Collagen IV (Human) AlphaLISA Detection Kit

Product number: AL3034 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

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Product Information

Application: This kit is designed for the quantitative determination of Collagen IV in serum, plasma, and cell culture supernatants using a homogeneous AlphaLISA assay (no wash steps). The assay shows 1% cross reactivity with COL1A1 and Collagen I, II, and III. Cross-reactivity with other species has not been tested.

Sensitivity:
- Lower Detection Limit (LDL): 155 pg/mL
- Lower Limit of Quantification (LLOQ): 570 pg/mL
- EC_{50}: 222 ng/mL

Dynamic range: 98 – 1 000 000 pg/mL (Figure 1).

![Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.](image)

Storage: Store kit in the dark at +4°C. Store reconstituted analyte at -20°C.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC_{50} and LDL were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and the instrument used, with no impact on LDL measurement.
**Analyte of Interest**

Collagen IV, as one of many extracellular matrix proteins, is produced by fibroblasts, endothelial cells, and epithelial cells. It is a heterotrimeric molecule containing two α1-like and one α2-like chain. The chain compositions of collagen IV are associated with six genes (COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6) suggesting that the existence of additional alpha-chains to collagen structures and functions. Collagen Type IV is found mainly in the basement membranes of many tissues and organs including liver, kidney, and skin. It functions to maintain the structural integrity of cells, tissues, and organs. Structural alterations of collagen IV is associated with many diseases such as nephritic syndrome, hemoptysis, liver fibrosis, subepidermal blistering diseases in the skin, and diabetic nephropathy. Concentrations of collagen IV in serum correlate with alcoholic liver disease.

**Description of the AlphaLISA Assay**

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

![AlphaLISA Assay Principle](image)

**Figure 2. AlphaLISA Assay Principle.**

**Precautions**

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. The analyte included in this kit is from a human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.
Kit Content: Reagents and Materials

<table>
<thead>
<tr>
<th>Kit components</th>
<th>AL3034HV (100 assay points***</th>
<th>AL3034C (500 assay points***</th>
<th>AL3034F (5000 assay points***</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlphaLISA Anti-Collagen IV Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2</td>
<td>40 µL @ 5 mg/mL (1 brown tube, white cap)</td>
<td>100 µL @ 5 mg/mL (1 brown tube, white cap)</td>
<td>1 mL @ 5 mg/mL (1 brown tube, white cap)</td>
</tr>
<tr>
<td>Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4</td>
<td>40 µL @ 5 mg/mL (1 brown tube, black cap)</td>
<td>100 µL @ 5 mg/mL (1 brown tube, black cap)</td>
<td>1 mL @ 5 mg/mL (1 brown tube, black cap)</td>
</tr>
<tr>
<td>Biotinylated Anti-Collagen IV Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN₃, pH 7.4</td>
<td>40 µL @ 500 nM (1 tube, black cap)</td>
<td>100 µL @ 500 nM (1 tube, black cap)</td>
<td>1 mL @ 500 nM (1 tube, black cap)</td>
</tr>
<tr>
<td>Lyophilized Collagen IV Analyte*</td>
<td>3 µg (1 tube, clear cap)</td>
<td>3 µg (1 tube, clear cap)</td>
<td>3 µg (1 tube, clear cap)</td>
</tr>
<tr>
<td>AlphaLISA Immunoassay Buffer (10X) **</td>
<td>2 mL, 1 small bottle</td>
<td>10 mL, 1 small bottle</td>
<td>100 mL, 1 large bottle</td>
</tr>
</tbody>
</table>

* Reconstitute Collagen IV in 100 µL Milli-Q® grade H₂O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. One vial contains an amount of Collagen IV sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3034S).

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL in 96- or 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should not be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

<table>
<thead>
<tr>
<th>Item</th>
<th>Suggested source</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>TopSeal™-A Plus Adhesive Sealing Film</td>
<td>PerkinElmer Inc.</td>
<td>6050185</td>
</tr>
<tr>
<td>EnVision®-Alpha Reader</td>
<td>PerkinElmer Inc.</td>
<td>-</td>
</tr>
</tbody>
</table>

www.perkinelmer.com
**Recommendations**

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H$_2$O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in the AlphaLISA Immunoassay Buffer for serum and/or plasma samples.

