Rhoa ELISA Kit (Rat)
(OKCD02814)
Lot# KD5533
Instructions for use

For the quantitative measurement of Rhoa in tissue homogenates, cell lysates and other biological fluids.

Lot to lot variations can occur. Use the manual provided with the kit packaging.

This product is intended for research use only.

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1. Background

Principle

Aviva Systems Biology Rhoa ELISA Kit (Rat) (OKCD02814) is based on standard sandwich enzyme-linked immuno-sorbent assay technology. An antibody specific for Rhoa has been pre-coated onto a 96-wellplate (12 x 8 Well Strips). Standards or test samples are added to the wells, incubated and removed. A biotinylated detector antibody specific for Rhoa is added, incubated and followed by washing. Avidin-Peroxidase Conjugate is then added, incubated and unbound conjugate is washed away. An enzymatic reaction is produced through the addition of TMB substrate which is catalyzed by HRP generating a blue color product that changes to yellow after adding acidic stop solution. The density of yellow coloration is read by absorbance at 450 nm and is quantitatively proportional to the amount of sample Rhoa captured in well.

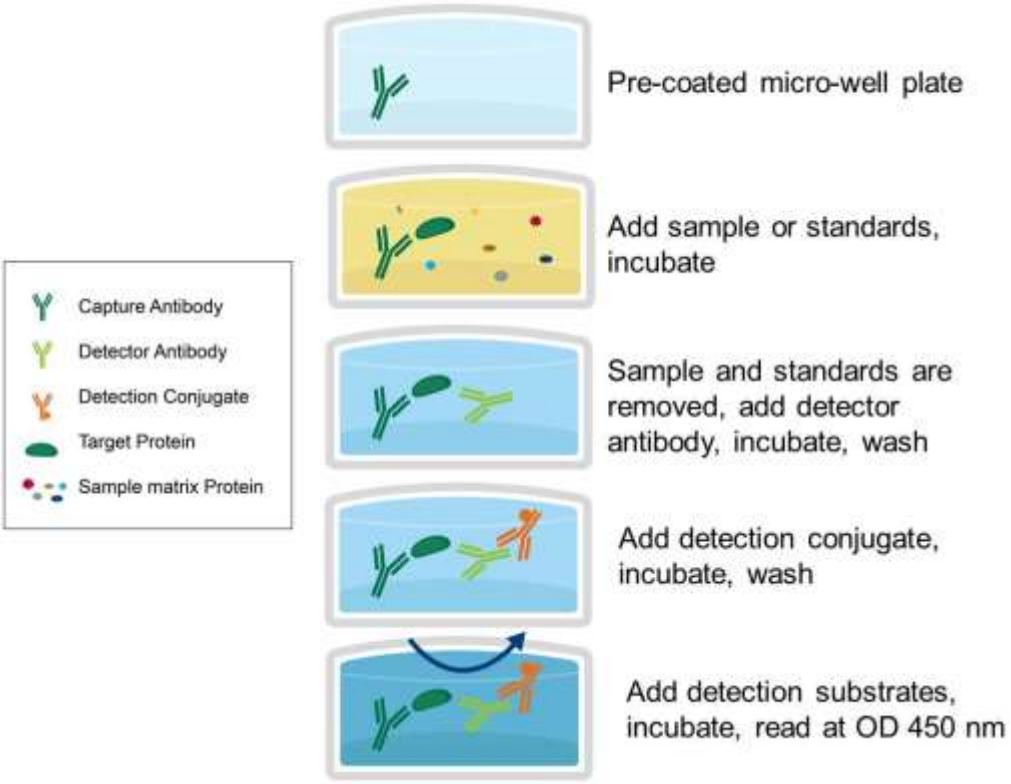
Background

Regulates a signal transduction pathway linking plasma membrane receptors to the assembly of focal adhesions and actin stress fibers. Involved in a microtubule-dependent signal that is required for the myosin contractile ring formation during cell cycle cytokinesis. Plays an essential role in cleavage furrow formation. Required for the apical junction formation of keratinocyte cell-cell adhesion. May be an activator of PLCE1. Activated by ARHGEF2, which promotes the exchange of GDP for GTP. Essential for the SPATA13-mediated regulation of cell migration and adhesion assembly and disassembly. The MEMO1-RHOA-DIAPH1 signaling pathway plays an important role in ERBB2-dependent stabilization of microtubules at the cell cortex. It controls the localization of APC and CLASP2 to the cell membrane, via the regulation of GSK3B activity. In turn, membrane-bound APC allows the localization of the MACF1 to the cell membrane, which is required for microtubule capture and stabilization. Regulates KCNA2 potassium channel activity by reducing its location at the cell surface in response to CHRM1 activation; promotes KCNA2 endocytosis.

