10.1 Reconstitute one vial of Angiotensin A with 1 mL assay buffer to yield a 40 ng/mL **stock standard**. Vortex to ensure the entire cake is dissolved.
- 10.2 Label six tubes with numbers 1 – 6 and another tube with B₀.
- 10.3 Add 750 µL assay buffer to all tubes
- 10.4 Prepare a 10,000 pg/mL **Standard 1** by adding 250 µL of the 40,000 pg/mL Stock Standard to tube 1. Mix thoroughly and gently.
- 10.5 Prepare **Standard 2** by transferring 250 µL from Standard 1 to tube 2. Mix thoroughly and gently.
- 10.6 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube 3. Mix thoroughly and gently.
- 10.7 Using the table below as a guide, repeat for tubes 4 through 6.
- 10.8 B₀ contains no protein and is the Blank Activity control.

ASSAY PREPARATION

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	250	10,000	2,500
3	Standard 2	250	250	2,500	625
4	Standard 3	250	250	625	156
5	Standard 4	250	250	156	39.1
6	Standard 5	250	250	39.1	9.8
B ₀	None	-	250	-	0



11. SAMPLE COLLECTION AND STORAGE

- The assay is suitable for the measurement of Angiotensin A serum and plasma. 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 plasma samples is 1:4 and from serum samples is 1:16. Due to differences in samples, users must determine the optimal sample dilution for their particular experiments.

11.1 Protocol for Plasma samples

- 11.1.1 Collect whole blood in vacutainer tubes containing EDTA.
- 11.1.2 Centrifuge at 1000 x g for 15 minutes at 4°C.
- 11.1.3 Remove supernatant (plasma) to a clean tube containing protease inhibitor cocktail to a final concentration of 0.05% and PMSF to a final concentration of 1mM.
- 11.1.4 Samples can be divided into aliquots and stored at or below -20°C, or used immediately in the assay.
- 11.1.5 Frozen samples may be stored for up to two weeks.
- 11.1.6 Avoid repeated freeze-thaw cycles.

11.2 Protocol for Serum Samples

- 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 1000 x g for 15 minutes at 4°C. Do not use brake.
- 11.2.4 Without disturbing the cell layer, place supernatant into a clean tube containing protease inhibitor cocktail to a final concentration of 0.05% and PMSF to a final concentration of 1mM.

- 11.2.5 The supernatant may be divided into aliquots and stored at or below -20°C , or used immediately in the assay.
- 11.2.6 Samples may be stored frozen for up to two weeks.
- 11.2.7 Avoid repeated freeze-thaw cycles.

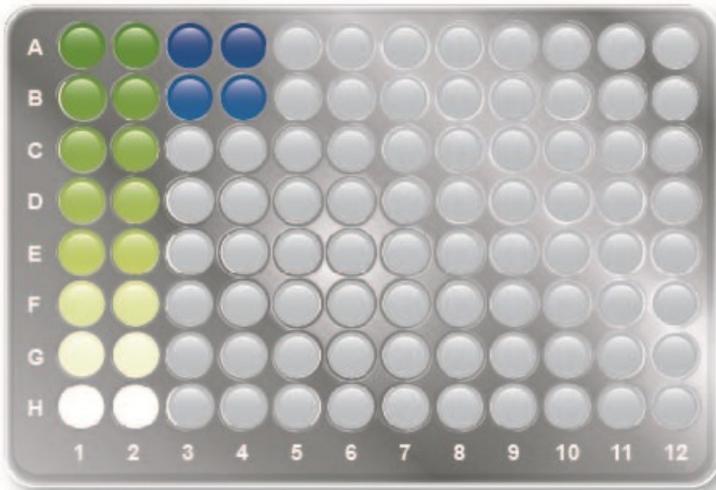
Angiotensins can be present in very low concentrations in some biological samples. In such cases, dilution of samples to avoid “non-specific” interference by any present factors is not productive because the angiotensin analyte is also diluted to levels far below the minimum detection concentration. 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); b) phenylsilylsilica cartridge extraction of Ang (1-7) from blood, eluted with methanol.

*Please note that the extraction methods described herein have not been validated for the Angiotensin A kit; researchers are strongly encouraged to optimize conditions for their specific samples.

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 returned to 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

Recommended plate layout



A1-2 - Standard #1
B1-2 - Standard #2
C1-2 - Standard #3
D1-2 - Standard #4
E1-2 - Standard #5
F1-2 - Standard #6
G1-2 - Standard #7 (Bo)
H1-2 - Blank Wells

A3-4 - Total Activity Wells (TA)
B3-4 - Non-Specific Binding Wells (NSB)

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 Prepare all reagents, working standards, and samples as directed in the previous sections.
 - 13.2 Add 100 μL of Assay Buffer into the B₀ well.
 - 13.3 Add 150 μL appropriate standard diluent (Assay Buffer or tissue culture media) into the NSB wells.
 - 13.4 Add 100 μL of prepared standards and diluted samples to appropriate wells.
 - 13.5 Add 50 μL of Angiotensin A Conjugate to all wells.
 - 13.6 Add 50 μL of Angiotensin A antibody to all wells.
 - 13.7 Seal the plate. Incubate the plate at room temperature for 2 hours on a plate shaker (~500 rpm).
 - 13.8 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.
 - 13.9 Add 200 μL of 1X Streptavidin-HRP to each well except the blank.
 - 13.10 Seal the plate. Incubate the plate at room temperature for 30 minutes on a plate shaker (~500 rpm).
 - 13.11 Wash as described in step 13.8.
 - 13.12 Add 200 μL of the TMB Substrate solution to every well. Incubate at room temperature for 30 minutes without shaking.