**Assay Procedure**

**IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE**

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.
<table>
<thead>
<tr>
<th>Format</th>
<th># of data points</th>
<th>Final</th>
<th>Sample</th>
<th>AlphaLISA Acceptor beads</th>
<th>Biotinylated Antibody</th>
<th>SA-Donor beads</th>
<th>Plate recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL3034HV</td>
<td>100</td>
<td>100 μL</td>
<td>10 μL</td>
<td>20 μL</td>
<td>20 μL</td>
<td>50 μL</td>
<td>White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>100 μL</td>
<td>10 μL</td>
<td>20 μL</td>
<td>20 μL</td>
<td>50 μL</td>
<td>White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)</td>
</tr>
<tr>
<td>AL3034C</td>
<td>500</td>
<td>50 μL</td>
<td>5 μL</td>
<td>10 μL</td>
<td>10 μL</td>
<td>25 μL</td>
<td>White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)</td>
</tr>
<tr>
<td></td>
<td>1 250</td>
<td>20 μL</td>
<td>2 μL</td>
<td>4 μL</td>
<td>4 μL</td>
<td>10 μL</td>
<td>Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)</td>
</tr>
<tr>
<td></td>
<td>2 500</td>
<td>10 μL</td>
<td>1 μL</td>
<td>2 μL</td>
<td>2 μL</td>
<td>5 μL</td>
<td>Light gray AlphaPlate-1536 (cat # 6004350)</td>
</tr>
<tr>
<td>AL3034F</td>
<td>5 000</td>
<td>50 μL</td>
<td>5 μL</td>
<td>10 μL</td>
<td>10 μL</td>
<td>25 μL</td>
<td>White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)</td>
</tr>
<tr>
<td></td>
<td>12 500</td>
<td>20 μL</td>
<td>2 μL</td>
<td>4 μL</td>
<td>4 μL</td>
<td>10 μL</td>
<td>Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)</td>
</tr>
<tr>
<td></td>
<td>25 000</td>
<td>10 μL</td>
<td>1 μL</td>
<td>2 μL</td>
<td>2 μL</td>
<td>5 μL</td>
<td>Light gray AlphaPlate-1536 (cat # 6004350)</td>
</tr>
</tbody>
</table>
3 Step Protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
   Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL Milli-Q® grade H2O.

2) Preparation of Collagen IV analyte standard dilutions:
   a. Reconstitute lyophilized Collagen IV (3 μg) in 100 μL Milli-Q® grade H2O.
   b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

<table>
<thead>
<tr>
<th>Tube</th>
<th>Vol. of Collagen IV (μL)</th>
<th>Vol. of diluent (μL) *</th>
<th>[Collagen IV] in standard curve (g/mL in 5 μL)</th>
<th>(pg/mL in 5 μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10 μL of reconstituted Collagen IV</td>
<td>90</td>
<td>3.0E-06</td>
<td>3 000 000</td>
</tr>
<tr>
<td>B</td>
<td>60 μL of tube A</td>
<td>120</td>
<td>1.0E-06</td>
<td>1 000 000</td>
</tr>
<tr>
<td>C</td>
<td>60 μL of tube B</td>
<td>140</td>
<td>3.0E-07</td>
<td>300 000</td>
</tr>
<tr>
<td>D</td>
<td>60 μL of tube C</td>
<td>120</td>
<td>1.0E-07</td>
<td>100 000</td>
</tr>
<tr>
<td>E</td>
<td>60 μL of tube D</td>
<td>140</td>
<td>3.0E-08</td>
<td>30 000</td>
</tr>
<tr>
<td>F</td>
<td>60 μL of tube E</td>
<td>120</td>
<td>1.0E-08</td>
<td>10 000</td>
</tr>
<tr>
<td>G</td>
<td>60 μL of tube F</td>
<td>140</td>
<td>3.0E-09</td>
<td>3 000</td>
</tr>
<tr>
<td>H</td>
<td>60 μL of tube G</td>
<td>120</td>
<td>1.0E-09</td>
<td>1 000</td>
</tr>
<tr>
<td>I</td>
<td>60 μL of tube H</td>
<td>140</td>
<td>3.0E-10</td>
<td>300</td>
</tr>
<tr>
<td>J</td>
<td>60 μL of tube I</td>
<td>120</td>
<td>1.0E-10</td>
<td>100</td>
</tr>
<tr>
<td>K</td>
<td>60 μL of tube J</td>
<td>140</td>
<td>3.0E-11</td>
<td>30</td>
</tr>
<tr>
<td>L</td>
<td>60 μL of tube K</td>
<td>120</td>
<td>1.0E-11</td>
<td>10</td>
</tr>
<tr>
<td>M ** (background)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N ** (background)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O ** (background)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P ** (background)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer). At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-Collagen IV Acceptor beads (100 μg/mL):
   a. Prepare just before use.
   b. Add 100 μL of 5 mg/mL AlphaLISA Anti-Collagen IV Acceptor to 4900 μL of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 5X Biotinylated Anti-Collagen IV Antibody (10 nM):
   a. Prepare just before use.
   b. Add 100 μL of 500 nM Biotinylated Anti-Collagen IV Antibody to 4900 μL of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (40 μg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 100 μL of 5 mg/mL SA-Donor beads to 12400 μL of 1X AlphaLISA Immunoassay Buffer.
6) In a white Optiplate (384 wells):

