RabBridge ELISA Kit

1 Plate Kit Catalog # AB-000222

Complete kit for the systematic Higher Order Structure (HOS) comparability analysis of rabbit polyclonal antibodies as Critical Reagents.

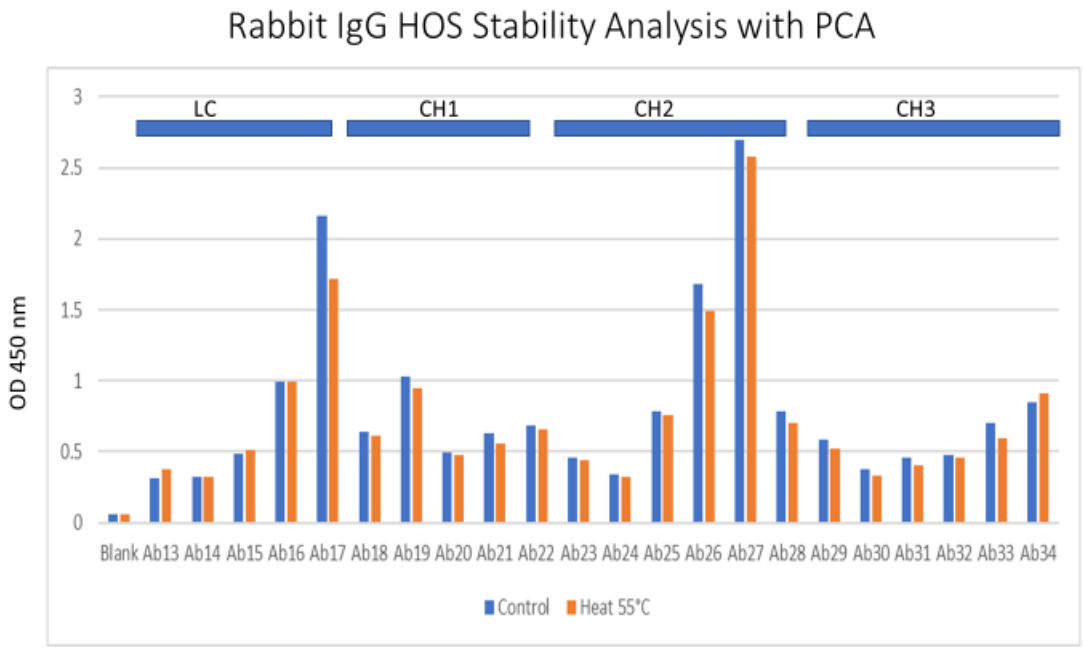
Please read this insert completely prior to performing the assay.
This kit is intended for research use only. Not for use in diagnostic procedures.

Assay Principle

The assay is in a Direct ELISA format where the plate is coated with a panel of antibodies raised against peptides derived from the constant regions of human IgG. Because of relative high homology between human and rabbit IgG constant regions, this antibody array developed from the human IgGs can analyze Higher Order Structure (HOS) changes of rabbit polyclonal antibodies with good sensitivity, the data can be used for the characterization and management of rabbit polyclonal antibodies as Critical Reagents.

For the Protein Conformational Array (PCA), taken individually, each of these antibodies is strongly antigenic to the peptide sequence that was used in its production. However, when these peptides are incorporated into a full length correctly folded protein, the antigenicity of many of them is masked by the three dimensional structure of the protein and only a limited number of the antibodies respond. The result is a histogram which can be likened to a 'fingerprint' for any given monoclonal antibody. Once that 'fingerprint' is established, then any changes that occur in that mAb during purification, storage or other manipulations will likely expose a new antigenic site which can be detected by the antibodies on the plate,

The assay is performed by making a 10 µg/ml solution of both test and reference rabbit polyclonal antibodies in PBS (phosphate-buffered saline), and adding to the 96-well plate. Following an overnight incubation at 4°C to allow coating of the antibodies to the microplate, 22 biotin-labeled antibodies covering the IgG constant regions are added to 22 columns on the microplate. After incubation, streptavidin conjugated with HRP (Horse Radish Peroxidase) is added and incubated for 1 hour to allow it to bind to any captured biotin-labeled antibodies. After this incubation, the plate is washed and TMB substrate is added and is converted by the captured HRP to a colored product in proportion to the amount of HRP bound to the plate. After a short incubation to allow color development, the reaction is stopped, and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The color development will be proportional to the captured biotin-labeled antibody, which in turn reflects epitope exposure from the testing samples initially coated on the microplate. A typical ELISA is shown in the page.



Supplied Components:

Clear 96 Well Plates
2 clear plastic microtiter plates are provided to coat the test and reference rabbit antibodies.
Kit AB-000222 (2 plates)

5x Dilution Buffer
Buffer used for dilution of antibodies and Streptavidin-HRP conjugate. The 20 ml of concentrate should be diluted to 100 ml with 80 ml deionized or distilled water.
Kit AB-000222 (20 ml)

10x PBS-T
After dilution, it is used as wash solution. The 50 ml of concentrate should be diluted to 500 ml with 450 ml deionized or distilled water.

