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**1,5-AG ELISA Kit
(OKCD02247)
Lot# KD5558**

Instructions for Use

For the quantitative measurement of 1,5-AG in serum, plasma and other biological fluids.

Variation between lots can occur. Refer to the manual provided with the kit.

This product is intended for research use only.

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1. Background

Principle

Aviva Systems Biology 1,5-AG ELISA Kit (OKCD02247) is based on a competitive enzyme immunoassay technique. The microtiter well-plate in this kit has been pre-coated with an anti-1,5-AG antibody. Sample or standards are added to the wells along with a fixed quantity of biotinylated 1,5-AG and incubated. The 1,5-AG found in the sample or standards competes with the biotinylated 1,5-AG for limited binding sites on the immobilized anti-1,5-AG antibody. Wells are washed and Avidin-HRP conjugate is added, incubated then washed. An enzymatic reaction is then produced through the addition of TMB substrate which is catalyzed by the immobilized HRP to generate a blue color product that changes yellow after adding acidic stop solution. The density of yellow coloration is measured by reading the absorbance at 450 nm which is quantitatively proportional to the amount of biotinylated 1,5-AG captured in the well and inversely proportional to the amount of 1,5-AG which was contained in the sample or standard.

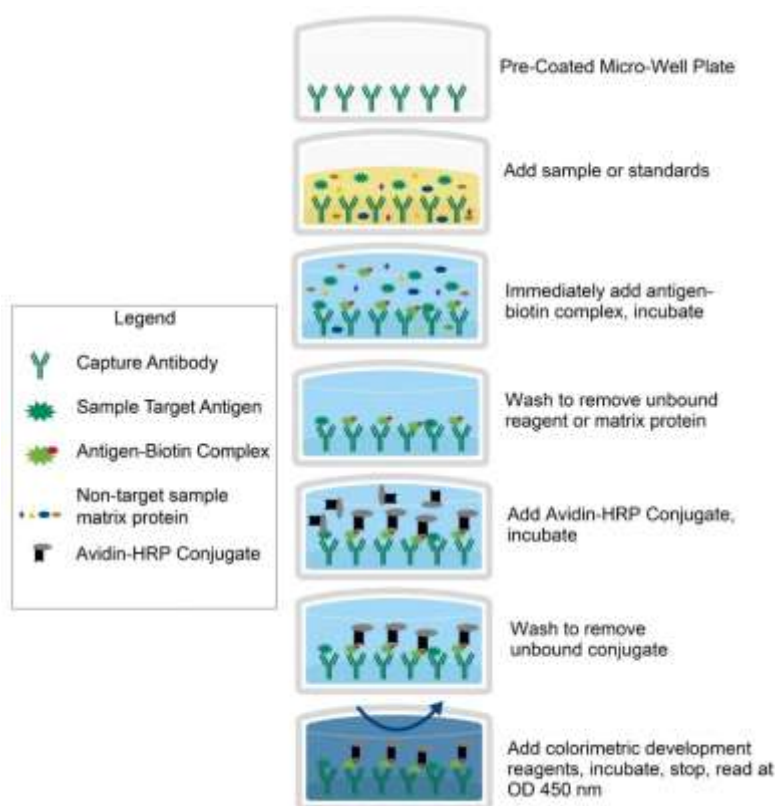
Target Background

1, 5-Anhydrosorbitol or 1, 5-anhydroglucitol (1, 5-AG) is a validated marker of short-term glycemic control. This substance is derived mainly from food, is well absorbed in the intestine, and is distributed to all organs and tissues. It is metabolically stable, being excreted in the urine when its level exceeds the renal threshold. It is reabsorbed in the renal tubules and is competitively inhibited by glucosuria, which leads to a reduction in its level in serum. The correlation between this reduction and the amount of glucose present in urine is so close that 1, 5 AG can be used as a sensitive, day-to-day, real-time marker of glycemic control. It provides useful information on current glycemic control and is superior to both hemoglobin A1C and fructosamine in detecting near-normoglycemia. 1, 5-AG in human plasma has been proposed for several years as a short-term, retrospective marker of glycemic control and seems to be the most suitable parameter for monitoring glucose excursions. The decrease in serum 1, 5-AG is very sensitive to urinary glucose excretion. It is a metabolically inert polyol that competes with glucose for reabsorption in the kidneys. Otherwise stable levels of 1, 5-AG are rapidly depleted as blood glucose levels exceed the renal threshold for glucosuria. 1, 5-AG is also more tightly associated with glucose fluctuations and postprandial glucose.

