**TECHNICAL HINTS & LIMITATIONS**

- We recommend the use of R&D Systems’ Reagent Diluent Concentrate 2 (Catalog # DY995) to prepare Reagent Diluent for use in this assay.
- The use of high quality Bovine Serum Albumin (BSA) for the Reagent Diluent is crucial for the optimum performance of the DuoSet® ELISA Development kit. Impurities such as proteases, binding proteins, soluble receptors or other interfering substances can be found to varying degrees in virtually all BSA preparations and can inhibit or interfere with the detection of certain analytes. If the standard curve appears suppressed, consider evaluating a different preparation of BSA.
- It is suggested to start Reagent Diluent optimization for serum and plasma samples by using PBS supplemented with 10-50% animal serum. Do not use buffers with animal serum to reconstitute or dilute the Detection Antibody or Streptavidin-HRP.
- It is important that the Reagent Diluent selected for reconstitution and dilution of the standard reflects the environment of the samples being measured.
- Avoid microbial contamination of reagents and buffers.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is recommended that all standards and samples be assayed in duplicate.
- The use of PBS from tablets may interfere in this assay.
- The use of PBS from tablets may interfere in this assay.

**TROUBLESHOOTING**

**Poor Standard Curve**
- Impure BSA used for Reagent Diluent preparation.
- Improper reconstitution and/or storage of standard.
- Improper dilution of highest standard and standard curve.
- Incorrect washing and/or aspiration of wells.
- Unequal volumes added to wells/pipetting error.

**Low or No color Development**
- Inadequate volume of substrate added to wells.
- Incorrect incubation times or temperatures.

**Poor Precision**
- Unequal volumes added to wells/pipetting error.
- Incomplete washing and/or aspiration of wells.
- Unequal mixing of reagents.

**Note:** For more detailed troubleshooting, please visit: www.RnDSystems.com/ELISADevelopment

**SPECIFICITY**

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

<table>
<thead>
<tr>
<th>Recombinant human</th>
<th>Natural proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibulin-3</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>Lumican</td>
<td></td>
</tr>
<tr>
<td>Tenascin R</td>
<td></td>
</tr>
</tbody>
</table>

**CALCULATION OF RESULTS**

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Aggrecan concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

**TYPICAL DATA**

This standard curve is only for demonstration purposes. A standard curve should be generated for each set of samples assayed.

![Graph showing optical density vs. human Aggrecan concentration](image)
DuoSet® Ancillary Reagent Kit 2 (5 plates):
(R&D Systems, Catalog # DY008) containing 96 well microplates, plate sealers, substrate solution, stop solution, plate coating buffer (PBS), wash buffer, and Reagent Diluent Concentrate 2.

The components listed above may be purchased separately:
96 well microplates: (R&D Systems, Catalog # DY990).
Plate Sealers: (R&D Systems, Catalog # DY992).
PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY006).
Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).
Reagent Diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY995).
Stop Solution: 2 N H₂SO₄ (R&D Systems, Catalog # DY994).

PRECAUTIONS
Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist. The Stop Solution suggested for use with this kit is an acid solution. The Color Reagent B suggested for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

CALIBRATION
This DuoSet® is calibrated against a highly purified NS0-expressed recombinant human Aggrecan G1-IGD-G2 Domains produced at R&D Systems.

MATERIALS PROVIDED & STORAGE CONDITIONS
Store the unopened kit at 2-8 ºC. Do not use past kit expiration date.

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>PART #</th>
<th># VIALS</th>
<th>STORAGE OF OPENED/ RECONSTITUTED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Aggrecan Capture Antibody</td>
<td>843416</td>
<td>1 vial</td>
<td>Refer to the lot-specific Certificate of Analysis (C of A) for storage conditions.</td>
</tr>
<tr>
<td>Human Aggrecan Detection Antibody</td>
<td>843417</td>
<td>1 vial</td>
<td></td>
</tr>
<tr>
<td>Human Aggrecan Standard</td>
<td>843418</td>
<td>3 vials</td>
<td></td>
</tr>
<tr>
<td>Streptavidin-HRP</td>
<td>#90003</td>
<td>1 vial</td>
<td></td>
</tr>
</tbody>
</table>

GENERAL ELISA PROTOCOL

Plate Preparation
1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure
1. Add 100 µL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

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