- Add 5 µL of each analyte standard dilution or sample
- Add 10 µL of 5X Anti-Collagen IV Acceptor beads (20 µg/mL final)
- Incubate 30 minutes at 23˚C
- Add 10 µL of 5X Biotinylated Anti-Collagen IV Antibody (2 nM final)
- Incubate 60 minutes at 23˚C
- Add 25 µL of 2X SA-Donor beads (20 µg/mL final)
- Incubate 30 minutes at 23˚C in the dark
- Read using EnVision-Alpha Reader (615 nm)

**Data Analysis**

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a 1/Y² data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the 3 step protocol in AlphaLISA Immunoassay Buffer (IAB) and cell culture medium containing 10% FBS. The analytes (standards) were prepared in IAB, DMEM, or RPMI, and all other components were prepared in IAB.

- **Assay Sensitivity:**

The LDL and LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL using the recommended assay conditions.

<table>
<thead>
<tr>
<th>LDL (pg/mL)</th>
<th>Buffer/Medium*</th>
<th># of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>IAB</td>
<td>6</td>
</tr>
<tr>
<td>159</td>
<td>DMEM + 10% FBS</td>
<td>6</td>
</tr>
<tr>
<td>1455</td>
<td>RPMI + 10% FBS</td>
<td>6</td>
</tr>
<tr>
<td>2461</td>
<td>RPMI</td>
<td>6</td>
</tr>
</tbody>
</table>

* Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by preparing standards in different matrixes. The standard prepared in RPMI with and without 10% FBS reduced the sensitivity to 1.5 ng/mL and 2.5 ng/mL, respectively, suggesting that biotin containing medium should be avoided when testing the cell culture supernatants.

- **Assay Precision:**

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in IAB, DMEM, or RPMI. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well format using IAB.

- **Intra-assay precision:**

The intra-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

<table>
<thead>
<tr>
<th>Collagen IV</th>
<th>IAB</th>
<th>DMEM</th>
<th>RPMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV (%)</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

- **Inter-assay precision:**

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 3 ng/mL sample. Shown as CV%.

<table>
<thead>
<tr>
<th>Collagen IV</th>
<th>IAB</th>
<th>DMEM</th>
<th>RPMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV (%)</td>
<td>5</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>
• **Spike Recovery:**

Three known concentrations of analyte were spiked in IAB, or in cell culture media. All samples, including non-spiked buffer or media were measured in the assay. The average recovery from three independent measurements is reported. Note that the standard curves were prepared in IAB, DMEM, and RPMI. Cell culture media was supplemented with 10% FBS.

<table>
<thead>
<tr>
<th>Spiked Collagen IV (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IAB</td>
</tr>
<tr>
<td>300</td>
<td>103</td>
</tr>
<tr>
<td>30</td>
<td>104</td>
</tr>
<tr>
<td>3</td>
<td>105</td>
</tr>
</tbody>
</table>

• **Specificity:**

Cross-reactivity of the human Collagen IV Kit was tested using human Collagen I, II, and III. The kit detected less than 1% cross reactivity with Collagen I, II, and III.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>0.9</td>
</tr>
<tr>
<td>Collagen II</td>
<td>0.8</td>
</tr>
<tr>
<td>Collagen III</td>
<td>0.7</td>
</tr>
<tr>
<td>COL1A1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Human Serum Experiments**

To validate the assay kit, commercially available human serum and plasma with unknown concentrations of Collagen IV was used to examine dilution linearity. Human Collagen IV (200 to 400 ng/mL) was detected in human serum and plasma. Good dilution linearity was obtained when the serum was diluted greater than 81-fold or when plasma was diluted greater than 9-fold.

<table>
<thead>
<tr>
<th>Serum Dilution Factor</th>
<th>Collagen IV Detected in serum (ng/mL)</th>
<th>Collagen IV Detected in Plasma (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>84</td>
<td>299</td>
</tr>
<tr>
<td>27</td>
<td>133</td>
<td>345</td>
</tr>
<tr>
<td>81</td>
<td>226</td>
<td>289</td>
</tr>
<tr>
<td>243</td>
<td>329</td>
<td>289</td>
</tr>
<tr>
<td>729</td>
<td>360</td>
<td>264</td>
</tr>
<tr>
<td>2187</td>
<td>396</td>
<td>298</td>
</tr>
</tbody>
</table>
Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at:


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