Kit AB-000222 (50 ml)

Reporting antibody
22 different biotin-labeled rabbit polyclonal antibody covering the human IgG constant regions. Immediately prior to the assay, dilute the entire 100 µl from each of the 22 vials into 22 vials of 0.9 ml of 1x Dilution buffer to give a 20 µg/ml working stock.
Kit AB-000222 (0.2 mg/ml, 100 µl / tube)

Streptavidin-HRP Conjugate
A Streptavidin – Horse Radish Peroxidase conjugate in a special stabilizing solution. Immediately prior to the assay, dilute the entire 750 µl into 30 ml of 1x Dilution buffer to give a 0.1 µg/ml working stock.
Kit AB-000222 (4 µg/ml, 750 µl / tube)

TMB Substrate
Use directly without dilution.
Kit AB-000222 (30 ml)

Stop Solution
A 1M solution of sulfuric acid. CAUSTIC. Use directly without dilution.
Kit AB-000222 (30 ml)

Plate Sealer
Kit AB-000222 (two)

Other Materials Required
Distilled or deionized water.
Single- and multi-channel micro-pipettes with disposable tips to accurately dispense volumes 5-250 µL.
Plastic tubes (i.e. 1.5 ml – 15 ml) for sample dilution
Reagent reservoirs for sample addition
Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on Page 4.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

Procedural Notes

Allow diluted reagents and buffers to reach room temperature (18-25°C) prior to starting the assay. Once the assay has been started, all steps should be completed in sequence and without interruption. You do not want the plate to dry out in between steps as this can cause high backgrounds or erroneous results. Make sure that required reagents and buffers are ready when needed. Prior to adding to the plate, reagents should be mixed gently (not vortexed) by swirling.

Avoid contamination of reagents, pipette tips and wells. Use new disposable tips and reservoirs, do not return unused reagent to the stock bottles / vials and do not mix caps of stock solutions.

Incubation time can affect results. All wells should be handled in the same order for each step.

Microplate washing is important and can affect results by giving erroneous results or high backgrounds. We recommend a multichannel pipette to add 250 µl of buffer to each well across the plate, followed by a dumping out of contents (to a sink or other receptacle) with a rapid wrist motion. The plate should then be tapped firmly on a paper towel to shake out any remaining liquid. Avoid prolonged incubation with wash buffer when performing wash steps.

When making additions to the plate, be careful to avoid damaging the antibody coating, for example by scratching the bottoms or the sides of the wells. One technique to avoid this is to make additions (for a right-handed person) from left to right across the plate, supporting the pipette tips on the right edge of the well with each addition and thus avoiding contact with the bottom or sides of the wells.

During the incubation times, the plate should be covered to minimize evaporation from the wells. This can be done with the adhesive covers provided or by stacking an empty plate on top.

After the last wash step and prior to adding the TMB substrate, wipe the bottom of the plate with a clean paper towel to ensure that moisture or fingerprints do not interfere with the OD reading.

Once the TMB substrate is added it will be converted by the captured HRP to a blue colored product. Generally we find that a 10 to 15 minute incubation is sufficient for enough color development to discern differences between the standards and the reaction should be stopped at this point. Bear in mind that, given sufficient time, even a small amount HRP is capable of converting all the TMB to product. Keeping OD_{450} values well below 2.0 will result in greatest accuracy as at high absorbance values very little light is reaching the detector and measurements are error prone. (Remember that at an OD of 1.0 only 10% of the light is being detected and at an OD of 2.0 only 1% of the light is reaching the detector).

Assay Protocol

1. Use the plate layout sheet on the back page to plan sample layout on plate and also aid in proper sample and antibody identification after the assay. Each plate is laid out as shown on the plate maps on the following pages, with each unique reporting antibody appearing in 6 positions on the plate (if duplicate assays are selected and the full microplate is used for 4 sample analysis, the reporting antibodies will be added to the 8 wells in each column). Rows A and H are not used in order to minimize edge effects. We recommend that assays are carried out in duplicate or (preferably) triplicate in order to minimize spurious results. For example, we have shown the plate layout for an experiment in triplicate, where the wells used for the reference antibody are highlighted and the three rows underneath are used for the test antibody. For an experiment in duplicate, use rows A to B for the reference antibody and rows C to H for three test antibodies.

2. Dilute the 10xPBS-T and 5x Dilution buffer with Milli-Q water to 1x-strength. Check both concentrate bottles for precipitates before proceeding and if found warm slightly in a water bath to dissolve before proceeding. The 50 ml of 10xPBS-T should be diluted to 500 ml with 450 ml water and the 20 ml of 5x Dilution Buffer should be diluted to 100 ml with 80 ml water.