General Specifications

General Specifications	
Range	0.156 – 10 ng/mL
LOD	< 0.063 ng/mL (Derived by linear regression of OD_{450} of the Mean Blank + 2xSD)
Specificity	Rat Transforming protein RhoA UniProt ID: P61589 GeneID: 117273 Target Alias: Arha, Arha2, MGC72339, Transforming protein RhoA
Cross-Reactivity	No detectable cross-reactivity with other relevant proteins

2. Assay Summary



3. Storage and Stability

• Open kit immediately upon receipt. Store components at -20°C (NOTE: exceptions below) for 6 months or until expiration date. Avoid any freeze/thaw cycles.

4. Kit Components

•The following reagents are the provided contents of the kit.

Description	Quantity	Storage Conditions
Anti-Rhoa Microplate	96 Wells (12 x 8 Well strips)	-20°C for 6 months
Rhoa Lyophilized Standard	2 x 20 ng	
100X Biotinylated Rhoa Detector Antibody	1 x 120 µL	
100X Avidin-HRP Conjugate	1 x 120 µL	
Standard Diluent	1 x 20 mL	Store at 4°C for 6 months
Detector Antibody Diluent	1 x 12 mL	
Conjugate Diluent	1 x 12 mL	
30X Wash Buffer	1 x 20 mL	
Stop Solution	1 x 6 mL	
TMB Substrate	1 x 9 mL	

5. Precautions

• Read instructions fully prior to beginning use of the assay kit.

• Any deviations or modifications from the described method or use of other reagents could result in a reduction of performance.

• Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.

• For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).

• Kit cannot be used beyond the expiration date on the label.

6. Required Materials Not Supplied

• Microplate reader capable of reading absorbance at 450 nm.

• Automated plate washer (optional).

• Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.

• Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.

• New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.

• Absorbent paper or paper toweling.

• Distilled or deionized ultrapure water.

• 37°C Incubator (optional)

7. Technical Application Tips

• Do not mix or substitute components from other kits.

• To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.

• Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.

• Prior to using the kit, briefly spin component tubes to collect all reagents at the bottom.

• Replicate wells are recommended for standards and samples.

• Cover microplate while incubating to prevent evaporation.

• Do not allow the microplate wells dry at any point during the assay procedure.

• Do not reuse tips or tube to prevent cross contamination.

• Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.

• Completely remove of all liquids when washing to prevent cross contamination.

• Prepare reagents immediately prior to use and do not store, with the exception of the top standard.

• Equilibrate all materials to ambient room temperature prior to use (standards exception).

• For optimal results for inter- and intra-assay consistency, equilibrate all materials to room temperature prior to performing assay (standards exception) and perform all incubations at 37°C.

• Pipetting less than 1 µL is not recommended for optimal assay accuracy.

• Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.

• Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.

• Samples containing precipitates, fibrin strands or bilirubin, or are hemolytic or lipemic might cause inaccurate results due to interfering factors.

• TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.

8. Reagent Preparation

• Equilibrate all materials to room temperature prior to use and use immediately.

8.1 Rat Rhoa Assay Standards

8.1.1 Prepare the **Rhoa** standards no greater than 2 hours prior to performing experiment. Standards should be held on ice until use in the experiment.

8.1.2 Reconstitute one vial of the provided **20 ng Lyophilized Rhoa Standard** for each experiment. Prepare the stock **20 ng/mL Standard** by reconstituting one tube of **Lyophilized Rhoa Standard** as follows:

8.1.2.1 Gently spin or tap the vial at 6,000 – 10,000 rpm for 30 seconds to collect all material at the bottom.

8.1.2.2 Add **1.0 mL** of **Standard Diluent** to the vial.

8.1.2.3 Seal the vial then mix gently and thoroughly.

8.1.2.4 Leave the vial at ambient temperature for 15 minutes.

8.1.3 Prepare a set of seven serially diluted standards as follows:

8.1.3.1 Label tubes with numbers 1 – 8.

8.1.3.2 Add 300 µL of **Standard Diluent** to Tube #'s 2 – 8.

8.1.3.3 Prepare a **10 ng/mL Rhoa Standard #1** by adding 500 µL µL of reconstituted **20 ng/mL Rhoa Standard** to 500 µL µL of **Standard Diluent** in Tube #1. Mix gently and thoroughly.

8.1.3.4 Prepare **Standard #2** by adding 300 µL of **10 ng/mL Rhoa Standard #1** (Tube #1) to Tube #2. Mix gently and thoroughly.

