EpoBridge ELISA Kit

1 Plate Kit Catalog # AB000210

Complete kit for the systematic 3-D conformational comparability analysis of Epoetin-A biosimilar molecules.

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

Background information

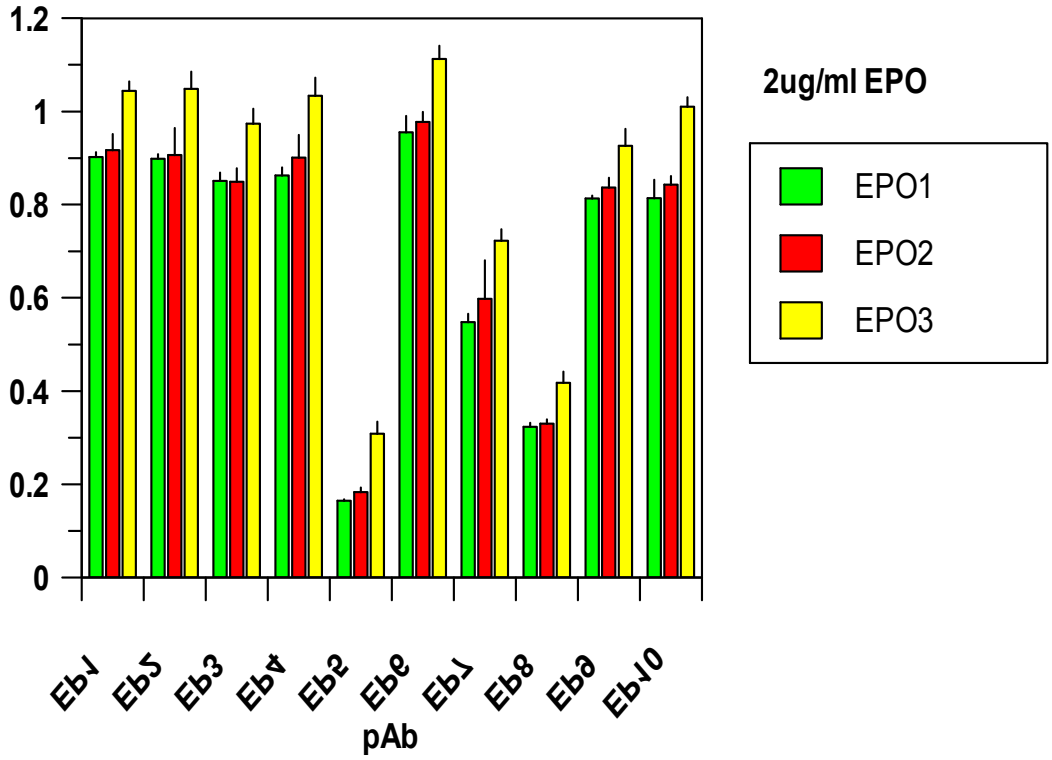
Epoetin α is a human erythropoietin produced in cell culture using recombinant DNA technology, it stimulates erythropoiesis (increases red blood cell levels) and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy. This kit will allow for 3-D conformational comparison (Higher-Order Structure) between EPO Biosimilars and authentic Epoetin.

Assay Principle

The assay is in a sandwich ELISA format where the plate is coated with a panel of antibodies raised against peptides derived from the full length protein sequence of Epoetin. 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 correctly folded Epoetin. For an EPO Biosimilar, if the protein is correctly folded and glycosylated, the 'fingerprint' will match that of Epoetin. If it is not correctly folded, previously masked peptide sequences will be exposed and will be recognized by the antibody made to that exposed sequence. In this way, changes in the 'fingerprint' generated by the ELISA will point out differences between the Biosimilar and authentic Epoetin.

The assay is performed by making a 2 µg/ml solution of EPO Biosimilar and Epoetin reference material respectively, and adding to the 96-well plate which was pre-coated with capturing antibodies the day before. It should be pointed out that in this sandwich ELISA assay, no blocking with BSA is needed except for the last step with the incubation with streptavidin-HRP. This is because the BSA contains low level of erythropoietin which will form a complex with the capturing antibody thus reducing the assay sensitivity. Following a 1 hour incubation to allow capture of the Biosimilar and Epoetin reference proteins by the panel of antibodies on the plate, a collection of reporting polyclonal anti- Epoetin antibodies, conjugated with biotin, is added and incubated for 45 min to allow it to bind to any captured proteins. After this incubation, the plate is washed and a Streptavidin-HRP (Horse Radish Peroxidase) conjugate is added and incubated for 30 minutes. The Streptavidin-HRP conjugate will be captured by any biotin labeled antibody bound to the plate. Following a wash step to remove unbound conjugate, 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 wavelengths. The color development will be proportional to the captured Biosimilar or Epoetin reference protein. A typical ELISA with Epoetin protein from three different manufacturers is shown in figure 1 below.