General Specifications

General Specifications	
Range	1.9 - 150 µg/mL
LOD	< 0.7 µg/mL (Derived by linear regression of OD ₄₅₀ of the Mean Blank + 2xSD)
Specificity	1,5-AG PubChem: 64960 Molecular Formula: C ₆ H ₁₂ O ₅ Target Alias: 1 1,5-Anhydro-D-glucitol; 1,5-Anhydroglucitol; 154-58-5; 1,5-ANHYDROSORBITOL
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-1,5-AG Microplate	96 Wells (12 x 8 Well strips)	-20°C for 6 months
CCK Standard	2 vials	
100X 1,5-AG Biotin Complex	1 x 120 µL	
100X Avidin-HRP Conjugate	1 x 120 µL	
Standard Diluent	1 x 20 mL	
Biotin Complex Diluent	1 x 12 mL	4°C for 6 months
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 μ L 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 in inter- 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 μ L 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 or fibrin strands or which 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.

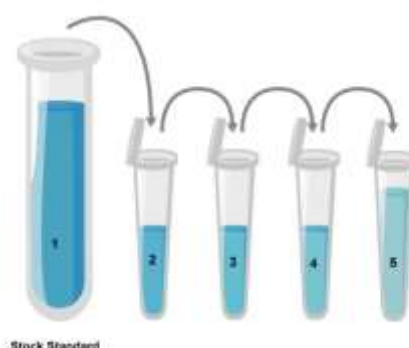
8. Reagent Preparation

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

8.1 1,5-AG Assay Standards

- 8.1.1 Prepare the 1,5-AG standards no greater than 2 hours prior to performing experiment. Standards should be held on ice until use in the experiment.
- 8.1.2 Prepare a stock 150 µg/mL Standard by reconstituting one tube of **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 **700 µL** 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 serially diluted standards as follows:
 - 8.1.3.1 Label tubes with numbers 2 – 6.
 - 8.1.3.2 Use the **150 µg/mL Standard** from step 8.1.2 as the high standard point (Tube #1).
 - 8.1.3.3 Add **400 µL** of **Standard Diluent** to Tube #'s 2 – 6.
 - 8.1.3.4 Prepare **Standard #2** by adding 200 µL of **150 µg/mL Standard** (Tube #1) to Tube #2. Mix gently and thoroughly.
 - 8.1.3.5 Prepare **Standard #3** by adding 200 µL of **Standard #2** from Tube #2 to Tube #3. Mix gently and thoroughly.
 - 8.1.3.6 Prepare further serial dilutions through Tube #6. Reference the table below as a guide for serial dilution scheme.
 - 8.1.3.7 Tube #6 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	1,5-AG Standard	NA	700	700	150 µg/mL
2	150 µg/mL	200	400	600	50 µg/mL
3	50 µg/mL	200	400	600	16.7 µg/mL
4	16.7 µg/mL	200	400	600	5.6 µg/mL
5	5.6 µg/mL	200	400	600	1.9 µg/mL
6	NA	0	400	400	0.0 (Blank)



8.2 **1X 1,5-AG-Biotin Complex**

- 8.2.1 Prepare the **1X 1,5-AG Biotin Complex** immediately prior to use by diluting the **100X 1,5-AG Biotin Complex** 1:100 with **Biotin Complex 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 1,5-AG Biotin Complex** to 990 µL **Biotin Complex 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 Avidin-HRP 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** as follows.
- 8.3.2 Briefly and gently mix the **100X Avidin-HRP Conjugate** prior to pipetting.
- 8.3.3 For each well strip to be used in the experiment (8-wells) prepare 1,000 µL **1X Avidin-HRP Conjugate** by adding 10 µL of **100X Avidin-HRP Conjugate** to 990 µL **Conjugate Diluent**.
- 8.3.4 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure.

8.4 **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.

8.5 **1X Wash Buffer**

- 8.5.1 If crystals have formed in the **30X Wash Buffer** concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.
- 8.5.2 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.
- 8.5.3 Seal and mix gently by inversion. Avoid foaming or bubbles.
- 8.5.4 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.

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:
 - **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1,000 x g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
 - **Plasma** - Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1,000 x g at 2-8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.
 - **Other biological fluids** – Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20°C or -80°C. 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.

- Dilute samples using **Standard Diluent**.
- Mix diluted samples gently and thoroughly.
- Pipetting less than 2 µL is not recommended for optimal assay accuracy.
- Optimal dilution must be determined by the user according to their specific samples.