8.1.3.5 Prepare **Standard #3** by adding 300 µL of **Standard #2** from Tube #2 to Tube #3. Mix gently and thoroughly.

8.1.3.6 Prepare further serial dilutions through Tube #7. Reference the table below as a guide for serial dilution scheme.

8.1.3.7 Tube #8 is a blank standard (only **Standard Diluent**), which should be included with every experiment.

Standard Number (Tube)	Standard To Dilute	Volume Standard to Dilute (µL)	Volume Standard Diluent (µL)	Total Volume (µL)	Final Concentration
1	20 ng/mL Reconstituted Rhoa Standard	500	500	1,000	10 ng/mL
2	10 ng/mL	300	300	600	5.0 ng/mL
3	5.0 ng/mL	300	300	600	2.5 ng/mL
4	2.5 ng/mL	300	300	600	1.25 ng/mL
5	1.25 ng/mL	300	300	600	0.625 ng/mL
6	0.625 ng/mL	300	300	600	0.312 ng/mL
7	0.312 ng/mL	300	300	600	0.156 ng/mL
8	NA	0	300	300	0.0 (Blank)

8.2 1X Biotinylated Rhoa Detector Antibody

8.2.1 Prepare the **1X Biotinylated Rhoa Detector Antibody** immediately prior to use by diluting the **100X Biotinylated Rhoa Detector Antibody** 1:100 with **Detector Antibody Diluent**.

8.2.2 For each well strip to be used in the experiment (8-wells) prepare 1,000 µL by adding 10 µL of **100X Biotinylated Rhoa Detector Antibody** to 990 µL **Detector Antibody Diluent**.

8.2.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure. Do not store at 1X concentration for future use.

8.3 1X HRP-Avidin Conjugate

8.3.1 Prepare the **1X Avidin-HRP Conjugate** immediately prior to use by diluting the **100X Avidin-HRP Conjugate** 1:100 with **Conjugate Diluent**.

8.3.2 For each well strip to be used in the experiment (8-wells) prepare 1,000 µL by adding 10 µL of **100X Avidin-HRP Conjugate** to 990 µL **Conjugate Diluent**.

8.3.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure. Do not store at 1X concentration for future use.

8.4 1X Wash Buffer

8.4.1 If crystals have formed in the **30X Wash Buffer** concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.

8.4.2 Add the entire 20 mL contents of the **30X Wash Buffer** bottle to 580 mL of ultra-pure water to a clean > 1,000 mL bottle or other vessel.

8.4.3 Seal and mix gently by inversion. Avoid foaming or bubbles.

8.4.4 Store the **1X Wash Buffer** at room temperature until ready to use in the procedure. Store the prepared **1X Wash Buffer** at 4°C for no longer than 1 week. Do not freeze.

8.5 Microplate Preparation

- Micro-plates are provided ready to use and do not require rinsing or blocking.
- Unused well strips should be returned to the original packaging, sealed and stored at 4°C.
- Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

9. Sample Preparation

9.1 Sample Preparation and Storage

• Store samples to be assayed at 2-8°C for 24 hours prior being assayed.

• For long term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

• Samples not indicated in the manual must be tested to determine if the kit is valid.

• Prepare samples as follows:

• **Tissue homogenates** - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS (0.01mol/L,pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5-10mL of PBS with a glass homogenizer on ice (Micro Tissue Grinders woks, too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5000 x g. Remove the supernate and assay immediately or aliquot and store at ≤ -20°C.

• **Cell Lysates** - Cells must be lysed before assaying according to the following directions.
1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly). Wash cells three times in cold PBS.
3. Resuspend cells in PBS (1 x) and the cells was subject to ultrasonication for 4 times (or Freeze cells at ≤ -20°C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for 3 times.)
4. Centrifuge at 1500 x g for 10 minutes at 2-8°C to remove cellular debris.

• **Other biological fluids** – Centrifuge samples for 20 minutes at 1,000 x g Collect the supernant and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

9.2 Sample Dilution

Target protein concentration must be estimated and appropriate sample dilution selected such that the final target protein concentration falls near the middle of the assay linear dynamic range. Samples exhibiting saturation should be further diluted.

• Dilute samples using **Standard Diluent**.

• Mix diluted samples gently and thoroughly.

• Pipetting less than 2 µL is not recommended for optimal assay accuracy.

10. Assay Procedure

• Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.

• Optimal results for intra- and inter-assay reproducibility will be obtained when performing incubation steps at 37°C as indicated below.