Figure 1. Epoetin Conformational Array ELISA



Supplied Components:

Clear 96 Well Plate
Clear plastic microtiter plate to be coated with the panel of antibodies against Epoetin peptides.
Kit AB-00210 (1 plate)

Dilution Buffer
Buffer used only for dilution of Streptavidin-HRP conjugate. Kit AB-00210 (15 ml)

PBS
Used as diluent for the capturing antibodies. Kit AB-00210 (15 ml)

10x PBS-T
After dilution, it is used as wash solution and also as diluent for the Epoetin testing samples and biotin-labeled reporting antibodies. The 50 ml of concentrate should be diluted to 500 ml with 450 ml deionized or distilled water.
Kit AB-00210 (50 ml)

Capturing antibody
10 different polyclonal antibodies against Epoetin peptides covering the whole molecule were provided. On the first day of testing, add 1 ml PBS to each of the antibody vial provided to make a 10 µg/ml working stock.
Kit AB-00210 (200 µg/ml, 50 µl / tube)

Reporting antibody
A biotin labeled rabbit polyclonal antibody against Epoetin protein. Immediately prior to the assay, dilute the entire 125 µl into 10 ml of 1x PBS-T buffer to give a 25 µg/ml working stock.
Kit AB-00210 (2 mg/ml, 125 µl / tube)

Streptavidin-HRP Conjugate
A Streptavidin – Horse Radish Peroxidase conjugate in a special stabilizing solution. Immediately prior to the assay, dilute the 375 µl into 15 ml of 1x Dilution buffer to give a 0.1 µg/ml working stock.
Kit AB-00210 (4 µg/ml, 375 µl / tube)

TMB Substrate
Use directly without dilution.
Kit AB-00210 (15 ml)

Stop Solution

A 1M solution of sulfuric acid. CAUSTIC. Use directly without dilution.
Kit AB-00210 (15 ml)

Plate Sealer
Kit AB-00210 (one)

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 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 antibody appearing in 6 positions on the plate. 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 control compound are highlighted and the three rows underneath are used for the test compound. For an experiment in duplicate, use rows B-C for the control and rows D-E and F-G for two test compounds.

2. Add 1 ml PBS to each of the 10 capturing antibody vials, mix well and coat each column of the 96-well plate provided with one antibody solution. For example, column 1 will be coated with Ab-1, column 2 coated with Ab-2 and so on. Only six rows will be coated (B, C, D, E, F and G), each well is coated with 100 µl of capturing antibody at 10 µg/ml. Incubate the plate at 4°C overnight with the cover sheet provided.

3. The next day, dilute the 10xPBS-T with water to 1x-strength. Check 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.

4. Wash the coated plate by emptying contents and adding 250 µl of PBS-T to each well. Empty wells again and tap the plate firmly upside down on a paper towel to fully empty well. Repeat.

5. Dilute your sample and Epoetin standard to a concentration of 2 µg/ml in PBS-T; prepare at least 4 ml of each if samples are to be run in duplicate, 5 ml of each if run in triplicate. Pipette 100 µL of 2 µg/ml sample or Epoetin standard into each row of the plate. For replicates use multiple rows, i.e. Epoetin standard in rows 2-3, sample 1 in rows 4-5 and sample 2 in rows 6-7. Cover plates and incubate 1 hour at room temperature.

6. During the above incubation, dilute the 1 mg/ml reporting antibody to 25 µg/ml by adding 125 µL to 10 ml of PBS-T.

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 25 µg/ml Reporting Antibody into each well. Cover plate and incubate plate 45 min at room temperature.

9. During the above incubation, dilute the 4 µg/ml Streptavidin-HRP conjugate to 0.1 µg/ml by adding 375 µL to 15 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 of 0.1 µg/ml Streptavidin-HRP conjugate into wells. Cover plate and incubate plate 30 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 of TMB substrate to each well. Allow color development to proceed for exactly 12 minutes and then stop reaction by adding 100 µL of Stop Solution to each well. 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 B11-G11 as blanks.

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 Epoetin standard. Any differences between your sample and the Epoetin standard should be apparent.

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10		
C	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10		
D	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10		
E	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10		
F	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10		
G	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10		
H												