10. Assay Procedure

- Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.
- For optimal control of small potential variations in micro well-plate and day to day ambient temperature fluctuations, equilibrate all reagents prior to use and perform all incubation steps at 37°C.

- 10.1** Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.
- 10.2** Retain at least one well as an absolute Blank without any samples or reagents.
- 10.3** Add 50 µL of serially titrated standards, diluted samples or blank into wells of the **Anti-1,5-AG Microplate**. At least two replicates of each standard, sample or blank is recommended.
- 10.4** Immediately add 50 µL of **1X 1,5-AG-Biotin Complex** to each well (excluding absolute Blank).
- 10.5** Cover the plate with the plate sealer and incubate at 37°C for 60 minutes.
- 10.6** Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
- 10.7** 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
- 10.8** Wash plate three times with **1X Wash Buffer** as follows:
 - 10.8.1 Add 350 µL of **1X Wash Buffer** to each assay well.
 - 10.8.2 Incubate for 2 minutes.
 - 10.8.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
 - 10.8.4 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.
 - 10.8.5 Repeat steps 10.8.1 through 10.8.4 **two** more times.
- 10.9** Add 100 µL of **1X Avidin-HRP Conjugate** to each well.
- 10.10** Cover the plate with the plate sealer and incubate at 37°C for 30 minutes.
- 10.11** Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
- 10.12** 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.
- 10.13** Repeat wash as in **step 10.8**.
- 10.14** 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 based on the standard, adjust incubation times.
(NOTE: optimal incubation time must be determined by the user. Optimal development can be visualized by blue shading in the bottom four standard wells, while the remaining standards still appear clearer.)
- 10.15** Add 50 µL of **Stop Solution** to each well. Well color should change to gradations of yellow immediately. Add the **Stop Solution** in the same well order as done for the **TMB Substrate**.
- 10.16** Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.15. 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 OD₄₅₀** 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 OD₄₅₀** of each standard serial dilution point vs. the respective standard concentration. The **1,5-AG** concentration contained in the samples can be interpolated by using linear regression of each mean sample **Relative OD₄₅₀** against the standard curve. This is best achieved using curve fitting software.

Note: if wavelength correction readings are 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

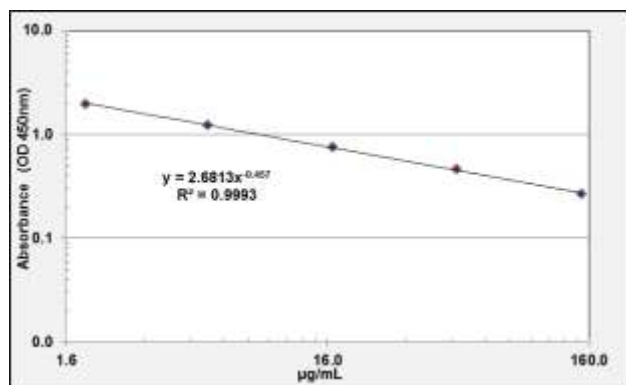
12.1 Reproducibility

Intra-assay Precision: 3 samples with known low, middle and high levels 1,5-AG were tested with 20 replicates on one plate, respectively. Inter-assay Precision: 3 samples with known low, middle and high level 1,5-AG 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 (µg/ml)	7.41	25.47	58.77	7.43	28.97	62.41
SD	0.496	1.579	3.291	0.505	1.883	3.807
CV (%)	6.7	6.2	5.6	6.8	6.5	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.



µg/mL	Absorbance		Mean Absorbance	Log of Concentration
	Rep 1	Rep 2		
150	0.265	0.267	0.266	2.176
50	0.458	0.456	0.457	1.699
16.7	0.756	0.762	0.759	1.223
5.6	1.222	1.214	1.218	0.748
1.9	1.968	1.946	1.957	0.279

12.3 Recovery

Matrices were spiked with 1,5-AG and recovery rates were calculated by comparing the measured values to the expected concentrations.

Sample	1:2	1:4	1:8	1:16
Serum (n=5)	84-97%	95-106%	94-103%	79-92%
EDTA Plasma (n=5)	87-105%	85-95%	82-94%	88-102%
heparin Plasma (n=5)	81-93%	79-92%	86-96%	89-99%

12.4 Linearity

Linearity of the kit was evaluated by spiking 1,5-AG into matrices, serially diluted and measured. Observed values were compared to the expected measurements.

Matrix	Recovery Range (%)	Average (%)
Serum (n=5)	90-99	94
EDTA plasma (n=5)	84-94	89
Heparin Plasma (n=5)	81-96	87

13. Technical Resources

Technical Support:

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

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