10.1 Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.

10.2 Add 100 µL of serially titrated standards, diluted samples or blank into wells of the **Anti-Rhoa Microplate**. At least two replicates of each standard, sample or blank is recommended.

10.3 Cover the plate with the plate sealer and incubate at 37°C for 60 minutes.

10.4 Remove the plate sealer and discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.5 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.6 Add 100 µL of prepared **1X Biotinylated Rhoa Detector Antibody** to each well.

10.7 Cover with the plate sealer and incubate at 37°C for 60 minutes.

10.8 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.9 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.10 Wash plate 3 times with **1X Wash Buffer** as follows:

10.10.1 Add 300 µL of **1X Wash Buffer** to each assay well.

10.10.2 Incubate for 1 minute.

10.10.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.

10.10.4 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.10.5 Repeat steps 10.10.1 through 10.10.4 **two** more times.

10.11 Add 100 µL of prepared **1XAvidin-HRP Conjugate** into each well, cover with plate sealer and incubate at 37°C for 30 minutes.

10.12 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.13 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.14 Wash plate **5 times** with **1X Wash Buffer** as in Step 10.10.

10.15 Add 90 µL of **TMB Substrate** to each well, cover with plate sealer and incubate at 37°C **in the dark** for 10-20 minutes. Wells should change to gradations of blue. If the color is too deep, reduce the incubation time.

(NOTE: optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the top four standard wells, while the remaining standards are still clear.)

10.16 Add 50 µL of **Stop Solution** to each well. Well color should change to yellow immediately. Add the **Stop Solution** in the same well order as done for the **TMB Substrate**.

10.17 Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.16. If wavelength correction is available, set to 540 nm or 570 nm.

11. Calculation of Results

For analysis of the assay results, calculate the **Relative OD_{450}** for each test or standard well as follows:

$$(\text{Relative } OD_{450}) = (\text{Well } OD_{450}) - (\text{Mean Blank Well } OD_{450})$$

The standard curve is generated by plotting the mean replicate **Relative OD_{450}** of each standard serial dilution point vs. the respective standard concentration. The **Rhoa** concentration contained in the samples can be interpolated by using linear regression of each mean sample **Relative OD_{450}** against the standard curve. This is best achieved using curve fitting software.

Note: If wavelength correction readings were available, subtract the readings at 540 nm or 570 nm from the readings at 450 nm. This may provide greater reading accuracy.

Note: If the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

12. Typical Expected Data

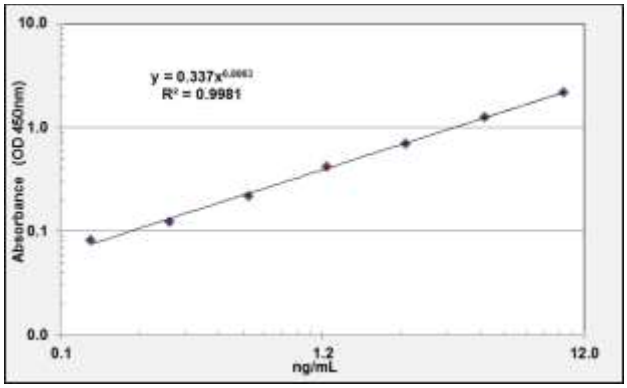
12.1 Reproducibility

Intra-assay Precision: 3 samples with known low, middle and high levels Rhoa were tested with 20 replicates on one plate, respectively. Inter-assay Precision: 3 samples with known low, middle and high level Rhoa were tested on 3 different plates, 8 replicates in each plate.

Sample	Intra-Assay			Inter-Assay		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	20	20	20	24	24	24
Mean (ng/ml)	0.23	1.21	4.98	0.25	1.26	4.85
SD	0.016	0.077	0.289	0.014	0.074	0.296
CV (%)	7.1	6.4	5.8	5.6	5.9	6.1

12.2 Typical standard curve

This standard curve is for demonstration purposes only. An assay specific standard curve should be performed with each assay.



ng/mL	Absorbance		Mean Absorbance	Blank Subtracted
	Rep 1	Rep 2		
10	2.178	2.198	2.188	2.141
5.0	1.258	1.292	1.275	1.228
2.5	0.697	0.689	0.693	0.646
1.25	0.415	0.407	0.411	0.364
0.625	0.217	0.221	0.219	0.172
0.312	0.123	0.127	0.125	0.078
0.156	0.082	0.084	0.083	0.036
Blank	0.048	0.046	0.047	NA

13. Technical Resources

Technical Support:

For optimal service please be prepared to supply the lot number of the kit used.

USA

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