ASSAY PROCEDURE

- 13.13 Add 50 μ L Stop Solution into each well. The plate should be read immediately.
- 13.14 Read the O.D. absorbance at 450 nm, preferably with correction between 570 and 590 nm.

14. CALCULATIONS

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.

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

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

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

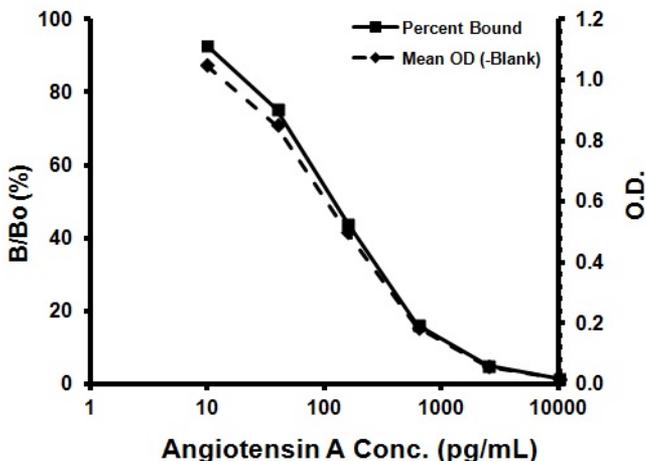
$$\text{Percent Bound} = (\text{Net OD} / \text{Net Bo OD}) \times 100$$

3. Plot both the Percent Bound and the Net OD versus Concentration of Angiotensin A for the standards. Sample concentrations may be calculated off of Net OD values using the desired curve fitting

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	Mean OD (-Blank)	% Bound	Angiotensin A pg/mL
Blank (mean)	(0.04)	-	-
NSB	0.004	0	-
Standard 1	0.017	1.5	10,000
Standard 2	0.057	5.1	2,500
Standard 3	0.186	16.4	625
Standard 4	0.499	44.1	156.3
Standard 5	0.851	75.4	39.1
Standard 6	1.048	92.8	9.8
Bo	1.130	100	0

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The sensitivity, minimum detectable dose of Angiotensin A using this Abcam ELISA kit was found to be 8.3 pg/mL. This was determined by the average optical density of the 0 pg/mL Standard and comparing to the average optical density for Standard 7. The detection limit was determined as the concentration of Angiotensin A measured at two standard deviations from the zero along the standard curve.

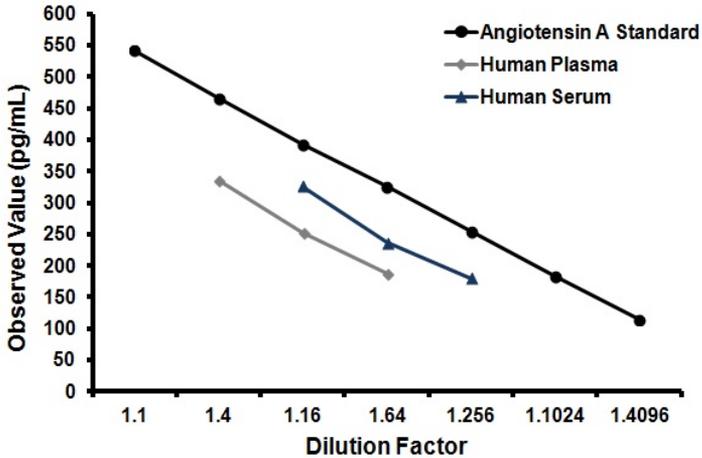
LINEARITY OF DILUTION –

Human samples containing Angiotensin A 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	
	Human Plasma	Human Serum
1:4	115	-
1:16	90	112
1:64	100	87
1:256	-	100

PARALLELISM –

Dose-response curves from human plasma and serum diluted into assay buffer were compared to the Angiotensin A standard curve. The parallel response indicates the standard effectively mimics the native protein.



PRECISION –

	Angiotensin A (pg/mL)	Intra-Assay %CV
Low	55.2	10.3
Medium	655.4	4.3
High	1297.9	7.5

	Angiotensin A (pg/mL)	Inter-Assay %CV
Low	55.6	17.7
Medium	612.2	5.2
High	1248.2	10.9

SAMPLE RECOVERY –

Recombinant Angiotensin A was spiked at high, medium and low concentrations to samples that had been diluted sufficiently to eliminate matrix interference. Endogenous Angiotensin A was subtracted from the spiked values and the average recovery in each of 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	Dilution	Spike Concentration (ng/mL)	% Recovery of Spike	Range of recovery %
Human Plasma	1:4	5,000	110	104-112
		500	113	107-117
		50	104	100-107
Human Serum	1:16	5,000	91	84-100
		500	85	81-90
		50	105	94-113

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	% Cross Reactivities in the range of 0.1pM – 500nM
Ang (1-12)	0.505
Ang I	0.000
Ang (1-9)	0.094
Ang II	0.000
Ang (1-7)	0.005
Ang A	100
Ang III	0.000
Ang IV	0.000
LVV-hemorphin 7	0.001
Bradykinin	0.025

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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