3. Coating the plate: dilute your test antibodies and reference rabbit polyclonal antibody to a concentration of 10 μg/ml in PBS; prepare at least 12 ml of each if samples are to be run in duplicate or triplicate. Pipette 100 µL of 10 μg/ml test or reference antibody solution into each row of the plate. For replicates use multiple rows, i.e. reference in rows 2 to 4, test sample in rows 5-7. Cover the plate and incubate at 4°C in a refrigerator overnight. If testing antiserum, dilute the antiserum with PBS at 1:100 and coat at 100 µL/well with the same coating condition as the purified antibody.

4. The next day, empty the coating solution and wash with 250 µL/well with wash buffer (PBS-T), repeat wash.

5. Add 250 µL/well of blocking buffer (1xDilutiopn Buffer, PBS-T with 1% BSA) to each of the 96-well plate and incubate at room temperature for 60 min.

6. During the above incubation, mark the 22 1.7 mL vials from 13 to 34 and add 0.9 mL dilution buffer to each vial. Prepare the reporting antibody stock solution by adding the entire 100 µL from each of the 22 vials supplied to each of the 1.7 mL vials just prepared.

7. Wash plate by emptying contents and adding 250 µl of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat.

8. Pipette 100 µL of Reporting Antibody into each well, one reporting antibody per column, the first column in each of the two plates is used as blank by adding blocking buffer only at 100 µL/well . Cover plate and incubate plate 60 min at room temperature.

9. During the above incubation, dilute the 4 µg/ml Streptavidin-HRP conjugate to 0.1 µg/ml by adding the entire 750 µL to 30 ml of Dilution Buffer.

10. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat.

11. Pipette 100 µL/well of 0.1 µg/ml Streptavidin-HRP conjugate into each well. Cover plate and incubate plate 45 min at room temperature.

12. Wash plate by emptying contents and adding 250 µL of wash buffer to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat 2 more times

13. Add 100 µL/well of TMB substrate to the plate. Allow color development to proceed for 15 minutes and then stop reaction by adding 100 µL of Stop Solution to each well (if the color develops too fast, stop the reaction earlier, if too slow add more incubation time). Upon addition of stop solution, developed color will change from blue to yellow.

14. Read the optical density generated from each well in a plate reader capable of reading at 450 nm, Use wells 1B to 1G as blank.

15. Export the plate reader data into Excel and calculate an average and variance for each set of replicates. If the variance is large inspect the raw data to determine the problem. With data in triplicate, one outlier may be evident, but if data is in duplicate, the higher value is generally suspect (it's easier to get a high value in error than a low value). Graph the data as a bar graph so that for each array antibody the response can be compared between your sample and standard. Any differences between your sample and the standard should be apparent.

Plate 1 Template (constant region-1)

Control compound suggested use in wells marked

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Blank	Ab13	Ab14	Ab15	Ab16	Ab17	Ab18	Ab19	Ab20	Ab21	Ab22	Ab23
C	Blank	Ab13	Ab14	Ab15	Ab16	Ab17	Ab18	Ab19	Ab20	Ab21	Ab22	Ab23
D	Blank	Ab13	Ab14	Ab15	Ab16	Ab17	Ab18	Ab19	Ab20	Ab21	Ab22	Ab23
E	Blank	Ab13	Ab14	Ab15	Ab16	Ab17	Ab18	Ab19	Ab20	Ab21	Ab22	Ab23
F	Blank	Ab13	Ab14	Ab15	Ab16	Ab17	Ab18	Ab19	Ab20	Ab21	Ab22	Ab23
G	Blank	Ab13	Ab14	Ab15	Ab16	Ab17	Ab18	Ab19	Ab20	Ab21	Ab22	Ab23
H												

Plate 2 Template (constant region-2)

Control compound suggested use in wells marked

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Blank	Ab24	Ab25	Ab26	Ab27	Ab28	Ab29	Ab30	Ab31	Ab32	Ab33	Ab34
C	Blank	Ab24	Ab25	Ab26	Ab27	Ab28	Ab29	Ab30	Ab31	Ab32	Ab33	Ab34
D	Blank	Ab24	Ab25	Ab26	Ab27	Ab28	Ab29	Ab30	Ab31	Ab32	Ab33	Ab34
E	Blank	Ab24	Ab25	Ab26	Ab27	Ab28	Ab29	Ab30	Ab31	Ab32	Ab33	Ab34
F	Blank	Ab24	Ab25	Ab26	Ab27	Ab28	Ab29	Ab30	Ab31	Ab32	Ab33	Ab34
G	Blank	Ab24	Ab25	Ab26	Ab27	Ab28	Ab29	Ab30	Ab31	Ab32	Ab33	Ab34
H												